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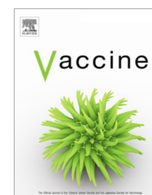

Immunization of pregnant cows with Shiga toxin-2 induces high levels of specific colostral antibodies and lactoferrin able to neutralize *E. coli* O157:H7 pathogenicity

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ABSTRACT

E. coli O157:H7 is a foodborne pathogen responsible for bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS). The objective of the present work was to evaluate the ability of colostral IgG obtained from Stx2-immunized cows to prevent against *E. coli* O157:H7 infection and Stx2 cytotoxicity. Hyperimmune colostrum (HC) was obtained from cows intramuscularly immunized with inactivated Stx2 or vehicle for controls. Colostral IgG was purified by affinity chromatography. Specific IgG antibodies against Stx2 and bovine lactoferrin (bLF) levels in HC and the corresponding IgG (HC-IgG/bLF) were determined by ELISA. The protective effects of HC-IgG/bLF against Stx2 cytotoxicity and adhesion of *E. coli* O157:H7 and its Stx2-negative mutant were analyzed in HCT-8 cells. HC-IgG/bLF prevention against *E. coli* O157:H7 was studied in human colon and rat colon loops. Protection against a lethal dose of *E. coli* O157:H7 was evaluated in a weaned mice model. HC-IgG/bLF showed high anti-Stx2 titers and high bLF levels that were able to neutralize the cytotoxic effects of Stx2 *in vitro* and *in vivo*. Furthermore, HC-IgG/bLF avoided the inhibition of water absorption induced by *E. coli* O157:H7 in human colon and also the pathogenicity of *E. coli* O157:H7 and *E. coli* O157:H7Δstx2 in rat colon loops. Finally, HC-IgG/bLF prevented in a 100% the lethality caused by *E. coli* O157:H7 in a weaned mice model. Our study suggests that HC-IgG/bLF have protective effects against *E. coli* O157:H7 infection. These beneficial effects may be due to specific anti-Stx2 neutralizing antibodies in combination with high bLF levels. These results allow us to consider HC-IgG/bLF as a nutraceutical tool which could be used in combination with balanced supportive diets to prevent HUS. However further studies are required before recommendations can be made for therapeutic and clinical applications.

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1. Introduction

Shiga toxin (Stx) producing *Escherichia coli* (STEC), first described about 35 years ago as Vero toxin producing *E. coli* (VTEC), is responsible for bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) [1]. STEC is an important bacterial pathogen involved in foodborne diseases [2]. STEC is endemic in Argentina, with approximately 400 new cases per year [3]. *E. coli* O157:H7 is associated worldwide with severe disease and is the most prevalent serotype isolated from HUS patients in Argentina [4]. The virulence of STEC is determined, in a large extent, by the production of Stx responsible for HUS development. STEC can produce two types of Stx, type 1 (Stx1) and/or type 2 (Stx2), and its variants, being Stx2 more toxigenic and epidemiologically more relevant than Stx1 [5]. *E. coli* O157:H7 is also characterized by

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other virulence-associated proteins responsible for the colonization of the intestinal mucosa of humans and animals. The characteristic attaching and effacing (A/E) lesion is a consequence of the intimately attached bacteria and effacement of the intestinal microvilli [6]. Most of the proteins responsible for the A/E lesion are encoded in a pathogenicity island called locus of enterocyte effacement (LEE). LEE encodes proteins that constitute the type three secretion system (T3SS), a virulence appendage that translocate virulence factors called effectors into the enterocytes. The main components of T3SS injectosome are EspA, EspB and EspD and one the major effector is Tir, a translocated receptor of Intimin, a bacterial outer membrane protein also encoded in LEE [7]. Many virulence factors of *E. coli* O157:H7 induce an immune response during the course of infections. Intimin, EspA and EspB elicit an immune response during human *E. coli* O157:H7 infection [8]. In this regard, studies of two STEC outbreaks correlated the presence of anti-Stx2 antibodies in human sera with protection against HUS development [9]. Consistent with these findings, a previous study reported anti-Stx2 seroreactivity both in healthy children and HUS patients [10]. Additionally, antibodies against Stx2 were found in adults, generally refractory to HUS, and in children without any sign of illness being part of a family STEC outbreak [10,11]. These data together with the extremely rare occurrence of a second episode of HUS in the same patient, suggest a protective role for anti-Stx antibodies against HUS development [12].

Antibodies against Stx2 may be also transferred from the mother to the baby through placenta and colostrums [13]. Studies carried out with colostrum samples from healthy Brazilian women have demonstrated the presence of antibodies reactive to the main virulence factors associated to enteropathogenic *E. coli* (EPEC) that exhibited a cross reaction and protection against STEC [14]. Secretory IgA antibodies against EPEC/STEC antigens were also found in colostrum of Brazilian breastfeeding women population living in an EPEC endemic area although were unable to neutralize Stx1 and Stx2 cytotoxic effects on Vero cells [15]. Altogether, these results suggest that anti-Stx2 antibodies could be relevant to protect infants from STEC infections. In this regard, it may be beneficial for children living in endemic areas for HUS, to access food containing bioactive compounds against Stx2.

It is well known that the consumption of bovine colostrum by human has therapeutics effects in several gastrointestinal infections, since it contains a range of peptides and proteins with direct antimicrobial and endotoxin-neutralizing effects [16]. In a previous study [17], we demonstrated that vaccination of pregnant cows with EspA, EspB, the C-terminal fragment of Intimin (γ-Intimin C_{280}) and inactivated Stx2 induced high levels of specific colostral antibodies that were efficiently transferred to newborn calves through lactation. Hence, biological activities of bovine colostrum benefit neonatal calves may also benefit children. However, only since the second half of the last century has it been possible to prepare stable and standardized preparations of colostrum [18].

The objective of the present work was to evaluate the ability of colostral IgG obtained from Stx2-immunized cows to neutralize STEC infection and Stx2 cytotoxicity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents and antibodies

Purified Stx2 (Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA) was checked for lipopolysaccharide contamination by Limulus amoebocyte lysate assay (Biowhittaker Inc., MD, USA). Toxin diluted with sterile phosphate-buffered saline (PBS) contained <10 pg LPS/ng of pure Stx2. Rabbit anti-bovine IgG conjugated with horseradish peroxidase (HRP-rabbit anti-bovine IgG, Bethyl Laboratories, Montgomery, USA) and goat anti-mouse conjugated with horseradish peroxidase (HRP-goat anti-mouse IgG, Bio-Rad Laboratories, California, USA) were used. Mouse monoclonal antibody (mAb 2E11) directed against the A-subunit of Stx2 (Stx2A) was kindly provided by Dr. Roxane M. F. Piazza (Butantan Institute, Sao Paulo, SP, Brazil). Colostrum from cows γ-Intimin C_{280}-immunized was previously obtained and used as a control [17].

2.2. Animals

Adult male Sprague-Dawley (SD) rats of 150–180 g of body weight (bwt) were obtained from the animal facility at the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Rats were individually housed under controlled conditions of illumination, humidity, and temperature, with food and water available *ad libitum*. Animals were allowed a minimum of 7 days to adapt to housing conditions before undergoing any manipulation.

Immature male BALB/c mice were used immediately after weaning (17–20 days of age, 8–10 g bwt). Mice were acquired from the animal facility at the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires.

Holstein dairy cows with an average age of 4 years and more than 2 lactations were bred at a herd of the Estación Experimental de Rafaela, INTA, Santa Fé, Argentina.

The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the School of Medicine of University of Buenos Aires (CICUAL, Res. N° 2954/10) and the Animal Welfare Committee of the National Institute of Agricultural Technology. All the procedures were performed in accordance with the EEC guidelines for care and use of experimental animals (EEC Council 86/609).

2.3. Immunization protocol and sample collection

Three pregnant dairy cows were immunized to obtain hyperimmune anti-Stx2 colostrum (HC). Prior to immunization, fecal samples were confirmed to be negative for *E. coli* O157:H7 by immunomagnetic separation performed as described elsewhere [19]. Sera samples were also confirmed to be negative for anti-Stx2 antibodies by ELISA. The animals received two intramuscular injections containing Stx2 (100 μg/dose) inactivated by 10 min at 75 °C diluted in 1 ml of PBS and mixed with 1 ml of a mineral oil-based adjuvant (Montanide ISA206, SEPPIC, France), at 40 and 20 days before delivery. Control colostrum (CC) was obtained from cows (n = 3) that received only adjuvant mixed with PBS. Colostrum samples were obtained within the first 24 h after parturition. Experimental colostral samples were pooled and delipidated by centrifugation at 3000 rpm at 4 °C for 45 min and the watery phase was stored at −20 °C until further analysis.

2.4. Purification of colostral IgG

Purification of colostral IgG was carried out by affinity chromatography under native conditions using a Protein-G-Sepharose column (GE Healthcare UK Ltd). Protein concentrations were determined with the BCA Protein Assay Kit (Pierce Biotech. Inc, USA). To determine the purity of colostral IgG, fractions were resolved on 12% SDS-PAGE, stained with Coomassie blue (Sigma Aldrich St Louis, USA) and analyzed by densitometry. The purification procedure resulted in approximately 10 μg/μl of colostral IgG. Purification of colostral IgG against γ-Intimin C_{280}-immunized cows was performed in the same experimental conditions.

2.5. Antibody titers and lactoferrin levels measured by ELISA

Colostral IgG samples were analyzed for the presence of specific antibodies against the B subunit of Stx2 (Stx2B) by ELISA [20]. Briefly, wells of 96-well ELISA plates were coated with 0.5 μg/well of Stx2B overnight at 4 °C. Then, uncoupled binding sites were blocked with 0.5% bovine serum albumin (BSA) in PBS-0.05% Tween 20 (PBS-T) for 2 h at 37 °C. After washing the plates, 50 μl of serial dilution of colostral IgG were added to the wells and the plates were incubated at 37 °C for 1:30 h. Negative and positive samples were included for analysis. After washing, 50 μl of HRP-rabbit anti-bovine IgG (1:1200) in PBS-T was added to each well and plates were incubated at 37 °C for 2 h. After three washes with PBS-T the color reaction was developed using OPD (o-phenylenediamine dihydrochloride) and the optical density (OD) was read at 492 nm (OD_{492}). Reactions were stopped after 10 min with 25 μl of H_2SO_4 2 N per well. Colostral antibodies against Stx2B were expressed in OD_{492}.

Bovine lactoferrin (bLF) levels were quantified by ELISA Quantification kit (Bethyl Laboratories, USA). Colostral IgG purified from Stx2-immunized cows (HC-IgG/bLF) had approximately 22 μg/ml of bLF.

2.6. Western blot

Pure Stx2 was separated by SDS-PAGE on a 12% gel and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were then blocked with 5% nonfat dry milk in PBS for 2 h under agitation, washed three times with PBS-T, and incubated 18 h with HC-IgG/bLF or control colostral IgG (CC-IgG), both diluted 1:2000. Following three washes with PBS-T, the nitrocellulose strips were incubated for 2 h with HRP-rabbit anti-bovine IgG (1:5000). MAb 2E11 (1:2000) used as positive control was detected with HRP-goat anti-mouse IgG (1:2000). Unbound materials were washed out with PBS-T. Finally, specific bands were detected by ECL system (Amersham Pharmacia Biotech, USA).

2.7. Cell line culture

Human colon carcinoma cell line (HCT-8, ATCC-CCL-244, Manassas, VA, USA) was grown in RPMI 1640 medium with 10% fetal calf serum (FCS), 2 mM L-glutamine, 25 mM glucose, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. For growth-arrested conditions, medium without FCS was used.

2.8. Stx2 neutralization assay

For the neutralization assay, Stx2 at a concentration required to kill 50% of HCT-8 cells (1 CD_{50} = 1.2 ng/μl) was co-incubated for 1 h at 37 °C with different concentrations of HC-IgG/bLF, CC-IgG or CC-IgG supplemented with 20 μg/ml bLF (CC-IgG+bLF). These mixtures were then added on HCT-8 cells and incubated for 72 h. Cell viability was determined using the neutral red assay adapted from a previously described protocol [21]. Cells cultured without mixture represented 100% of viability. The percentage of neutralization of Stx2-cytotoxicity was calculated by the following formula: [1 - (Stx2-cytotoxicity with colostral IgG/Stx2-cytotoxicity without colostral IgG)] × 100.

2.9. Bacterial strains and growth conditions

Properties of *E. coli* strains used in this study have been previously described. Briefly, *E. coli* O157:H7 strain 125/99wt (O157: H7) was isolated from a patient with HUS. The derivative of this strain with a deletion mutation in *stx2* gene (O157:H7Δstx2) was previously obtained [22]. A commensal nonpathogenic wild-type *E. coli* strain isolated from the feces of a healthy human (commensal *E. coli*) was used as negative control. Bacterial strains were grown in Luria Broth medium (LB) for 18 h at 37 °C with shaking at 150 rpm and then diluted 1/50 in RPMI medium and grown to exponential phase (OD_{600} = 0.6–0.8) at 37 °C with shaking at 50 rpm. The culture was then centrifuged and the bacteria re-suspended at a concentration of 10^8 CFU/ml in PBS pH 7.4.

For neutralization assays, 200 μl of freshly-grown O157:H7 and O157:H7Δstx2 cultures were previously incubated with 200 μl of HC-IgG/bLF or CC-IgG for 1 h at 37 °C.

2.10. In vitro adherence assay

E. coli O157:H7 adherence to HCT-8 was assayed as previously described [23]. Briefly, HCT-8 cells were grown until 80% of confluence in 24-well plates, washed twice with sterile PBS to remove traces of antibiotic containing medium and incubated for 4 h at 37 °C and 5% CO_2 with O157:H7 (10^8 CFU/ml) cultures alone or pre-incubated with HC-IgG/bLF or CC-IgG. HCT-8 cells were then washed with PBS to remove non-attached bacteria and lysed using 0.2% Triton X-100 in PBS. Serial dilutions of this cell suspension were spread on LB plates and incubated at 37 °C for 24 h in order to determine bacterial adhesion (CFU/ml).

2.11. Measurement of net water transport in human colon

Colon fragments (n = 3) used in this study were obtained from surgeries performed on three adult cancer patients (informed consent was obtained). The Ethics Committee of the Universidad de Buenos Aires approved the use of human tissues for research purposes (Res. (CD) N° 2864/10). The intestinal tissue was removed immediately after ablation from macroscopically unaffected regions inside the "security zone" adjacent to the pathologically affected zone and the mucosa and submucosa layers were then dissected from the underlying tissue (kept at 4 °C) and placed as a diaphragm on a modified Ussing chamber as it was previously described [24]. The water absorption (Jw) was recorded automatically across the Ussing chamber connected to a special electro-optical device [25]. The sensitivity of this instrument is approximately 50 nl. Once Jw was stabilized, HC-IgG/bLF or CC-IgG, at a final concentration of 1 μg/μl, was added to the luminal side of the colonic mucosa and incubated for 30 min. After that, 200 μl of 10^8 CFU/ml of either O157: H7 or commensal *E. coli* was added to the mucosal side (time 0), and Jw was recorded for the following 1 h. Because of tissue variability, data are analyzed as ΔJw where ΔJw = Jw (at a given time) – Jw (at time 0). The results are reported as mean ± 1 standard error (SEM).

2.12. Rat colon loop experiments

A total of 18 SD rats were used in this study, fasted overnight and then anesthetized for surgery with an i.m. injection of 100 μg ketamine/g bwt and 10 μg diazepam/g bwt. Body temperature was kept at 37 °C by a lamp. The abdominal cavity was opened by a midline incision and three 2-cm long colon segments (loops) separated by 1-cm interloops were prepared in each rat. An inoculum of 200 μl of O157:H7 alone or pre-incubated with HC-IgG/bLF or CC-IgG, was injected into the lumen of the experimental colon loops and PBS into the control. For each animal, treatments were assigned randomly, one loop received PBS (Control), the second group O157:H7, and the third O157:H7 pre-incubated with either HC-IgG/bLF or CC-IgG. After inoculation, colon loops were replaced in the abdomen, and the laparotomy incision was closed. Animals were housed for 16 h under controlled conditions of light and

temperature. In selected experiments, O157:H7 was replaced by O157:H7Δstx2. Each inoculum was tested in at least 3 rats.

2.13. Measurement of fluid accumulation and histological damage in rat colon loops

Following the specified treatment period, rats were euthanized using carbon dioxide chamber. After the abdominal cavity was opened, loops were excised in the same order as inoculated, and the fluid content was measured with a 1 ml tuberculin syringe. The loops were then opened along their anti-mesenteric margins, fixed and prepared for histological studies. Briefly, pieces of the colon loops were fixed for at least 24 h in cold (4 °C) fixative PBS containing 4% formaldehyde. After fixation, tissues were carefully dehydrated, and embedded in paraffin to provide sections perpendicular to the mucosa. Sections of 5 μm were made by a microtome (Leica RM 2125, Wetzlar, Germany) and mounted on 2% silane coated slides. Sections were then stained with hematoxylin–eosin (H&E) and observed by light microscopy (Nikon Eclipse 200, NY, USA). The slides were then examined by two pathologists in a blinded fashion. A subjective quantitative scoring system used was based in the identification of histological parameters typically observed in colon loops treated with O157:H7 or O157:H7Δstx2: detachment of the surface epithelium, loss of goblet cells, necrosis, edema and inflammation. In this scoring, 0 indicates no histological damage while 1, 2, 3, 4 indicate increasingly severe damage. At least six photomicrographs fields at ×200 magnification were analyzed for each experimental condition.

2.14. Mice infection

The weaned mice were randomly divided into five experimental groups (4–6 mouse per group per experiment). After 8 h of fasting, mouse groups received intragastrically (i.g.) PBS or 1.5 mg of HC-IgG/bLF, CC-IgG, or CC-IgG+bLF, 1 h before and 2 h after bacterial inoculation. A single lethal dose of O157:H7 (2×10^{10} CFU/g bwt) or was i.g. administered between treatments [26]. Control group only received PBS. Food and water were provided to mice *ad libitum* 4 h after the ingestion of the bacteria suspension. Survival of mice was observed daily. Survival percentages corresponding to at least three experiments are shown.

2.15. Statistical analysis

Results are reported as mean ± SEM, and the significance of any differences was determined using a Mann-Whitney test and one-way analysis of variance (ANOVA). Bonferroni test was used as *a posteriori* test. For bacterial adhesion assay Tukey's multiple comparison test was used. Survival curves were compared using log-rank test. Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). Statistical significance was set at a $P < 0.05$.

3. Results

3.1. Lactoferrin levels in bovine colostrum from cows immunized with Stx2

Colostrum from pregnant cows immunized with Stx2 exhibited significant higher levels of bLF compared to colostrum from γ-Intimin C_{280}-immunized and Control cows (Table 1). The significant increase in bLF was maintained despite the purification process and it was specific for HC-IgG/bLF because it was not observed in hyperimmune colostrum from cows immunized with γ-Intimin-C_{280} obtained previously [17] (Table 1). Hyperimmune IgG anti-Intimin purified with an identical protocol as that used to purify HC-IgG/bLF and CC-IgG was mostly depleted in bLF (Table 1). These results suggest that the immunization with Stx2 may contribute to the appearance of bLF. Stx2 cytotoxicity determined on Vero cells by neutral red uptake viability assay after 72 h of incubation showed that 0.1 μg/ml Stx2 kept around 20% of activity after exposure for 10 min at 75 °C (data not shown).

Table 1
Concentration of lactoferrin in bovine colostrum samples.

Cow condition	Colostrum μg/ml	Colostral IgG μg/ml
Stx2-immunized	3314 ± 162*	22 ± 2*
γ-Intimin C_{280}-immunized	459 ± 2	0.4 ± 0.1
Non-immunized control	512 ± 24	2.2 ± 0.2

* $P < 0.001$ compared with the other conditions.

3.2. Hyperimmune colostral IgG from pregnant Stx2-immunized cows developed anti-Stx2 specific IgG antibodies with high neutralizing capacity

A significant immunological response was induced in pregnant cows immunized with Stx2. The HC-IgG/bLF showed specific IgG antibodies against Stx2B compared to CC-IgG (Fig. 1A). Anti Stx2A antibodies in HC-IgG/bLF were detected by Western blot. HC-IgG/bLF revealed a band similar in size to Stx2A recognized by mAb 2E11 (Fig. 1B). Furthermore, HC-IgG/bLF showed a high capacity to neutralize the cytotoxic effects of Stx2 on HCT-8 cells. The protective effect was observed when 1 CD_{50} of Stx2 (1.2 ng/μl) was pre-incubated for 1 h at 37 °C with serial dilutions of HC-IgG/bLF up to 1:5000 compared to CC-IgG. A significant although less neutralization was observed with CC-IgG+bLF indicating that not only Stx2-specific antibodies but also bLF contributes to neutralize the cytotoxic effects of Stx2 on HCT-8 cells (Fig. 1C).

3.3. Neutralization of O157:H7 effects in human colon and inhibition of O157:H7 and O157:H7ΔStx2 adhesion on HCT-8 cells

A standardized functional test to evaluate bacterial diarrheagenicity in human colon was used [27]. Briefly, an absorptive water flux (Jw, μl/min·cm^2) was measured across the colonic human mucosa placed between two identical Ringer solutions in the Ussing chamber. Addition of O157:H7 to the luminal side of the colonic mucosa (time 0) resulted in a significant decrease in the absorptive Jw (negative ΔJw) as it was previously described [22]. However, this inhibition was neutralized when HC-IgG/bLF (1 μg/μl), was added on the luminal side 30 min prior to O157:H7 (Fig. 2A). The pretreatment with CC-IgG (1 μg/μl) prior to O157:H7 did not show neutralization activity. The time-response was similar to that found with O157:H7 alone (Fig. 2A). Jw was not inhibited in the presence of a commensal *E. coli* used as negative control.

The bacterial adhesion on HCT-8 cells was similar when the intestinal cells were incubated for 4 h with O157:H7 compared to O157:H7Δstx2 (Fig. 2B). The degree of adhesion decreased significantly ($P < 0.001$) when both bacteria strains were pre-incubated with HC-IgG/bLF (Fig. 2B). In contrast, CC-IgG did not inhibit the bacterial adherence under the same experimental conditions.

3.4. Neutralization of O157:H7 and O157:H7Δstx2 damage in rat colon loops

Rat colon loops were used to evaluate the neutralizing effects of HC-IgG on bacterial intestinal damages (Fig. 3). The results showed that loops treated with O157:H7 were distended and filled with abundant hemorrhagic fluid (Fig. 3B) whereas loops treated with

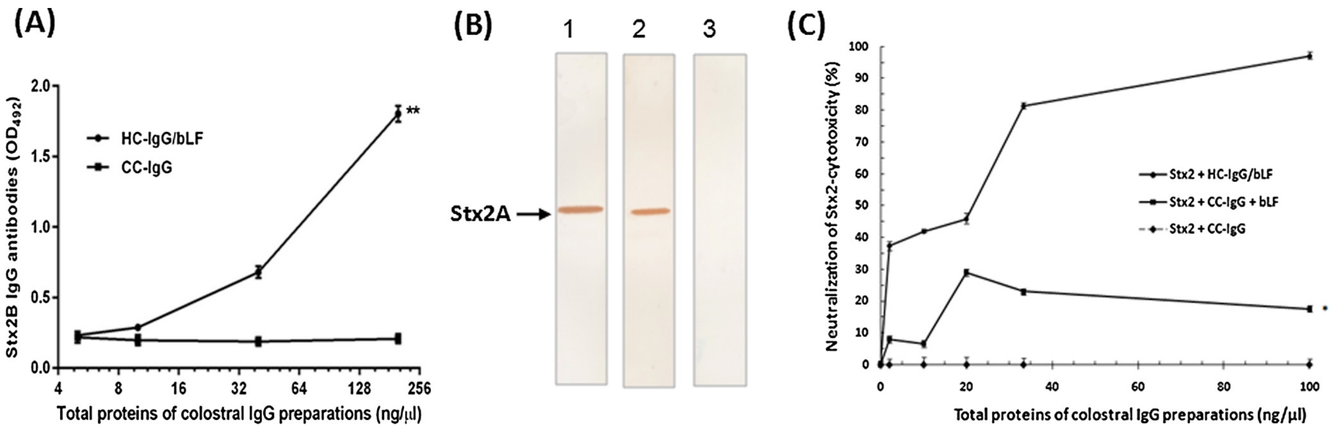

Fig. 1. Colostral IgG antibody responses to Stx2B and Stx2A and neutralization of Stx2 in HCT-8 cells. (A) Antibodies against Stx2B were determined in colostral IgG from Stx2-immunized (HC-IgG/bLF) and control (CC-IgG) cows by ELISA, as detailed in *Materials and Methods*. Results are representative of three separate experiments. **P < 0.01 *vs* CC-IgG. (B) Reactivity against Stx2A, HC-IgG/bLF (lane 1) and mAb 2E11 (lane 2), CC-IgG (lane 3). (C) Stx2 at a concentration required to kill 50% of HCT-8 cells (1.2 ng/μl) was preincubated with bovine colostral IgG preparations from immunized (HC-IgG/bLF) or control (CC-IgG) cows for 1 h at 37 °C. These cocktails were then assayed in HCT-8 cells. Viability was determined after 72 h by the incorporation of neutral red. Results are expressed as percentage of cell neutralization of Stx2-cytotoxicity. Data are shown as mean ± standard error of the mean of 3 experiments performed in sextuplicate. * P < 0.05 versus CC-IgG and HG-IgG/bLF**.** (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

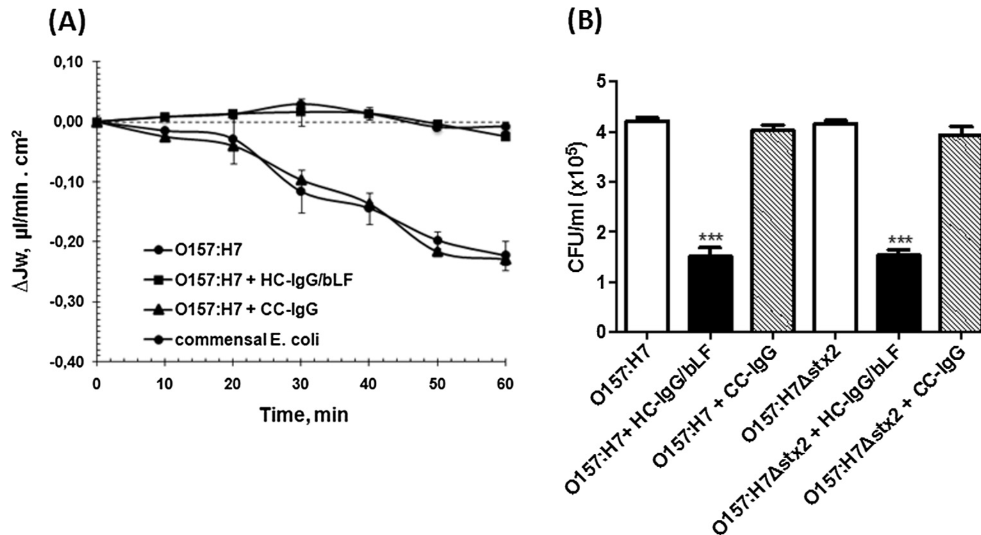

Fig. 2. Neutralization of O157:H7 effects in human colon and inhibition of O157:H7 and O157:H7ΔStx2 adhesion on HCT-8 cells. (A) Human colonic mucosa was preincubated with colostral IgG from Stx2-immunized (HC-IgG/bLF) or control (CC-IgG) cows at a final concentration of 1 μg/μl for 30 min before the addition of 200 μl of O157:H7 (10^8 CFU/ml) to the mucosal side (time 0). Tissue incubated with O157:H7 alone was used as positive control and a commensal *E. coli* was used as negative control. Because of tissue variability, data are analyzed as ΔJw where ΔJw = Jw (at a given time) – Jw (at time 0). Values are the means of 3 experiments for each time point. (B) Bacterial adhesion was determined after 4 h of HCT-8 incubation with O157:H7 or O157:H7Δstx2 alone or preincubated 1 h at 37 °C with colostral IgG from Stx2-immunized (HC-IgG/bLF) or control (CC-IgG) cows as described in Materials and Methods. Data are expressed as CFU/ml of adhered bacteria from triplicate cell wells and are the mean of at least three separate experiments. The statistical difference was expressed as the P value determined by a Tukey's Multiple Comparison (***P < 0.001).

O157:H7Δstx2 presented accumulation of luminal aqueous fluid (Fig. 3C) compared to PBS-treated loops (Fig. 3A). At the microscopic level, a notable histological damage consisted of detachment of the surface epithelium, loss of goblet cells and severe necrosis of the muscle layer was observed with O157:H7 incubation (Fig. 3F) compared with Control (Fig. 3E). After O157:H7Δstx2 treatment, a significant minor damage with moderate edema in mucosa and submucosa was detected (Fig. 3G). In contrast colon loops treated with either O157:H7 or O157:H7Δstx2 previously incubated with HC-IgG/bLF did not show fluid accumulation in the intestinal lumen (Fig. 3D) nor develop significant histological damage (Fig. 3H). Fig. 4 shows quantitative analysis of luminal fluid accumulation and histological damage of colon mucosa 16 h after treatment. Loops incubated with O157:H7 showed a significant hemorrhagic fluid accumulation compared to Control. Interestingly, loops incubated with O157:H7Δstx2 showed a significant fluid accumulation similar to O157:H7 but with aqueous appearance (Fig. 4A). In addition, histological studies showed more severe lesions in loops incubated with O157:H7 compared to O157:H7Δstx2 (Fig. 4B). Fig. 4 also shows a statistically significant prevention of luminal fluid accumulation and colonic mucosa damage when either O157:H7 or O157:H7Δstx2 were previously incubated with HC-IgG/bLF. In contrast, CC-IgG was unable to neutralize intestinal bacterial damage under similar experimental conditions (Fig. 4 A, B).

3.5. Mice protection against O157:H7 infection

HC-IgG/bLF totally protected weaned mice survival from a lethal dose of O157:H7 (Fig. 5). On the contrary, all the mice receiv-

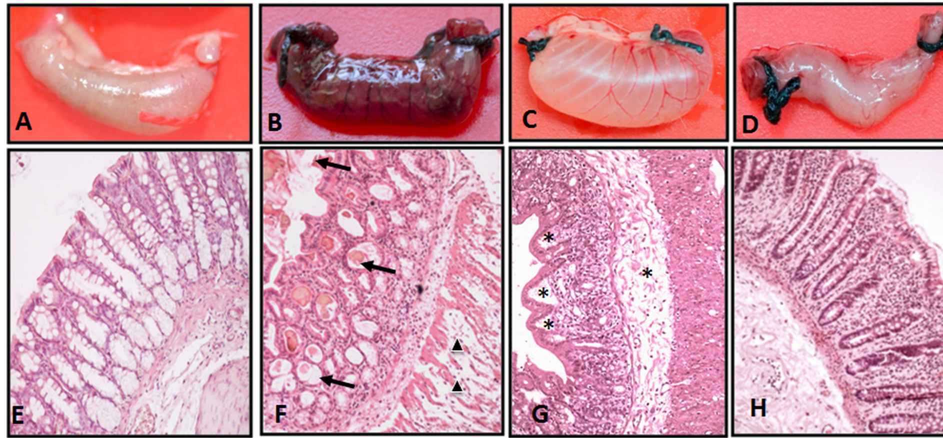

Fig. 3. Neutralization of O157:H7 and O157:H7Δstx2 damage in rat colon loops. Colon loops treated for 16 h with O157:H7 were distended and filled with abundant hemorrhagic fluid (B) and loops treated with O157:H7Δstx2 were filled with aqueous fluid (C) compared to loops treated with PBS (Control, A) or with bacterial strains previously incubated with colostral IgG from Stx2-immunized cows (HC-IgG/bLF, D). Histological examination showed detachment of the surface epithelium, loss of goblet cells (black arrows) and severe necrosis of the muscle layer (black triangle) in loops treated with O157:H7 (F). Marked edema in mucosa and submucosa (asterisks) was observed in loops treated with O157:H7Δstx2 (G) that were absent in matching Control loops (E). Loops treated with HC-IgG/bLF did not show histological alterations (H). Original magnification: ×200. Pictures are representative of three independent experiments.

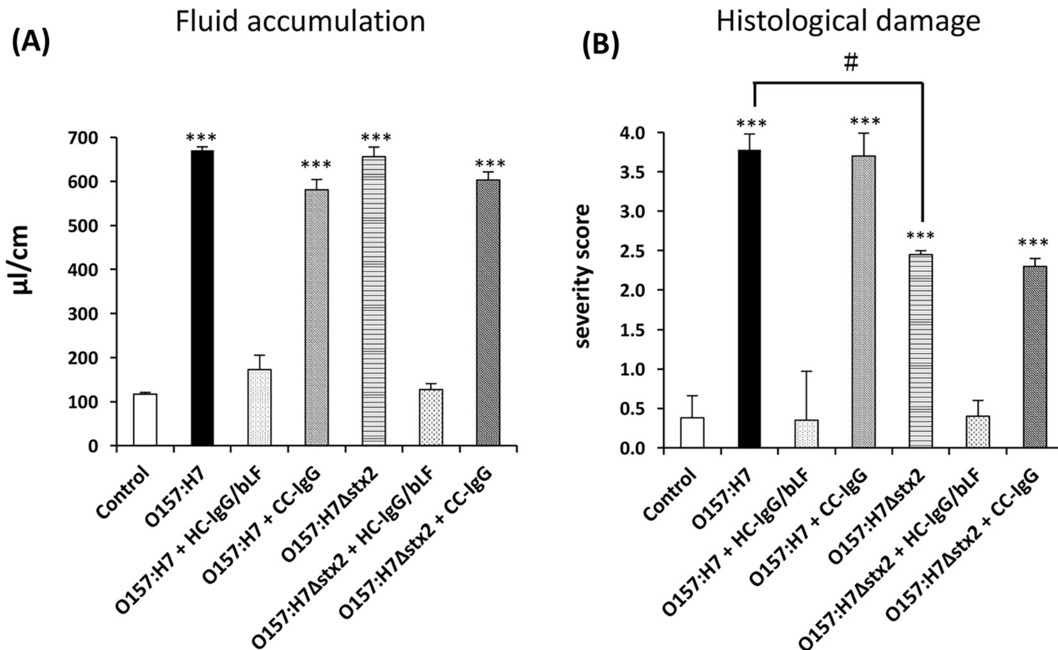

Fig. 4. Quantitative effects of colostral IgG on bacterial damage in rat colon loops. (A) Luminal fluid accumulation (µl/cm) in colon loops after 16 h of treatment with O157:H7 or O157:H7ΔStx2 alone or pre-incubated 1 h at 37 °C with colostral IgG from Stx2-immunized (HC-IgG/bLF) or control (CC-IgG) cows. (B) Histological damage severity was based on a four-point scale, with 4 representing the most severe damage (see Material and Methods). Results shown are the mean ± standard error of the mean for 3 rats. (***compared to the Control ($P < 0.001$), #O157:H7 *vs* O157:H7Δstx2 ($P < 0.01$).

ing CC-IgG died between 72 h and 96 h after O157:H7 challenge (Fig. 5) similarly to mice challenge with O157:H7 without treatment. Although supplementation of CC-IgG with bLF (20 µg/ml) significantly delayed the mortality of weaned mice compared to those treated with PBS or CC-IgG, mice finally died at 120 h suggesting that specific anti-Stx2 IgG antibodies are essential for protection. The survival rate in mice receiving only PBS without O157:H7 challenge was 100% (Control) (Fig. 5).

4. Discussion

In the present study, we have demonstrated that purified colostral IgG from pregnant cows i.m. immunized with two doses of inactivated Stx2 contains anti-Stx2 specific antibodies and bLF with high capacity to neutralize the Stx2 cytotoxic effects on HCT-8 cells. The presence of antibodies that react with and neutralize Stx2 has been previously described in colostrum from cows vaccinated with three doses of recombinant EspB and γ-Intimin C_{280}, and inactivated Stx2 which were efficiently transferred to the newborn calves by feeding with hyperimmune colostrum [17,28]. The objective of the present work was to evaluate colostral IgG from Stx2-immunized cows and characterize their properties against STEC infection and Stx2 cytotoxicity.

Diarrhea is a consequence of an imbalance of water absorption and secretion. In this regards, we have previously demonstrated that *E. coli* O157:H7 caused a significant inhibition of water absorp-

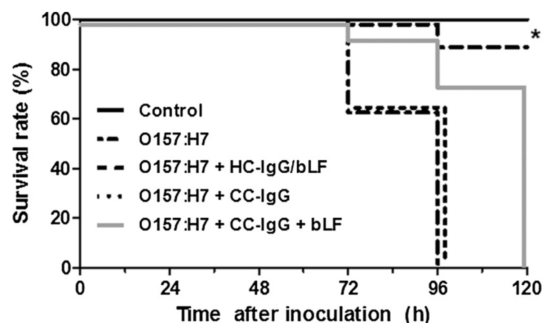

Fig. 5. Protection of mice against a lethal O157:H7 dose. Weaned mice were intragastrically (i.g.) inoculated with a lethal dose of O157:H7 (2×10^{10} CFU/g of body weight). Mice orally received 150 μl (10 μg/μl) of bovine colostral IgG from Stx2-immunized (HC-IgG/bLF), control cows (CC-IgG) or CC-IgG supplemented with 20 μg/ml bLF (CC-IgG+bLF), 1 h before and 2 h after of bacterial inoculation. PBS was orally administered to control mice. Survival of mice was then observed daily. Survival percentages corresponding to at least three experiments are shown (4–6 mouse/group/experiment). *O157:H7 *vs* experimental groups ($P < 0.05$).

tion and histological damages in the human colonic mucosa at time less than 60 min [22]. In the current study we demonstrated that HC-IgG/bLF added to the luminal side 30 min prior to *E. coli* O157:H7 infection showed an effective neutralizing capacity against the inhibition of water absorption.

Our studies have also shown that HC-IgG/bLF strongly inhibited the adherence of *E. coli* O157:H7 on HCT-8 cells and their effects in rat colon loops. Since the neutralizing capacity of HC-IgG/bLF was absent when CC-IgG was used, these findings appear to indicate that anti-Stx2 antibodies and bLF present in HC-IgG/bLF were responsible for these findings. The HC-IgG/bLF treatment also showed an inhibition of the adherence of the Stx2-negative mutant, *E. coli* O157:H7Δstx2 on HCT-8 cells and a significant neutralizing capacity on rat colon loops. The reason for this result may be that HC-IgG/bLF contains additional substances apart of anti-Stx2 antibodies able to prevent *E. coli* O157:H7 pathological effects. It is well established that bLF, an iron-binding glycoprotein abundant in bovine colostrum and milk, has antimicrobial, antiinflammatory, and immunomodulatory functions [29]. Exhaustive evidence exists regarding bLF involved in a protecting role during EPEC/STEC infections. Vilte et al. [30] reported that bLF can neutralize the red blood cells lysis caused by an EPEC strain. Yekta et al. [31] demonstrated that bLF can reduce the attachment of a non-toxin producing *E. coli* O157:H7 strain to Caco-2. Kieckens et al. [32] reported a clearance of O157:H7 infection in calves by rectal administration of bLF and recently have shown that this protein affects the release of Stx2 from STEC and causes degradation [33]. We have found increased levels of bLF in hyperimmune calostrum from Stx2-immunized cows, probably due to the development of an inflammation process. Pregnant cows were immunized with Stx2 which had been partially inactivated by exposure for 10 min at 75 °C. It is well-established that Stx2 is relatively heat resistant and that B subunit has toxin activity. Rasooly R and Do PM [34] reported that thermal treatment at 100 °C for 5 min completely inactivated Stx2 but at lower temperatures failed to inactivate the toxin. Furthermore, we and others have reported that Stx2B produced apoptosis [35–38]. It is very likely that Stx2 triggers inflammation in cows after immunization leading to the appearance of bLF in colostrum. The high bLF levels could contribute to the efficacy of HC-IgG/bLF to prevent Stx2 cytotoxicity. Additional experiments using CC-IgG supplemented with bLF at the same level as that found in the HC-IgG/bLF preparations demonstrated that bLF partially neutralized the Stx2 cytotoxicity *in vitro*. However, anti-Stx2 antibodies were necessary to completely neutralize Stx2 cytotoxicity. The effectiveness of HC-IgG/bLF against the entire bacteria was confirmed in the *in vivo* weaned mice model after oral infection with *E. coli* O157:H7 [39]. Treatment with HC-IgG/bLF exhibited a full mice protection against a lethal dose of *E. coli* O157:H7. Although CC-IgG supplemented with bLF delayed mortality of weaned mice compared to mice treated with CC-IgG, these finally died. In consequence, we propose that specific IgG anti-Stx2 antibodies together with other bioactive components, as bLF, could be responsible for the protection. In line with these findings, Kuribayashi et al. [40] reported that treatment with hyperimmune colostrum against Stx1 or Stx2, increases mice survival rates from a lethal challenge with *E. coli* O157:H7. However these authors did not discuss if other bioactive compounds could contribute to this protection.

In conclusion, our studies suggest that bovine colostral IgG from pregnant cows immunized with Stx2 could have protective effects against *E. coli* O157:H7 infection. Our results lead us to investigate if HC-IgG/bLF could be an important tool to be used in combination with balanced supportive diets to prevent HUS. However further studies are required before recommendations can be made for therapeutic and clinical applications.

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Author Contributions

Conceived and designed the experiments: A.A., F.S., E.A.S., D.A. V., G.F., R.F.B.

Performed the experiments: A.A., F.S., E.A.S., D.A.V., G.F., R.F.B.

Analyzed the data: A.A., F.S., D.A.V., M.S.P., A.C., C.I.

Contributed reagents/materials/analysis tools: D.A.V., M.S.P., M. M.A., A.C. E.Z., C.I.

Wrote the paper: F.S., M.S.P., M.M.A., E.Z., E.C.M., A.C., C.I.

Conflicts of Interest and Funding Sources

The authors declare no conflict of interest.

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