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ORIGINAL ARTICLE

Oral immunization with attenuated *Salmonella* vaccine expressing *Escherichia coli* O157:H7 intimin gamma triggers both systemic and mucosal humoral immunity in mice

Aline Ferreira Oliveira[1,2,3], Silvia Almeida Cardoso[2,3], Fausto Bruno dos Reis Almeida[2], Leandro Licursi de Oliveira[4], André Pitondo-Silva[5], Sandro Gomes Soares[3] and Ebert Seixas Hanna[2,3]

[1]Department of Genetics, Evolution and Bioagents, Institute of Biology, Rua Charles Darwin s/n, Campinas State University, Campinas, SP 13083-862, [2]Departament of Molecular and Cellular Biology, and Pathogenic Bioagents, Faculty of Medicine in Ribeirão Preto, University of São Paulo, Av. Bandeirantes 3900, Ribeirão Preto, SP 14049-900, [3]Invent Biotecnologia Ltd., Business Incubator Supera, Rua dos Técnicos s/n, Ribeirão Preto, SP 14040–900, [4]Department of General Biology, Federal University of Viçosa, Av. PH Rolfs, s/n, Viçosa, Minas Gerais, CEP 36570–000, and [5]Department of Clinical Chemistry, Toxicology and Bromatology, Faculty of Pharmaceutical Sciences in Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, 14040–903, Brazil

ABSTRACT

Human infections with EHEC such as O157:H7 have been a great concern for worldwide food-industry surveillance. This pathogen is commonly associated with bloody diarrhea that can evolve to the life-threatening hemolytic uremic syndrome. Animals are the natural reservoir where this pathogen remains asymptomatically, in steps of ingestion and colonization of the bowel. The bacterium is shed in the feces, contaminating the surroundings, including water and food that are directed for human consumption. A major player in this colonization process is intimin, an outer membrane adhesion molecule encoded by the *E. coli* attachment and effacement (*eae*) gene that has been shown to be essential for intimate bacterial attachment to eukaryotic host cells. In an attempt to reduce the colonization of animal reservoirs with EHEC O157:H7, we designed a vaccine model to induce an immune response against intimin gamma. The model is based on its recombinant expression in attenuated *Salmonella*, used as a suitable vaccine vector because of its recognized ability to deliver recombinant antigens and to elicit all forms of immunity: mucosal, systemic, and humoral responses. To test this model, mice were orally immunized with a *S. enterica* serovar Typhimurium strain carrying the pYA3137*eaeA* vector, and challenged with *E. coli* O157:H7. Here we show that immunization induced the production of high levels of specific IgG and IgA antibodies and promoted reduction in the fecal shedding of EHEC after challenge. The live recombinant vaccine reported herein may contribute to the efforts of reducing animal intestinal mucosa colonization.

Key words *Escherichia coli*, *Salmonella enterica* Typhimurium, Vaccines.

Enterohemorrhagic *Escherichia coli* (EHEC), a Gram-negative bacillus, is able to colonize the large intestine, living as part of the bowel microbiota of animals, mainly in cattle. However, some EHEC strains, such as O157:H7, are major food-borne infectious pathogens that cause diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome in humans (1). The transmission occurs when the bacteria are shed in the feces of infected animals, directly contaminating animal handlers or the food and water used for human consumption (2).

Correspondence
Ebert Seixas Hanna, Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Ribeirão Preto, SP 14049-900, Brazil.
Tel: +55 16 3602 3066; fax: +55 16 3633 1786; email: itahanna@usp.br

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List of Abbreviations: CFU, colony-forming unit; DNA, deoxyribonucleic acid; EHEC, Enterohemorrhagic *Escherichia coli*; kDa, kiloDalton; O.D., optical density; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TMB, tetramethylbenzidine and SEM, standard error of the mean.

Due to the increasing global incidence of EHEC human infections, since the early 1990s, associated with the severity of the disease, and the problematic treatment using conventional antibiotics (3,4), considerable interest has been raised in this pathogen (5). Many studies, with different animal models, have been developed and they are encouraging investigators to design strategies to prevent EHEC infections by reducing intestinal colonization of the animals by these bacteria (6–11).

Escherichia coli O157:H7 strain produces intimin gamma, an outer membrane protein encoded by the *eae* (*E. coli* attach and efface) gene. It corresponds to an adhesion molecule, essential for bacterial attaching to mammalian cells and for colonization of the intestinal mucosa in newborn piglets, calves, adult cattle, as well as in humans (12, 13). The carboxy-terminal portion of intimin mediates the intimate attachment of the bacteria to the eucaryotic cell surface, through the binding of the bacterium-encoded translocated intimin receptor (Tir) and the nucleolin, a host cell receptor (14–16).

There is evidence that intimin gamma *per se* was not capable of stimulating an effective immune response so as to reduce the enteric colonization of *E. coli* O157:H7 (17). On the other hand, earlier studies have shown that patients infected with EHEC exhibited high titers of anti-intimin antibodies (18, 19). Moreover, the expression of intimin gamma by live attenuated bacteria, particularly *Salmonella*, has been associated with the development of an effective and specific immunity against the natural source of this antigen (10, 11). Several studies have reported the feasibility of using attenuated *Salmonella* strains as live vectors for the oral delivery of recombinant vaccine antigens (20–25). These strains, submitted to attenuation procedures, lost their pathogenicity but remained invasive and are able to induce protective mucosal, humoral, and systemic immune responses against heterologous antigens, in a variety of animal models (20–28). Besides that, oral vaccines are easy to administer, safe, adequate for large-scale immunization, and stable without refrigeration (if lyophilized) (26). Nevertheless, the *Salmonella*-based vaccines are generally promises, and most work remains unaccomplished, partly because of the dilemma of establishing that they are safe for vaccine development.

This article offers further evidence that the use of *Salmonella* could be a prototype of vaccine for controlling EHEC infections. In this sense, the aim of the present study was to use a live attenuated *S. enterica* serovar Typhimurium strain, for expressing the EHEC intimin gamma antigen, and evaluate its capacity to: (a) induce a consistent systemic, mucosal, and humoral immunity; (b) reduce intestinal mucosa colonization in mice model.

MATERIAL AND METHODS

Animals

All animals used in this study were 4–6-week-old female Balb/c mice, which were housed under specific-pathogen-free conditions of the Animal Research Facilities in the Medical School of Ribeirão Preto-USP. This project has been approved by the Ethics in Animal Experimentation Committee of the Faculty of Medicine in Ribeirão Preto, University of São Paulo, with the protocol number 020/2008, in accordance to the Ethical Principles in Animal Experimentation of the "Brazilian College for Animal Experimentation."

Bacterial strains, cultures, and plasmids

Throughout this work, the *Salmonella enterica* serovar Typhimurium χ3987 strain was denominated *S.* Typhimurium.

All the strains carrying loss-of-function mutations in *asd* (Δ*asd*) and the pYA3137 plasmid were kindly provided by Dr Roy Curtiss 3rd (Center for Infectious Diseases and Vaccinology, The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, USA), except the H683 strain, which was kindly supplied by Dr David M. Hone (Aeras Global TB Vaccine Foundation, Rockville, MD 20850, USA). The virulent *E. coli* O157:H7 strain was kindly donated by Dr Alfredo Caprioli (Istituto Superiori di Sanità, Rome, Italy).

Bacterial strains were grown in 10 mL Luria Broth (LB) medium (Oxoid, Hampshire, UK), in a rotary shaker at 250 rpm, 37°C. To prepare the vaccine strains for mice inoculation, 700 μL of overnight cultures of *S.* Typhimurium strains were inoculated in 20 mL of LB medium and incubated in shaker (200 rpm) at 37°C. The culture was monitored until it reached the OD_{600} around 0.7 and pelleted by centrifugation (3000 *g*; 15 min). The pellet was then resuspended in 4 mL of PBS and serial diluted for bacterial counting on MacConkey Agar (Oxoid), so as to determine a correlation between the OD and CFU/mL. For immunization, *S.* Typhimurium suspensions were prepared to a final cell density of 1–5 $\times$ 10^{10} CFU/mL. The number of inoculated bacteria was confirmed by CFU counts from the injection suspension (29).

For the challenge, the *E. coli* O157:H7 strain cultures were monitored until the OD_{600} of 0.5 was reached. Bacterial cultures were then washed twice in PBS and all mice were orally challenged with 10^8 CFU of *E. coli* O157:H7, by gavage needle.

Plasmid minipreparations were performed using the GeneJET Plasmid Miniprep kit (Fermentas, Burlington, ON, Canada), and plasmid midipreparations were

performed using the Eppendorf plasmid purification kit (Eppendorf, Hamburg, Germany).

Salmonella Intimim Gamma Recombinant Strains

Escherichia coli χ6212 Δ*asd* strain (30) has been used in cloning experiments involving the pYA3137 plasmid (20, 31). The *S.* Typhimurium Δ*cya* Δ*crp* Δ*asd* (23, 32) and H683 Δ*aro* Δ*asd* (31) attenuated strains were used here for expression of the intimin gamma antigen. This was accomplished by cloning the *eaeA* gene into the pYA3137 plasmid.

To this end, a DNA fragment corresponding to the 2805-bp *eaeA* gene for intimin gamma was amplified by PCR using the *E. coli* O157:H7 genomic DNA as a template. The primers INTG1 (5′-GCGGCGGATC-CATGATTACTCATGGTTGTTATACC-3′) and INTG2 (5′-GCGGCAAGCTTTTATTCTACACAAACCGCATAG-3′) were used to create the *Bam*HI and *Hind*III sites flanking this gene. Amplification was reached using 30 cycles at the following temperatures: 94 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 2 min. Finally, the reaction was maintained at 72 °C for 10 min. The PCR product was then cloned into the same sites of the pYA3137 plasmid. The presence of mutations in the *eaeA* gene was ruled out by sequencing the entire gene.

Next, the pYA3137 and pYA3137*eaeA* plasmids were transferred to the *S.* Typhimurium strain through a transductional method using the bacteriophage P22 HT (33).

To determine the specific growth rate, one isolated colony on LB agar of *S.* Typhimurium pYA3137 and pYA3137*eaeA* was inoculated in 50 mL of LB medium and cultured in a rotary shaker (250 rpm) at 37°C. Samples were taken and analyzed to generate growth curves. Viable counts were performed by spreading 100 μL of the diluted cell culture onto MacConkey agar plates. Counts were recorded as the number of CFU/mL. Absorbance measurements were made at 600 nm using a spectrophotometer (Ultrospec 3000 pro, Amersham Pharmacia Biotech, Cambridge, United Kingdom). All growth determinations were performed in triplicate.

SDS-PAGE and Western blotting analysis

Bacterial extracts were analyzed by SDS-PAGE using 12% polyacrylamide gels. Gels were stained with Coomassie blue or electroblotted onto nitrocellulose membranes for immunological analysis with rabbit anti-intimin polyvalent antibodies, kindly provided by Dr Antônio Fernando Pestana de Castro (Instituto de Ciências Biomédicas, Universidade de São Paulo, Brazil), at the dilution of 1:2000. Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO, USA) was used for detection. Western blotting conditions were conducted as previously described (34).

Mice immunization and sampling

Groups of five BALB/c mice were orally immunized by gavage needle, on days 0 and 14, with the *S.* Typhimurium strain carrying the pYA3137*eaeA* vector or the pYA3137 empty vector. Each inoculum consisted of a volume of 200 μL of a PBS suspension containing 1×10^9 CFU. Another group of mice were inoculated with 200 μL of the PBS vehicle only. Thus, during all this work, mice that had received the intimin-producing *S.* Typhimurium strain were denoted as immunized, while the mice inoculated with the empty vector-carrying *S.* Typhimurium strain or with PBS were denoted as strain control or vehicle control mice, respectively.

Fecal samples were collected in different days after immunization and monitored for the presence of *Salmonella* strains, as previously described (6), with slight modifications. For this, fresh fecal pellets were obtained from each animal and transferred to pre-weighed tubes. Feces were homogenized in PBS, serially diluted, and plated onto MacConkey agar. The plates were incubated overnight, at 37°C, for bacterial counting. Isolates suspected to be *Salmonella* were tested for agglutination with anti-*Salmonella* somatic antigen, as per the manufacturer's instructions (Probac, São Paulo, SP, Brazil).

The assay of recovery of *Salmonella* vaccine strains from lymphoid organs was performed (eight 7-week-old female BALB/c mice, inoculated orally as described above) on day 10 following oral inoculation with the χ3987-pYA3137*eae*A or the χ3987-pYA3137 control strain. For that, groups of four mice were killed, and spleens and Peyer's patches were removed. Tissues were homogenized in PBS, and aliquots of 100 μL were plated onto MacConkey agar. Plates were incubated at 37°C for 18 h, for bacterial counting.

Detection of specific antibodies

Fecal and blood samples were collected from five mice of each immunized, strain control, and vehicle control mouse groups on days 7, 14, 21, and 28 after the second immunization, and assessed for the detection of IgA and IgG specific antibodies, respectively, by ELISA. Fecal samples were obtained as previously described (6), with slight modifications. Briefly, fresh fecal pellets were collected from each animal, transferred to pre-weighed tubes, and then homogenized in PBS, pH 7.2. The resuspended fecal material was centrifuged for 5 min, and each supernatant was then transferred to a clean tube and stored at –20°C until assayed. To collect the blood samples, mice were bled by the retro-orbital sinus (29). Briefly, polystyrene 96-well flat-bottom microtiter plates

(Greiner-GmbH, Kremsmunster, Austria) were coated with 100 ng/well purified recombinant intimin-gamma (courtesy of Alice O'Brien, Uniformed Services University of the Health Sciences, Bethesda, USA), dissolved (100 μL/well) in 0.06 M sodium carbonate-bicarbonate coating buffer (pH 9.6). The coated plates were incubated at 4 °C overnight. Free binding sites were blocked with blocking buffer (PBS [pH 7.4], 0.1% Tween 20, and 1% BSA). Individual feces and serum samples from immunized mice were diluted at 1:20 in PBS-1% gelatin, added in duplicate (100 μL/well), and plates were incubated at 37 °C for 2 h. Goat anti-mouse IgG or rabbit anti-mouse IgA antibodies, conjugated with horseradish-peroxidase (Sigma), were then added at 1:1,000, and the plates were incubated for 1 h at 37 °C. Color was allowed to develop for 15 min at 37°C with 3,3',5,5'-tetramethylbenzidine substrate (TMB) prepared according to the manufacturer's instructions (Pierce Chemical, Rockford, IL, USA). The reaction was stopped with 2 M H_2SO_4 before readings were taken at 450 nm in a Microplate Scanning Spectrophotometer (PowerWave X, Bio-Tek Instruments, Winooski, VT, USA).

E. *coli* O157:H7 colonization challenge

The groups of five mice described above were orally inoculated, by gavage needle, with an inoculum of 10^8 CFU of *E. coli* O157:H7 14 days after the last immunization. Next, fecal pellets were collected, on different days following the challenge from each individual mouse, and weighed. Serial dilutions of fecal pellet homogenates were prepared in PBS, as described previously (6). These homogenates were plated on MacConkey agar with 100 μg of streptomycin per mL to determine the CFU per gram of feces.

Statistical analysis

Statistical analysis was performed using analysis of variance followed by the parametric Tukey-Kramer test (INSTAT software, GraphPad, San Diego, CA, USA). Results are presented as the mean and SD. A P-value < 0.05 was considered statistically significant.

RESULTS

Cloning of intimin gamma eaeA gene into the pYA3137 vector

The *eaeA* gene was PCR-amplified from the genomic DNA of the EHEC O157:H7 strain and cloned into the pYA3137 plasmid. Figure 1a illustrates the structure of the resulting plasmid, pYA3137*eaeA*. The success of the cloning process is demonstrated by endonuclease digestions of the recombinant vector, and the observation that a band, correspondent to the amplified *eaeA* gene, is visualized in the agarose gel (Fig. 1b). This vector displays the *asd* gene

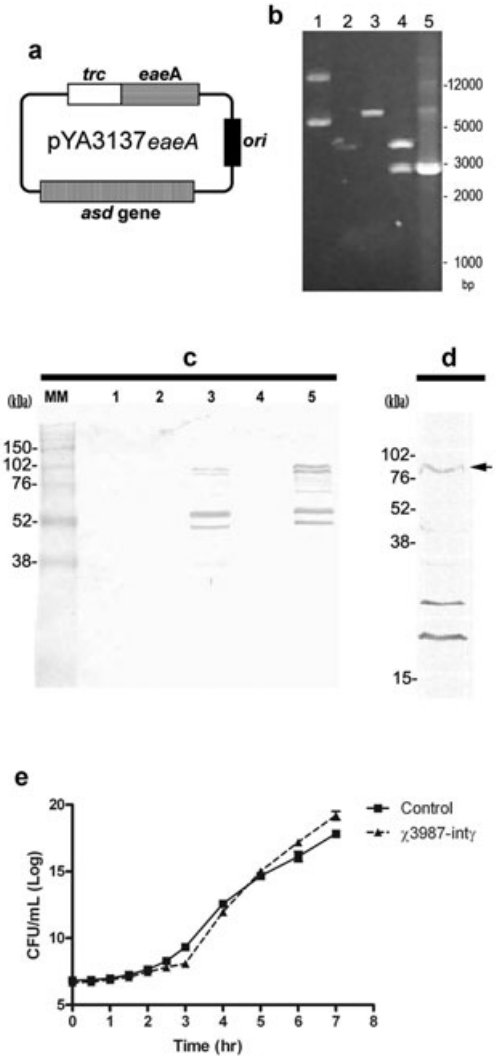

Fig. 1. Construction of the attenuated *Salmonella* vaccine. The gene *eaeA* from EHEC O157:H7 strain, encoding for intimin gamma, was cloned into the vector pYA3137, and *Salmonella* Δ*asd* strains were transformed with this recombinant vector. (a) diagram of the recombinant vector, containing the 2.8 kb fragment of the *eaeA* gene; (b) agarose gel electrophoresis (1%) of the control and recombinant vectors. Non-linearized pYA3137 control vector (b1); pYA3137 control vector linearized with *Bam*H1 and *Hind*III (b2); non-linearized pYA3137*eaeA* vector (b3); pYA3137*eaeA* vector linearized with *Bam*H1 and *Hind*III, releasing the 2.8 kb fragment of intimin gamma (b4); and the *eaeA* gene PCR-amplified from EHEC (b5). (c,d) Western blot of the crude extracts *Salmonella* vaccine strains, cultivated in LB medium (c) or in M9 minimal medium (d). Primary antibody was the polyvalent serum anti-intimin. Pre-stained molecular weight standard (MM); EHEC *strain* O157:H7 (c1 and d); *Salmonella* strain H683 control, carrying the pYA3137 control vector (c2); strain H683, carrying the pYA3137*eaeA* vector (c3); *Salmonella* strain χ3987, carrying the pYA3137 control vector (c4); and strain χ3987, carrying the pYA3137*eaeA* vector (c5). (e) Bacterial growth was monitored by optical density at 600 nm (OD 600) and the determination of the number of CFU at the time points indicated. The number of viable recovered colonies was similar between the control (-■-) and the χ3987 pYA3137*eaeA* (-▲-) strains.

and integrates the lethal balanced system when introduced in Δ*asd Salmonella* strains.

Intimin gamma expression by the Salmonella vaccine strains

The recombinant plasmid was introduced into two Δ*asd Salmonella* strains, H683 and χ3987. Western blotting reaction, using rabbit antibodies anti-intimin gamma, showed that both strains, carrying the pYA3137*eaeA* vector, were capable of expressing the recombinant protein (Fig. 1c). The blotting membrane showed a typical profile of intimin gamma recombinant expression, with multiple bands that ranged from 97 kDa to lower molecular weight byproducts of degradation (Fig. 1c, lanes A3 and A5). The χ3987 transformed strain was chosen because of the previous experience of our group with it. These recombinant strains were later used to deliver intimin gamma, the chosen EHEC virulence factor, to the optimal site of infection.

Intimin gamma expression by *E. coli* O157:H7

It was previously described that intimin gamma production is very low or not detected when EHEC O157:H7 is grown in conventional media such as LB (35) (Fig. 1c, lane A1). However intimin gamma production is detectable when the EHEC strain is grown in minimal media, as we verified with DMEM cultivation (Fig. 1d). Indeed, among all the fractions of the crude extract, the typical 97-kDa band was revealed by the anti-intimin antibody. It is important to highlight that lower molecular weight bands or multiple bands are typical for the natural or recombinant expression analyses, respectively.

Growth *in vitro* of the S. Typhimurium strains

The growth in LB broth of the *S.* Typhimurium pYA3137*eaeA* mutant was compared with that transformed with the empty vector, namely *S.* Typhimurium pYA3137 (control). Note that expression of intimin gamma did not substantially affect the growing of the bacterial cell, as measured hourly by viable count (Fig. 1e). The specific growth rates of the control and of the intimin gamma-expressing *Salmonella* were 2.243 ± 0.17 and 3.039 ± 0.13, respectively.

Detection of anti-intimin antibodies after oral immunization with the Salmonella vaccine strain

Antibody production against intimin gamma in the sera and feces of all mice was evaluated on different days after the second immunization. Serum IgG response to intimin gamma was significantly greater by immunized

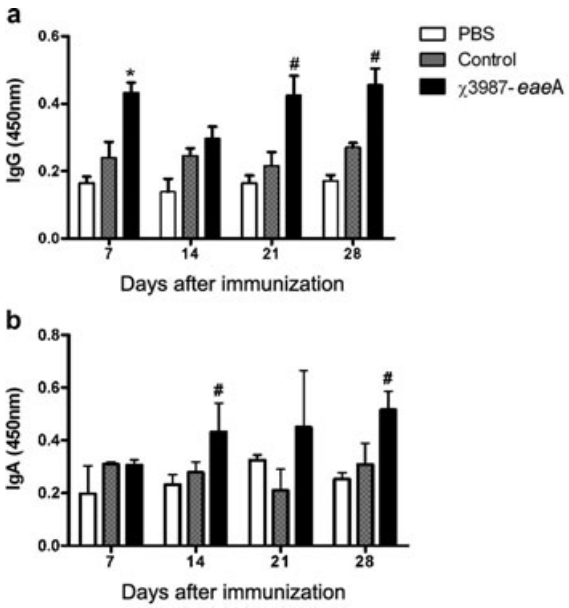

Fig. 2. IgG and IgA antibody responses. Groups of BALB/c mice were orally immunized on days 0 and 14 with *Salmonella enterica* χ3987-pYA3137*eaeA*, or inoculated with either *S. enterica* χ3987-pYA3137 or PBS. On days 7, 14, 21, and 28 after the second immunization, sera and feces were collected for measurement of specific IgG (a) and IgA (b) antibodies by ELISA. Results are expressed as the mean of OD450 nm values of five mice per group (immunized, vector control, and vehicle control) and are a representative experiment of two assays. **P* value < 0.05, in relation to the two control groups, and #*P* value < 0.05, in relation to the vehicle control group PBS.

mice than that observed for the other two control groups of mice. The highest production of antigen-specific IgG in immunized mice was detected on days 7, 21, and 28 post-immunization, when the obtained ELISA absorbance values were two orders of magnitude higher than those detected by the strain control or vehicle control (PBS) mice (Fig. 2a).

A mucosal IgA response was also detectable in the fecal extracts from immunized mice. The IgA-specific levels produced by these mice were higher than those verified in the feces of strain control mice mainly on days 14 and 28 following the second immunization (Fig. 2b). Together, these results indicate that oral immunization with live attenuated *Salmonella* expressing intimin gamma was able to trigger both systemic and mucosal humoral immunity.

Host tissue colonization by transformed strains of S. Typhimurium

We next evaluated the colonization and persistence of the *S.* Typhimurium strains in the mouse tissues by recovering bacterial colonies from the spleen, Peyer's patches,

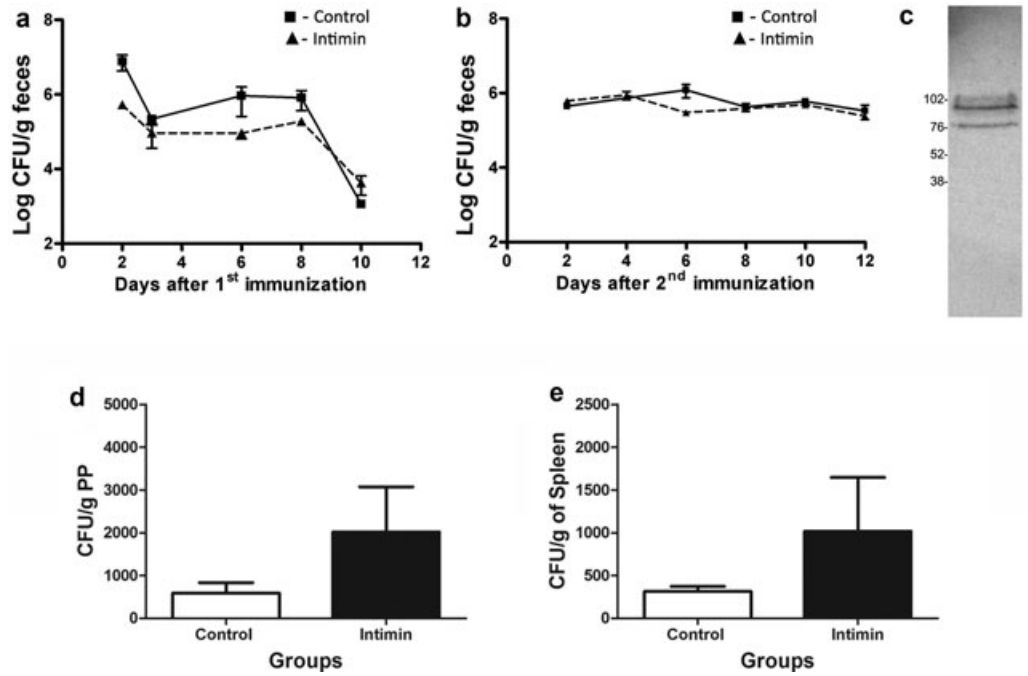

Fig. 3. Colonization and persistence of the *Salmonella* strains in the mouse tissues and lymphoid organs. BALB/c mice were inoculated orally with 10^9 CFU of *Salmonella enterica* χ3987-pYA3137*eaeA* (-▲-) or *S. enterica* χ3987-pYA3137 (-■-) and their feces were monitored for the presence of the organisms over a period of 10 days following the first immunization (a) and 12 days following the second immunization (b). The mean values ± SD of five mice per group (immunized, vector control, and vehicle control) are shown. Some of the recovered colonies were incubated with anti-*Salmonella* polyvalent antiserum, and the intimin expression was confirmed by Western blot, using an anti-intimin antibody (c). Colonization of Peyer's patches (d) and spleen (e) 10 days following oral inoculation with the control χ3987-pYA3137 (empty bars), or χ3987-pYA3137*eaeA* (black bars) *Salmonella* vaccine strains. Results of Peyer's patches (PP) and spleen colonization are shown as CFU recovery per gram of organ (CFU/g), and represent the mean values ± SD of four mice per group.

and feces of immunized and strain control mice. Following oral inoculation with *S.* Typhimurium carrying the pYA3137 empty vector or *S.* Typhimurium carrying the pYA3137*eaeA* recombinant vector, no significant difference was observed in the number of bacteria recovered from both groups of animals during the whole period analyzed (Fig. 3). Figure 3a shows that the number of CFU recovered from the fecal extracts decreased from 2 to 10 days following the first immunization. However, after the second immunization, CFU number was maintained during the whole time period studied (Fig. 3b). On day 10 after oral inoculation, the vaccine strains could also be detected on the Peyer's patches (Fig. 3d), and spleen (Fig. 3e), indicating that the vectorized *Salmonella* is presenting the recombinant intimin gamma in the context of these organs, and it is possibly triggering an immune response against this antigen.

Some colonies were tested for agglutination with anti-*Salmonella* polyvalent antiserum (data not shown) and for reaction with the anti-intimin antibody in Western blot analysis (Fig. 3c). These results indicate that we could track the fecal shedding of the recombinant strains after the oral immunization, and that the *in vivo* trafficking did not affect the stability of the recombinant vector.

Fecal shedding following challenge with *E. coli* O157:H7

Next, we investigated whether the immune response triggered by immunization with *Salmonella* expressing intimin gamma was able to prevent mouse intestinal mucosa colonization with *E. coli* O157:H7. To this end, immunized and strain control mice were orally challenged with 10^8 CFU/mL of *E. coli*, 2 weeks after the last immunization. The feces of all mice were collected on different days after challenge and monitored subsequently for the presence of *E. coli* O157:H7 by counting the number of CFUs. Compared to the strain and vehicle control groups, immunized mice exhibited lower mean fecal loads of *E. coli* O157:H7 during the whole time period studied (Fig. 4). The highest difference in fecal shedding was observed between the immunized and the vehicle control groups, on day 5 after challenge. Nevertheless, the fecal shedding from immunized mice was always lower than that verified in both

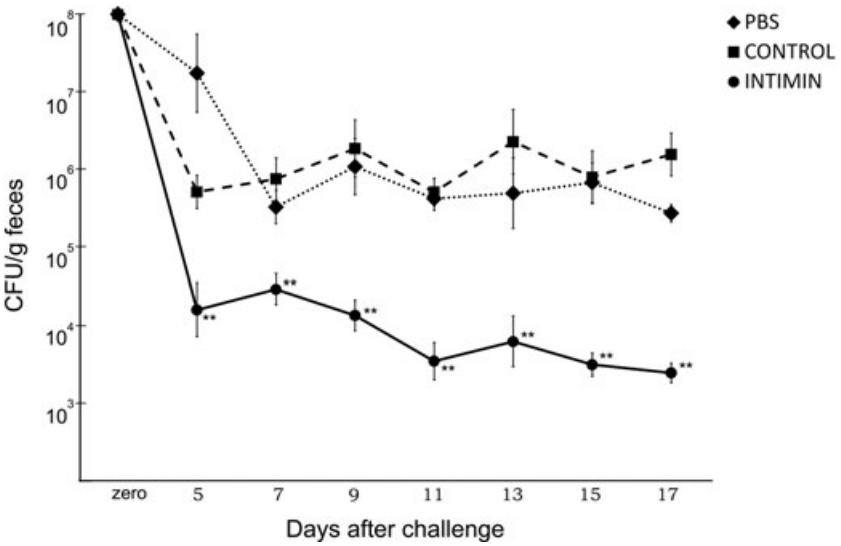

Fig. 4. Fecal shedding of *Escherichia coli* O157:H7 by immunized mice. BALB/c mice were orally immunized with *Salmonella enterica* χ3987-pYA3137*eaeA* (●), or inoculated with either *S. enterica* χ3987-pYA3137 (-■-) or PBS (-◆-), as controls, on days 0 and 14. All mice were orally challenged with 10^8 CFU of *E. coli* O157:H7 14 days after the last immunization, and their feces were monitored for the presence of the *E. coli*. Results are expressed as the mean CFU ± SD per gram of feces for each group of five mice. Similar results were obtained in two independent experiments. ***P* value < 0.05.

vector and vehicle control groups (Fig. 4), which was associated with a higher production of IgA and IgG specific antibodies by these immunized mice.

DISCUSSION

In the present study we have demonstrated the expression and delivery of intimin gamma by an attenuated *S.* Typhimurium strain and its effects in the immune response against *E. coli* O157:H7 colonization in mice.

Enterohemorrhagic *E. coli* infections in humans are commonly acquired via contact with ruminant feces. There are several methods being explored to reduce the bovine carriage rate of *E. coli* O157:H7 (36, 37) and vaccination is one of them (38). However, the few trials using EHEC vaccines presented variable degrees of protection (38–40).

Previous studies have already demonstrated the immunogenic properties of intimin (6, 19, 34). In the present work we have cloned the intimin encoding gene *eaeA* into the pYA3137 vector. This vector encodes the *asd* gene and is capable of integrating the lethal balanced system, allowing a high and stable expression of the heterologous protein (23). The attenuated Δ*asd S.* Typhimurium strain, transformed with the pYA3137*eaeA* vector, was able to express the recombinant protein, which was not toxic and did not affect the growth of the bacterial cell, since both control and intimin-expressing *Salmonella* strains presented similar growth rates.

The vaccine model design started from the notion that live attenuated *Salmonella* is a desirable vaccine vector because of its recognized ability to deliver recombinant antigens and to elicit various types of immune responses (20, 21, 24, 30, 41). Indeed, the results reported herein reveal that mouse immunization with *S.* Typhimurium expressing intimin gamma causes a strong systemic, humoral, and mucosal immunity. These responses to vaccination have been featured by detection of high and persistent levels of serum IgG and fecal IgA antigen-specific, respectively. The occurrence of gastrointestinal immunity represents an important effect of the immunization procedure, because secretory immunoglobulins (IgA) constitute the frontline defense against microorganisms that attach and colonize the host mucosal surfaces, as is the case of *E. coli* O157:H7 infection.

Previous studies reported that the immunization of animals with *Salmonella*-expressing EHEC antigen(s) resulted in a strong humoral response, as well as in the reduction of EHEC colonization (10, 11). Both studies have used the intimin gamma as deliverable for the intestinal mucosa. In our work, we have used a distinct strain of *Salmonella* vaccine, expressing solely the whole intimin gamma, but the results of protection were somewhat comparable. In this context, our results have contributed to the notion that *Salmonella* vaccines are effective, safe, easier to scale-up and cheaper to produce, since it is not necessary to undertake the downstream steps of purification of recombinant proteins (42).

It is known that some recombinant *Salmonella* strains can lose their colonization capacity or can weakly colonize host tissues (43). On the other hand, oral immunization with *S.* Typhimurium expressing heterologous antigens has been associated with long term bacterial colonization of the host Peyer's patches, mesenteric lymph nodes, and spleen (21, 29, 44). In the present study, *S.* Typhimurium transformed with either pYA3137 or pYA3137*eaeA* plasmids were orally inoculated in mice and monitored for their *in vivo* capacity to colonize the intestinal mucosa. In

both cases, bacteria were persistently detected in the feces of mice. Following the first immunization, the number of CFU decreased from 2 to 10 days. However, after the second immunization CFU numbers were maintained during the whole time period studied, indicating that intimin gamma expression did not affect the ability of *Salmonella* to colonize these tissues.

Nevertheless, the *Salmonella* vaccine strains could reach lymphoid organs after oral inoculation. This is particularly important because EHEC can fairly trigger any immune response in mice or any other possible reservoir. The expression of intimin gamma in the context of the *Salmonella*, in steps of colonization, invasion and persistence in lymphoid organs, could trigger an immune response against this antigen so as to effectively reduce the colonization of the intestinal mucosa of animals with EHEC.

In fact, lines of evidence have indicated that antibodies to intimin may prevent the initial steps of EHEC colonization in the gastrointestinal tract (7, 45, 46). In an attempt to determine whether the high production of specific antibodies was associated with reduced colonization of EHEC, BALB/c mice that had been orally immunized with *Salmonella* expressing intimin gamma and control animals were challenged with *E. coli* O157:H7. Our results show that mice previously exposed to *Salmonella* expressing intimin gamma have lower *E. coli* intestinal colonization as demonstrated by the decrease of EHEC CFU in fecal shedding, clearly visible on Figure 4. Interestingly, the strain control only decreased bacterial counts in the first day of the analyzed period (day 5 following inoculation), when compared with the vehicle control (P 0.05), and maintained similar counts in subsequent days. A possible explanation for this result involving the immunization with this *Salmonella* control strain is that *E. coli*, and more importantly the O157:H7 strain, may share some common antigens (47), which could result in a cross immune response. Nevertheless, the immunization with the *Salmonella* vaccine strain was shown to reduce bacterial counts of the challenged bacteria during all analyzed period.

Intervention on beef and dairy feedlots has been the main strategy to decrease the exposure of humans to *E. coli* O157:H7. A study by Sargeant and coworkers (48) fully reviewed the intervention efficacies of many strategies developed to increase resistance to colonization of *E. coli* O157 in animals. Of particular interest is the vaccine preparation with type-III secreted proteins (39), consisting of Esp proteins and the cellular intimin receptor Tir, which are secreted to and purified from the culture supernatant (19). This vaccine has been submitted to a large-scale clinical trial and the results demonstrated an effective reduction of *E. coli* O157:H7 colonization in cattle (9) and has therefore been approved by the Canadian authority for unrestricted use by cattle producers and their veterinarians (49). However, its effect on human cases of disease by *E. coli* O157:H7 in Canada remains to be reported.

Although the vaccine constructed by the Canadian research group had been effective, there is still a place for a live recombinant vaccine against *E. coli* O157:H7, because such an approach is easier to scale-up, when compared to the purification of secreted proteins from culture media. Furthermore, *Salmonella* strains are more feasible for this purpose, as they are natural enteric pathogens and can elicit a strong mucosal immunity, besides other types of immunity. Indeed, promising results were obtained with an attenuated *Salmonella* strain expressing the *R. equi* VapA (20, 21).

In summary, in the present study we showed that a recombinant *Salmonella* strain, expressing the intimin gamma from *E. coli* O157:H7, increased the clearance of colonized EHEC in the mouse intestine, a response that appears to be mediated by anti-intimin systemic IgG and mucosal IgA antibodies. Also, these results are in accordance to other works with two different strains of attenuated *Salmonella*, contributing for its potential to vaccination strategy against EHEC.

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DISCLOSURE

The authors have nothing to disclose.

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