



Article

Nasal Immunization with the C-Terminal Domain of BclA3 Induced Specific IgG Production and Attenuated Disease Symptoms in Mice Infected with *Clostridioides difficile* Spores

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Abstract: *Clostridioides difficile* is a Gram-positive, spore-forming bacterium that causes a severe intestinal infection. Spores of this pathogen enter in the human body through the oral route, interact with intestinal epithelial cells and persist in the gut. Once germinated, the vegetative cells colonize the intestine and produce toxins that enhance an immune response that perpetuate the disease. Therefore, spores are major players of the infection and ideal targets for new therapies. In this context, spore surface proteins of *C. difficile*, are potential antigens for the development of vaccines targeting *C. difficile* spores. Here, we report that the C-terminal domain of the spore surface protein BclA3, BclA3_{CTD}, was identified as an antigenic epitope, over-produced in *Escherichia coli* and tested as an immunogen in mice. To increase antigen stability and efficiency, BclA3_{CTD} was also exposed on the surface of *B. subtilis* spores, a mucosal vaccine delivery system. In the experimental conditions used in this study, free BclA3_{CTD} induced antibody production in mice and attenuated some *C. difficile* infection symptoms after a challenge with the pathogen, while the spore-displayed antigen resulted less effective. Although dose regimen and immunization routes need to be optimized, our results suggest BclA3_{CTD} as a potentially effective antigen to develop a new vaccination strategy targeting *C. difficile* spores.

Keywords: mucosal vaccine; exosporium proteins; *Bacillus subtilis*; immune response; *Clostridium difficile*; gastrointestinal infection; recombinant spores

1. Introduction

Clostridioides difficile is a Gram-positive, spore-forming and obligate anaerobe gastrointestinal bacterium, responsible for the most common nosocomial infection in industrialized countries [1]. In recent years the incidence and severity of *C. difficile* infections (CDI) has increased worldwide due to the emergence of antibiotic-resistant and hyper-virulent strains. According to the Centers for Disease Control and Prevention (CDC) in 2017 there were an estimated 223,900 cases in hospitalized

patients and 12,800 deaths in the United States by CDI [2]. Elderly people and frequent hospitalized patients have been notified as the groups of major risk to develop CDI [3]. In addition, about 20% of the infected people develop a second CDI episode within 2 months and in the case of more than two episodes, the frequency of further recurrences increases dramatically up to 60% [4–6]. Nowadays, CDI is not only a major concern, but also an economic burden. Recent data indicated that CDI is more common than methicillin-resistant *Staphylococcus aureus* infections [7] and estimated a cost per CDI episode ranging from EUR 5000 to 12,000 in the European Union [8] and approximately USD 21,000 in the United States [9].

CDI is mainly transmitted by *C. difficile* spores through the fecal-oral route. Ingested spores survive the transit through the stomach, interact with intestinal epithelial cells and persist in the host gut. When gut conditions are favorable, i.e., when the number of other members of the gut microbiota is severely reduced, *C. difficile* spores germinate and massively colonize the gut. Growing cells of *C. difficile* then produce virulence factors that induce a strong immune response and the symptoms associated to *C. difficile* infections [10,11]. Being infection vehicles, mediators of the initial interaction with intestinal cells and responsible of the persistence of the pathogen in the animal gut [12], *C. difficile* spores are key players of CDI and ideal targets of anti-*C. difficile* therapeutic treatments.

It is known that the hydrophobicity of *C. difficile* spores, due to the proteinaceous exosporium, contributes to adhesion to hospital surfaces and to intestinal epithelial cells (IECs) [13,14] and members of the BclA family of collagen-like glycoproteins are abundantly present in the *C. difficile* exosporium. In the hyper-virulent strain R20291 of *C. difficile* the BclA proteins showed 56% similarity with the BclA protein of *Bacillus anthracis* [15], known to be highly immunogenic and to act as spore surface ligand for the $\alpha 2\beta 1$ integrin present in IECs, driving spore entry into the epithelial barrier [16]. Further investigation needs to be done in order to fully understand the role of BclA proteins in *C. difficile* spores; however, evidence suggest that these proteins are involved in the formation of the hair-like projections in most *C. difficile* strains such as the hyper-virulent strain R20291 [17,18] and their spore surface location propose them as potential antigens [19]. While the antigenicity of BclA1 and BclA2 has been recently tested [20–23], BclA3 has not been directly evaluated to date.

The *C. difficile* collagen-like BclA3 exosporium protein is composed by an N-terminal domain, possibly oriented to the inside, a collagen-like domain formed by GXX repeats, which is highly glycosylated [15], and a C-terminal domain that is presumably faced outwards of the exosporium [22]. A recent study has shown that several glycosylated peptides of the collagen-like region of BclA3 were able to induce humoral immune response in mice [24]. However, it is unclear whether the C-terminal domain of BclA3 could be used as an antigen in a *C. difficile* spore-based vaccination strategy.

In this study, the BclA3 amino acid sequence was analyzed in silico and the C-terminal domain, BclA3_{CTD}, was identified as a potential epitope. BclA3_{CTD} antigenicity was then tested in vivo in a murine model as a free protein or displayed on *Bacillus subtilis* spores, a well-established antigen delivery system [25], proven to efficiently interact with antigen-presenting cells (APCs) leading to the induction of humoral, local and cellular responses [26,27]. Mice immunized with recombinant spores or with the pure antigen were able to produce BclA3_{CTD}-specific Immunoglobulin G (IgG). The immunization with pure BclA3_{CTD} also impaired weight loss after a challenge with *C. difficile* spores and induced a decrease in the *C. difficile* spore load in feces one day after the infection.

2. Results

2.1. In Silico Analysis of BclA3 and Construction of the Recombinant Strain Expressing the Chimera Protein CotBΔ- BclA3_{CTD}

To predict the most immunogenic domain of BclA3 we used the Immune Epitope Database (IEDB) to analyze BclA3 amino acid sequence and predict continuous linear B and T cell Major Histocompatibility complex (MHC)-I and MHC-II epitopes. As shown in Figure 1A, the C-terminal 170 amino acid residues of BclA3 showed the highest B cell antigenicity score. The prediction of T cell epitopes also suggests this part of the protein as the most immunogenic (Tables S1–S3). Based on that

and on a previous report suggesting that the C-terminus of BclA3 is faced outwards of the exosporium of *C. difficile* spores [22], we selected the C-terminal part of BclA3 (BclA3_{CTD}) (Figure 1B) as a putative antigen to be tested in vivo as a free protein and for display on the surface of *B. subtilis* spores. Hence, His-tagged BclA3_{CTD} was overexpressed in *Escherichia coli* BL21(DE3) and purified by affinity chromatography with Ni-sepharose columns as described in the Methods section. Upon purification, the protein was loaded on a Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1C) next to pure BclA2_{CTD}, an exosporium protein already purified by our group [23]. Whilst pure BclA2_{CTD} migrates on SDS-PAGE gel as a single band of approximately 14 kDa, as expected, pure BclA3_{CTD} consistently migrates in two bands, one with a molecular weight of about 20 kDa and the other of about 60 kDa that match with the predicted sizes of a monomer and a trimer, respectively, which may indicate protein aggregation.

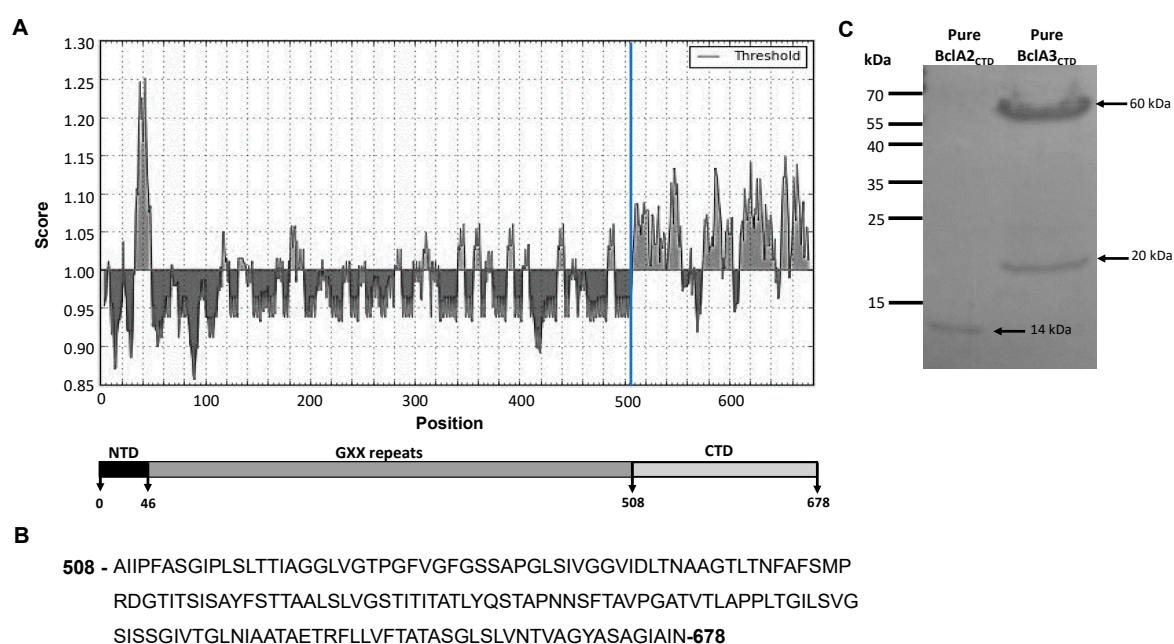


Figure 1. Analysis of the glycoprotein BclA3 from *C. difficile* R20291. **(A)** The C-terminal domain (CTD) of BclA3 (last 170 amino acid residues) shows higher B cell epitope propensity score compared with the rest of the protein (Kolaskar & Tongaonkar Antigenicity Method from Immune epitope database). The X- and Y-axes represent the sequence position and antigenic propensity score, respectively. The threshold value was generated by default by Immune epitope database (<http://tools.iedb.org/bcell/>). The regions above the threshold are antigenic. **(B)** The correspondent aminoacidic sequence of BclA3_{CTD} is shown. **(C)** Coomassie stained SDS-PAGE gel; 2 and 5 μ g of purified C-terminal domains of the exosporium proteins BclA2 and BclA3 were loaded, respectively. BclA2_{CTD} migrates as 14 kDa band while BclA3_{CTD} migrates as 2 bands of approximately 20 and 60 kDa.

DNA coding for the last 170 amino acid residues of BclA3 (BclA3_{CTD}) was used not only to over-express and purify the protein fragment but also to construct a gene fusion with DNA coding for the *B. subtilis* spore surface protein CotB, a coat protein already used to anchor heterologous antigens in other studies [28–32]. In particular, we used a truncated version of CotB, CotB Δ , by deleting 105 C-terminal amino acids, thus removing a region with repeated sequences avoiding potential structural instability of the genetic constructs [28]. As previously reported [28], the gene fusion was cloned into an integrative vector adjacent to a chloramphenicol-resistant gene cassette (Cm^R) and used to transform competent cells of the *B. subtilis* strain PY79 [33]. Chloramphenicol-resistant clones were the result of a double cross-over integration event, schematically indicated in Figure 2A. Chloramphenicol-resistant clones were tested for the site of chromosomal integration by PCR (not shown) and clone AZ703 was selected for further analysis.

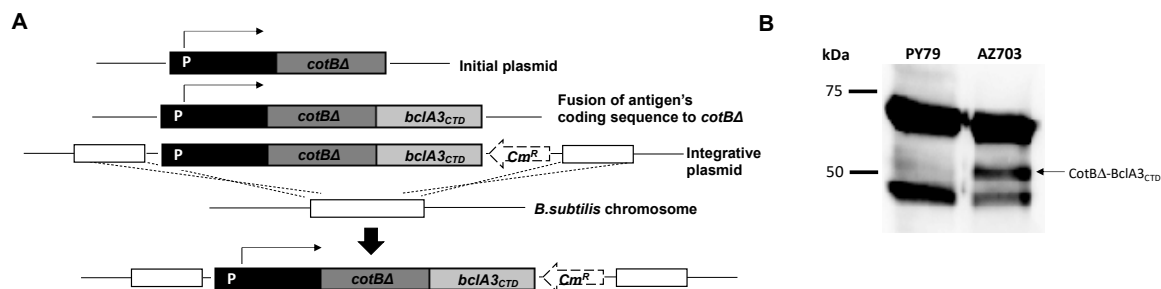


Figure 2. Construction of *B. subtilis* recombinant strain AZ703 expressing the chimera protein CotBA-BclA3_{CTD}. (A) Schematic representation of the strategy for the integration of the gene fusion *cotBA::bclA3_{CTD}* in the chromosomal DNA of *B. subtilis* PY79. The Western blot analysis of proteins extracted from spores of *B. subtilis* laboratory strain PY79 (lane 1 in B) and from the recombinant strain AZ703 (lane 2 in B) show bands of 66 and 46 kDa, which correspond to the endogenous CotB protein. The lane of recombinant strain AZ703 also displays a band of about 50 kDa which corresponds to the chimera protein CotBA-BclA3_{CTD}; 5×10^8 of spores were resuspended in 100 μ L of loading buffer and 20 μ g of protein extract was loaded in SDS-PAGE gel. The immunoreaction was performed with anti-CotB antibodies and anti-rabbit secondary antibody conjugated with horseradish peroxidase.

Purified spores of strain AZ703 were used to extract surface proteins by the SDS-DTT procedure [34] and extracted proteins were analyzed by Western blot with anti-CotB antibody. CotB has a deduced molecular mass of 46 kDa but it is known to migrate on SDS-PAGE in two forms: a predominant form of 66 kDa and a minor form of 46 kDa [35]. Both forms were extracted from *B. subtilis* laboratory strain PY79 and AZ703 spores with only the latter also showing an additional protein, slightly bigger than 50 kDa (Figure 2B). The additional protein was recognized by the anti-CotB antibody and conformed well with the expected size for the fusion protein, since the truncated form of CotB and the BclA3 fragment have predicted sizes of 36 kDa [23] and 20 kDa (Figure 1C), respectively.

2.2. Mice Intranasal Immunization

A mucosal immunization experiment was performed in a murine model to test the efficiency of BclA3_{CTD} as an antigen, both as a free protein and upon display on *B. subtilis* spores. Mice were divided into four experimental groups and nasally immunized three times either with Phosphate buffered saline (PBS) pH 7 ($n = 11$), 2×10^9 spores of *B. subtilis* PY79 ($n = 11$) (Sp), 2×10^9 spores of AZ703 ($n = 11$), or 4 μ g of purified BclA3_{CTD} ($n = 11$). The animal serum was collected one day before the first (pre-immunization day (PI)), second (day 13) and third (day 27) immunizations as well as two days before *C. difficile* infection (day 42). One day before the infection with 5×10^7 spores of the *C. difficile* strain R20291, mice were treated with Clindamycin as previously reported [36] and schematically shown in Figure 3.

BclA3_{CTD} immunogenicity was measured by ELISA analyzing the presence of anti-BclA3_{CTD} IgG in animal serum throughout the experiment. As shown in Figure 4A mice immunized with pure BclA3_{CTD} produced BclA3_{CTD}-specific IgG upon nasal administration even on the 13rd day (immunized only once). After the second and third immunizations (d27 and d41, respectively) the increase was significant ($p < 0.0001$ compared to Pre-immune serum and with d13). The recombinant spores AZ703 also induced significant levels of anti-BclA3_{CTD} IgG after the third immunization ($p = 0.0380$ compared to Pre-immune serum). Neither PBS nor *B. subtilis* PY79 spores were able to raise an anti-BclA3_{CTD} IgG response in mice, as expected.

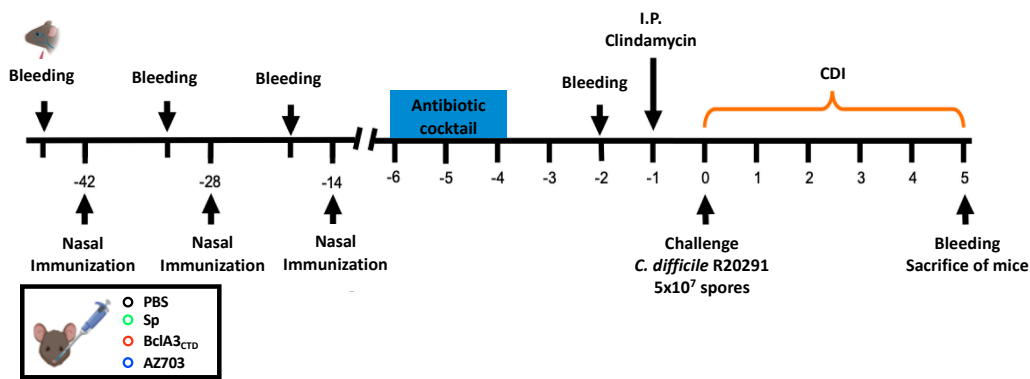


Figure 3. Schematic overview of the experimental design for the prevention of *C. difficile* infection in a murine model. C57BL/6 mice were nasally immunized three times (42, 28 and 14 days before challenge with *C. difficile* R20291 spores) with PBS, spores of *B. subtilis* PY79, purified BclA3_{CTD} or spores of *B. subtilis* displaying the chimera protein CotBΔ-BclA3_{CTD} (AZ703). Prior to the infection with *C. difficile* R20291, the animals were submitted to an antibiotic cocktail (days 4–6 before challenge) and clindamycin administration (1 day before challenge). On day 0, mice were infected with 5×10^7 of *C. difficile* R20291 spores and were monitored from day 0 to day 5 for CDI symptoms. Serum was collected one day before each immunization, two days before *C. difficile* infection and on the day of sacrifice.

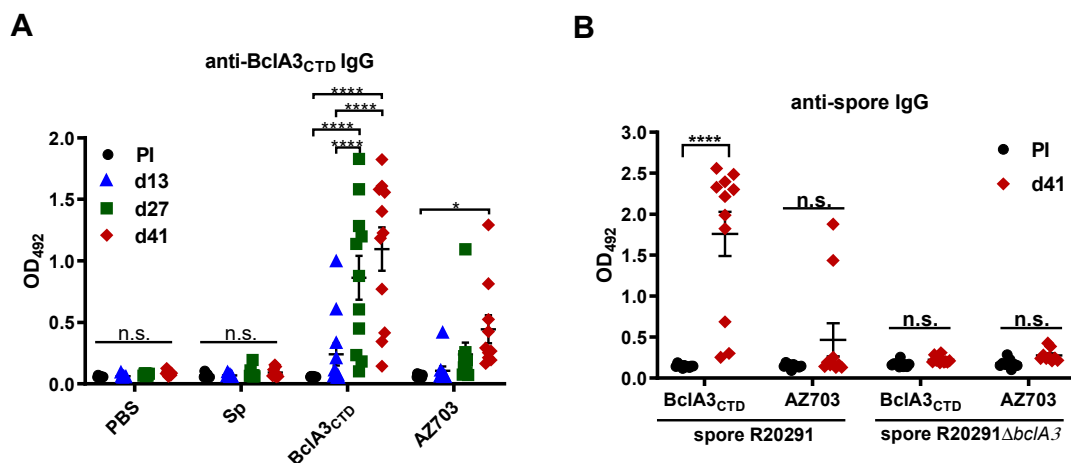


Figure 4. Evaluation of IgG anti-BclA3_{CTD} and anti-*C. difficile* spores R20291 and R20291Δ*bclA3* titers on mice serum. ELISA plates coated with 50 ng/well of purified BclA3_{CTD} (A) or 1.6×10^7 spores/well of *C. difficile* R20291 or R20291Δ*bclA3* spores (B) were incubated with 1:100 of mice serum immunized on days 0 (Pre-Immune serum, PI), 13 (d13, before second immunization), 27 (d27, before third immunization) or day 41 (d41, before infection with *C. difficile* spores). The assessment of specific IgG anti-BclA3_{CTD} and anti-spores was obtained using the secondary antibody 1:5000 anti-IgG mouse HRP. Results are reported as optical density (OD) units at 492 nm. The geometric mean plus standard error of the mean for each cohort are shown. Comparisons between days in the same group were obtained using two-way ANOVA and Tukey's multiple comparison test; statistical significance ($p < 0.05$) is indicated by asterisks. * $p < 0.05$ and **** $p < 0.0001$. No significance (n.s).

The immune reactivity of the serums collected 2 days before *C. difficile* R20291 infection when incubated either with spores of the hyper-virulent strain R20291 or with the isogenic *bclA3* mutant strain (R20291Δ*bclA3*) was also tested. As shown in Figure 4B, mice immunized with pure BclA3_{CTD} produced IgG able to recognize R20291 spores ($p < 0.0001$ in comparison to the Pre-immune serum) but not R20291Δ*bclA3* spores indicating the specificity of the response. As expected by the low immune

response induced by spore-displayed BclA3 (Figure 4A), only two out of eleven mice immunized with spore-displayed BclA3_{CTD} produced IgG able to specifically recognize R20291.

In conclusion, results of Figure 4 show that BclA3_{CTD} is an antigen able to induce the production of BclA3_{CTD}-specific IgG in a murine model and serum of animals immunized with the pure antigen were also able to recognize spores of the hypervirulent strain R20291 of *C. difficile*. When displayed on *B. subtilis* spores BclA3_{CTD} is still able to induce the production of BclA3-specific IgG, even if at a lower level.

2.3. Effect of Nasal-BclA3_{CTD} Immunization against *C. difficile* R20291 Infection

Figure 5A shows that intranasal immunization with purified BclA3_{CTD} prevented weight loss after the challenge with 5×10^7 spores of the R20291 strain of *C. difficile*. In particular, on days 1 and 2 post-infection a statistically significant difference ($p = 0.0177$ and $p = 0.0099$, respectively) was observed between mice immunized with pure BclA3_{CTD} and those immunized with wild type spores of *B. subtilis* (Figure 5A). No statistically significant differences were observed concerning the appearance of diarrhea caused by challenge with R20291 spores, indicating that the nasal immunization with pure BclA3_{CTD} or with the recombinant strain displaying CotBΔ-BclA3_{CTD} did not halt all CDI symptoms (Figure 5B). Plus, the severity of diarrhea, associated with a high score (zero meaning normal stool and three liquid stool), did not vary significantly between groups in the same day (Figure 5C).

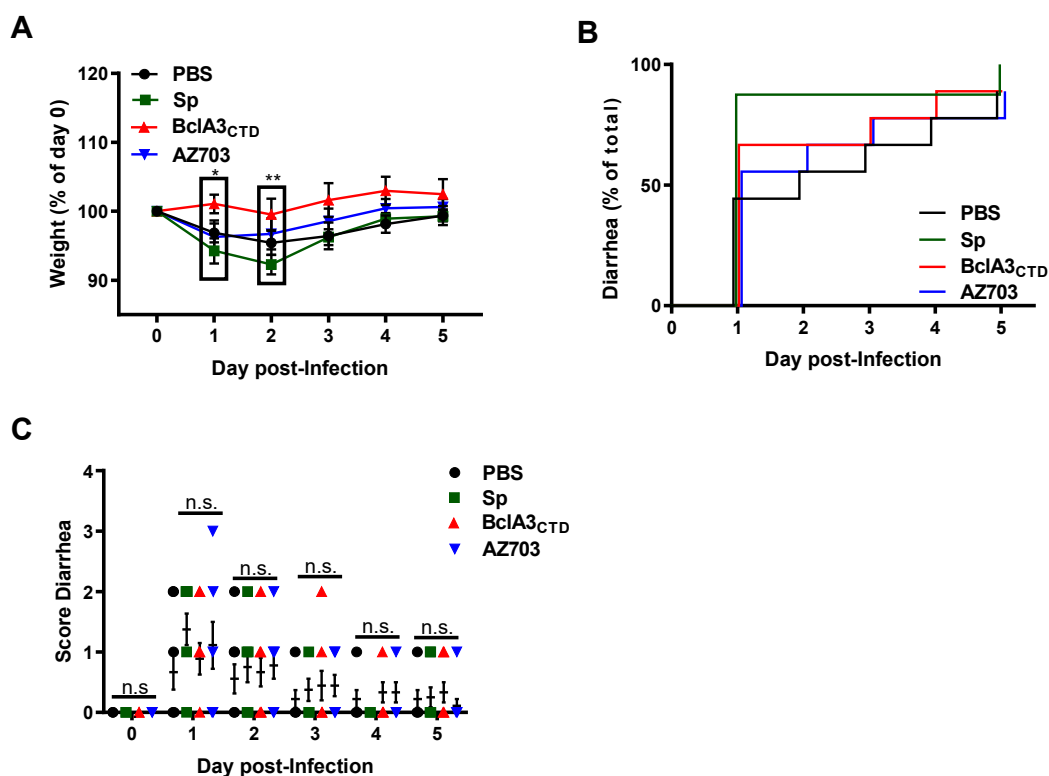


Figure 5. Protective efficacy of intranasal administration of BclA3_{CTD} and AZ703 against CDI in a murine model. C57BL/6 mice were nasally immunized with PBS, *B. subtilis* PY79 spores, purified BclA3_{CTD} or AZ703 spores and challenged with *C. difficile* R20291 spores. Mice were monitored in the following 5 days after infection for (A) Weight loss presented as the relative % of the weight to the day of infection (day 0 or D0); (B) Time of occurrence of diarrhea, presented as the relative % of diarrhea in a group to the total mice and (C) Score of diarrhea per day. Error bars are standard error of the mean. Two-way ANOVA Tukey's multiple comparisons test (A and C); Log-rank (Mantel-Cox) test (B). Statistical significance ($p < 0.05$) is indicated by asterisks. * $p < 0.05$ and ** $p < 0.01$. No significance (n.s.).

In order to evaluate if the immunization influenced *C. difficile* sporulation inside the host and spore clearance, we compared the spore levels present in stools within 5 days post-infection. We observed a statistically significant lower spore load in the feces of mice immunized with BclA3_{CTD} one day post-infection ($p < 0.0001$ with respect to mice immunized with PBS or PY79 spores and $p = 0.0002$ with mice immunized with AZ703 spores) (Figure 6A) suggesting that animals immunized with the pure antigen were able to quickly eliminate *C. difficile* spores. However, from day 2 to day 5 all other groups of mice were similarly able to eliminate *C. difficile* spores (Figure 6A). No differences were observed in the *C. difficile* spore load in the ileum, proximal, middle or distal colon tissue (not shown). Finally, we measured toxin levels in mice feces, a sign of *C. difficile* colonization inside the cecum. As shown in Figure 6B, no statistically significant differences were observed suggesting that the immunization strategies could not prevent colonization and cytotoxicity in the cecum.

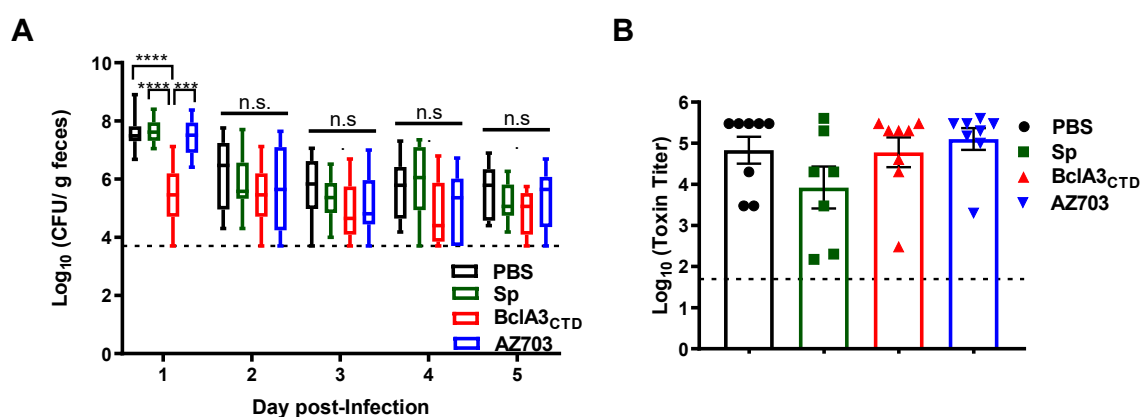


Figure 6. Analysis of spore load in feces and cecal toxin titers. (A) The load of *C. difficile* spores in the feces was evaluated on the following 5 days after infection as log₁₀ Colony Forming Unit (CFU)/g of feces. (B) The cecum content toxicity was measured and represented as log₁₀ toxin titer. Two-way ANOVA Tukey's multiple comparisons test in A and Mann–Whitney non-parametric tests in B were used; Statistical differences ($p < 0.05$) are indicated by asterisks. *** $p < 0.001$ and **** $p < 0.0001$. The bars are the geometric mean \pm standard error of the mean. No significance (n.s).

3. Discussion

Current treatment options for CDI rely on the use of antibiotics, fecal microbiota transplantation, probiotic administration or monoclonal antibodies against *C. difficile* toxins [37]. The spore, form in which *C. difficile* persists inside the host, possibly modulating its immune system, is still not considered as a main target for therapies. We have recently demonstrated that the C-terminal domain of *C. difficile* exosporium protein BclA2 elicits an elevated humoral response after nasal immunization in mice [23]. Here, we have identified as a potential antigen to induce an anti-spore immune response, the C-terminal domain of the exosporium protein BclA3 (BclA3_{CTD}) of the hyper-virulent strain R20291 of *C. difficile*.

Here, we show that mice nasally immunized with pure BclA3_{CTD}, even only two times, were able to produce BclA3_{CTD}-specific IgG (Figure 4A), indicating that the antigen was able to elicit a specific humoral immune response and therefore could be exploited for a vaccination strategy against *C. difficile*. Moreover, upon challenge with *C. difficile* spores, mice immunized with pure BclA3_{CTD} not only maintained their weight but (Figure 5A) also had a reduction in *C. difficile* spore load in feces even one day after the infection (Figure 6A), improvements that so far have not been observed in mice nasally immunized with BclA2_{CTD} [23]. An interesting study from Ghose et al. on 2016 using mice immunized intraperitoneally with BclA1 and challenged with spores of *C. difficile* strain UK1 have shown that despite the raise of specific IgG anti-BclA1, the immunization failed to provide protection against challenge with the pathogen [20]. In the present work, the fact that the nasally immunized animals with BclA3_{CTD} were able to show some improvements on CDI symptoms may suggest that this protein may be a strong candidate for vaccine strategies against CDI. Considering an induction of a robust

specific systemic immune response, as suggested by the high titers of IgG anti- BclA3_{CTD}, it is tempting to assume that animals immunized with the pure peptide have activated the complement signaling cascade leading to the opsonization and neutralization of *C. difficile* spores, phagocytic elimination and therefore experienced spore clearance earlier than the other groups of animals [38,39]. Effective mucosal vaccines should also induce cellular and local immune responses able to persist and sustain a protection in case of future infection with the pathogen. It is expected that exposition to BclA3_{CTD} will enhance the homing of antigen-reactive lymphocytes to sites of primary immunization and T cell activation should prompt B cell class switching, affinity maturation, and memory establishment [40]. However, noteworthy, the immunization with pure BclA3_{CTD}, without adjuvant, was not able to halt neither the occurrence and intensity of diarrhea (Figure 5B,C) nor to reduce the spore load in colonic tissues (data not shown) or to avoid *C. difficile* spore colonization in mice cecum (Figure 6B). These results may indicate that the systemic immune response was not strong enough to prompt local immunity and a protective immune response. A change in the administration route and/or the optimization of the antigen dose could possibly overcome these limitations. The measurement of mucosal Immunoglobulin A (IgA) will be addressed in the following experiments for a better understanding of local immune response and the phenotype of the induced humoral immune response will be examined by analyzing IgG subclasses.

We have also immunized mice with *B. subtilis* spores recombinantly engineered to display BclA3_{CTD} anchored to a highly abundant coat protein, CotB. Due to its safety and robustness *B. subtilis* spores have been widely used as mucosal vaccine vehicles [41–43]. Recombinant *B. subtilis* spores administered by the nasal route have been shown able to expose the antigen to mucosal-associated lymphoid tissue (MALT) and therefore prompt a strong immune response [44–46]. Plus, it is shown that antigens are more protected from enzymatic lysis and have increased stability when displayed into *B. subtilis* spore surface than free antigen [26,47]. Nevertheless, our work only showed a slight increase in specific anti-BclA3_{CTD} IgG in mice immunized with recombinant spores and only after three shots of immunizations (Figure 4A). Consequently, it is not surprising that we have not observed and impairment in CDI symptoms (Figures 5 and 6). These results contrast with our previous study, where we observed that immunizing mice through nasal delivery with *B. subtilis* spores adsorbed with BclA2_{CTD} on the spore surface yielded similar titers as with pure BclA2_{CTD} [23]. This could explain, in part, the low titers against BclA3_{CTD} observed by the ELISA using sera from mice that were immunized with *B. subtilis* spores expressing chimera CotBΔ-BclA3_{CTD} protein. Although, immunoblot analysis (Figure 2B) indicates that the chimeric protein CotBΔ-BclA3_{CTD} is expressed and present as a fusion in the spore coat of *B. subtilis* spores, it is unclear whether the fusion expressed in the spore coat is displayed on the spore surface and accessible to antibodies or buried in the spore coat layer. At this time, we are unable to assess the amount of surface displayed CotBΔ-BclA3_{CTD} and surface accessibility to antibodies by fluorescence-activated cell sorting (FACS) or quantitative Western. We acknowledge this limitation and will assess quantitative surface display of chimeric CotBΔ-BclA3_{CTD} protein in further studies seeking to optimize *B. subtilis* recombinant surface display and/or adsorption of *C. difficile* exosporium proteins. In this context, it is known that recombinant spores of *B. subtilis* administered by gavage germinate in the stomach and small intestine and due to low oxygen present in the large bowel environment they re-sporulate displaying the heterologous antigen again [48–50] therefore, the oral delivery of recombinant *B. subtilis* spores would be also considered and interesting option since it allows higher doses and number of immunizations. Additionally, the use of adjuvants with immunostimulatory capacities, such as Toll-Like Receptors (TLR) and NOD-Like Receptor (NLR) ligands [51] or probiotics [52] might boost the expected immune response. A recent study has shown that humoral and cellular immune responses observed in mice nasally immunized with *B. subtilis* spores displaying the C fragment of the tetanus toxin (TTFC) on the surface were enhanced in mice previously submitted to *Bacillus toyonensis* as probiotic [52]. Therefore, the use of these kinds of strategies would contribute to an improvement in this work.

In conclusion, the induction of humoral immune response and the partial protective effects observed in animals nasally immunized with the purified protein BclA3_{CTD} clearly indicate that BclA3_{CTD} is a promising antigen to be tested in future in vivo trials.

4. Materials and Methods

4.1. Bacterial Strains and Spore Purification

E. coli strains DH5 α and BL21 (DE3) (Invitrogen, Agawam, MA, USA) were used for cloning and BclA3_{CTD} overexpression, respectively. *B. subtilis* PY79 [33] was used as a parental strain of AZ703. The hyper-virulent strain R20291 of *C. difficile* was used for mice infection. To test BclA3_{CTD}-specific immunogenicity an R20291 Δ bclA3 knockout mutant was used (Paredes-Sabja, unpublished work).

Sporulation of *B. subtilis* PY79 and AZ703 was induced by the exhaustion method [53]. Briefly, after 35 h of growth in Difco Sporulation (DS) medium at 37 °C with vigorous shaking, spores were collected, washed and purified. The purification was performed using KCl 1 M, lysozyme 10 mM, NaCl 1 M, SDS 0.05% and several washes with water.

C. difficile spores were purified as described elsewhere [54]. Spore suspensions were prepared by plating a 1:100 dilution of an overnight culture onto a 70:30 medium (63 g Bacto peptone (ThermoFisher, Agawam, MA, USA), 3.5 g proteose peptone (ThermoFisher), 0.7 g ammonium sulfate (NH₄)₂SO₄, 1.06 g Tris base, 11.1 g brain heart infusion extract (ThermoFisher) and 1.5 g yeast extract (ThermoFisher) for 1 L) and incubating it for 5 days at 37 °C under anaerobic conditions [55]. After incubation, plates were removed from the chamber and the surface was scraped up with ice-cold sterile water. Next, the spores were washed five times gently with ice-cold sterile water in micro centrifuge at 14,000 rpm for 5 min. Spores were loaded onto a 45% Nycodenz solution, centrifuged (14,000 rpm, 40 min). After centrifugation, the spore pellet was washed five times (14,000 rpm, 5 min) with ice-cold sterile water to remove Nycodenz remnants.

The spores were counted in Neubauer chamber and volume adjust at 5×10^9 spores per mL. Spore suspensions were purified until they were > 99% free of vegetative cells, sporulating cells and cell debris as determined by phase-contrast microscopy.

4.2. BclA3_{CTD} Over-Production and Purification

The chromosomal DNA of *C. difficile* R20291 was used for the amplification of *bclA3* C-terminal domain (CTD) (513 bp) with the oligonucleotides BclA3_{CTD}^{sense} (ggtagcggatccGCAATAATACCTTTTGCATCAGG, in lower case is the recognition site for KpnI, NcoI and BamHI restriction enzymes) and BclA3_{CTD}^{anti} (tctagactgcagCTAATTTATTGCAATTCCTGCAC in lower case is the recognition site for XbaI and PstI restriction enzymes) to prime the reaction. The coding sequence of BclA3_{CTD} was cloned in the plasmid pGEMT-easy (Promega) and posteriorly cleaved with BamHI/PstI restriction enzymes and inserted in-frame to an N-terminal polyhistidine tag in the expression vector pRSETA (Invitrogen), previously digested with the same enzymes. Upon transformation of *E. coli* BL21(DE3) with pRSETA::*bclA3*_{CTD}, the strain was incubated in ampicillin-supplemented (50 μ g/mL) TY medium. Once reached an optical density of 0.7 at 600 nm the culture was added to an autoinduction medium (T7 promoter induction by lactose) and incubated for 16 h at 37 °C with shaking. The six-His-tagged BclA3_{CTD} protein was purified under native conditions using the His-Trap column as recommended by the manufacturer (GE Healthcare Life Science, Darmstadt, Germany). The purified protein was desalted and concentrated with the Centricon cutoff 10 kDa (Merck Millipore, Darmstadt, Germany). The purity of the protein was analyzed by SDS-PAGE and Western blot using Anti-His antibodies.

4.3. Construction of the Recombinant Strain AZ703

DNA coding for BclA3_{CTD} and for the N-terminal 275 amino acids of CotB were PCR amplified using the *C. difficile* R20291 and *B. subtilis* PY79 chromosome as template, respectively. To prime *cotBA* the oligonucleotides B1 (acatgcatgcACGGATTAGGCCGTTTGTCC in lower case there is the recognition site for SphI restriction enzyme) and B3 (gaaagatctGGATGATTGATCATCTGAAG in lower case there is the recognition site for BglII restriction enzyme) were used. The obtained amplification products were cloned in pGEMT-easy (yielding pGEMT-easy::*bclA3_{CTD}* and pGEMT-easy::*cotBA*). The *bclA3_{CTD}* gene was digested from pGEMT-easy::*bclA3_{CTD}* with BamHI/PstI restriction enzymes and cloned in-frame to the 3' end of the *cotBA* gene carried by plasmid pGEMT-easy::*cotBA* previously digested with BglII/PstI, yielding plasmid pGEMT-easy::*cotBA::bclA3_{CTD}*. The fusion *cotBA::bclA3_{CTD}* gene was digested with the restriction enzymes SphI/SalI and ligated to the previously digested integrative plasmid pDG364 which contains a coding region for chloramphenicol resistance. Competent *B. subtilis* PY79 cells were transformed with the previously linearized integrative vector with NdeI and plated in medium with chloramphenicol. The antibiotic-resistant clones were the result of double-crossover recombination with *amyE* gene on the *B. subtilis* chromosome. The chromosomal DNA was extracted from the positive clones and tested by PCR. Sporulation of PY79 and recombinant strain (AZ703) was induced by nutrient exhaustion in DS medium. After 35 h incubation at 37 °C, spores were collected, washed and purified as described before. The coat proteins from 5×10^8 spores of PY79 and AZ703 were extracted by SDS-DTT treatment. To verify the expression of the chimera protein CotBΔ-BclA3_{CTD}, extracted proteins were analyzed by Western blot using anti-CotB antibodies.

4.4. Western Blot Analysis

The coat proteins from 5×10^8 spores of *B. subtilis* PY79 and AZ703 were extracted by SDS-DTT treatment [34] and quantified by Bradford assay (BioRad, Milan, Italy). In total, 20 µg of protein extract, 5 µg and 2 µg of pure BclA3_{CTD} and BclA2_{CTD}, respectively, were treated with protein sample buffer 2× [56], incubated at 100 °C for 7 min and loaded onto a 12% or 15% SDS-PAGE gel. Proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech, Milan, Italy) and used for Western blot analysis by standard procedures. To identify the recombinant protein CotBΔ-BclA3_{CTD} it was used anti-CotB 1:7000 as primary antibody and anti-rabbit secondary antibody conjugated with horseradish peroxidase 1:7000. To identify pure BclA3_{CTD} it was used the antibody anti-His 1:7000.

4.5. Animals

Mice 8-12 weeks old C57BL/6 (male or female) were obtained from a breeding colony at Facultad de Ciencias Biológicas, Universidad Andres Bello (Santiago, Chile), established using animals purchased from Jackson Laboratories (Protocol number 0035/2018, project identification code: Fondef ID18/10230; approval date: 22 January 2018; approval act code 0035/2018; approval committee: Comité de Bioética de la Facultad de Ciencias Biológicas). Water, bedding and cages were previously autoclaved, and mice had a 12-h cycle of light and darkness. All experimental protocols were conducted in strict accordance with and under the formal approval of the Biologicals Sciences Faculty of Universidad Andrés Bello.

4.6. Immunization Regimen in Mice

Mice were randomly assigned to four experimental groups (11 animals each group) according to the type of immunization received. Mice were intranasally immunized on days 0, 14 and 28 with 20 µL (10 µL per nostril) of PBS pH 7, 2×10^9 spores of *B. subtilis* PY79, 2×10^9 spores of AZ703 or 4 µg of purified BclA3_{CTD}. The day before each immunization, two days before infection and on the day of the sacrifice blood was collected.

4.7. Animal Infection Model

Prior to infection, mice were pre-treated with an antibiotic cocktail of kanamycin (40 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA), gentamicin (3.5 mg/kg body weight; Sigma-Aldrich), colistin (4.2 mg/kg body weight; Sigma-Aldrich), metronidazole (21.5 mg/kg body weight; Sigma-Aldrich) and vancomycin (40 mg/kg body weight; Sigma-Aldrich) for 3 days by oral administration [36]. Two days after the antibiotic treatment, mice were intraperitoneally administered with a single dose of clindamycin (10 mg/kg) and on the next day were infected orogastrically with 100 μ L of PBS containing 5×10^7 spores of *C. difficile* strain R20291. Mice were housed individually in sterile cages with *ad libitum* access to food and water. All procedures and mouse handling were performed aseptically in a biosafety cabinet to contain spore-mediated transmission.

The clinical condition of mice was monitored daily with a scoring system. The presence of diarrhea was classified according to severity as follows: (i) normal stool (score = 0); (ii) color change/consistency (score = 1); (iii) presence of wet tail or mucosa (score = 2); (iv) liquid stools (score = 3). A score higher than 1 was considered as diarrhea [57]. The animals were weighted daily after infection and other clinical symptoms as physical aspect (i.e., abnormal/hunched gait, piloerection), spontaneous behavior (i.e., lethargy, inactivity or lack of mobility) and emaciation were monitored as described [58]. Moribund mice or mice displaying overt signs of disease were sacrificed. At the time of sacrifice, ileum, proximal, median and distal colon were collected as well as the cecal content.

4.8. Evaluation of BclA3_{CTD}-Specific IgG Levels in Mice Serum

The blood collected the day before each immunization, two days before infection and at the time of sacrifice was incubated at 37 °C for 30 min and then centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant, containing the serum fraction was stored at -20 °C until use. To assess the production of IgG against BclA3_{CTD}, an Enzyme-linked immunosorbent assay (ELISA) was performed. Purified BclA3_{CTD} (50 ng/well), spores of *C. difficile* R20291 (1.6×10^7 spores/well) or *C. difficile* R20291 Δ bclA3 (1.6×10^7 spores/well) were coated onto 96-wells plates and incubated overnight at 4 °C. Then, the samples were blocked with PBS-0.05% Tween-20 (PBS-T) containing 2% BSA for 1 h at 37 °C. After several washes, the wells were next incubated with 1:100 of animal serum (in 1% BSA in PBS-T). The plates were incubated 2 h at 37 °C. After the removal of non-adherent IgG by several washes, the plates were incubated with 1:5000 secondary antibody anti-IgG mouse HRP, for 1 h at 37 °C. Finally, the colorimetric reaction was initiated upon the addition of 50 μ L of reaction buffer containing 0.05 M citric acid, 0.1 M disodiumhydrogen phosphate, 2 mg/mL of o-phenyldiamine (Sigma-Aldrich, USA) and 0.015% of H₂O₂ (Merck, Darmstadt, Germany). The reaction was stopped after 20 min with 25 μ L of 4.5 N of H₂SO₄ and absorbance was measured at 492 nm. The experiment was performed in duplicate.

4.9. Quantification of *C. difficile* Spores from Feces and Colon Samples

Fecal samples were collected in the following five days after infection and were stored at -20 °C until *C. difficile* spore quantification. On the day of the analysis, 10 μ L of PBS was added for each mg of stools, mixed and incubated for 30 min at room temperature. Then, 50 μ L of absolute ethanol (Sigma-Aldrich) was added to 50 μ L of feces and incubated for 30 min at room temperature. Samples were serially diluted and plated onto selective media supplemented with Taurocholate (0.1% *w/v*), Cefoxitin (16 μ g/mL) and L-cycloserine (250 μ g/mL) (TCCFA plates). The plates were incubated anaerobically at 37 °C for 48 h, the *C. difficile* colonies were counted, and the results were expressed as the Log₁₀ of CFU/g of feces.

Proximal, median and distal colons were collected from mice upon sacrifice and washed with PBS with a syringe. They were posteriorly resuspended and homogenized with 2.5 μ L of PBS for each mg of tissue. Upon incubation at room temperature with absolute ethanol and serial dilution, they were

plated onto TCCFA plates. The plates were incubated anaerobically at 37 °C for 48 h. Finally, the colony count was expressed as the Log₁₀ of CFU/g of tissue.

4.10. Cytotoxicity Assay

Vero cell cytotoxicity was performed as described previously [59]. Briefly, 96-well flat-bottom microtiter plates were seeded with Vero cells at a density of 10⁵ cells/well. Mice cecal contents were kept at −20 °C prior use. At the time of the experiment cecal contents were suspended in PBS (10 µL of PBS per mg of cecal content), vortexed and centrifuged (14,000 rpm, 5 min). The filter-sterilized supernatant was serially diluted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. In total, 100 µL of each dilution was added to wells containing Vero cells. Plates were screened for cell rounding 16 h after incubation at 37 °C. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in at least 80% of Vero cells per gram of luminal samples under ×200 magnification.

4.11. Statistical Analysis

Prism 8 (GraphPad Software, Inc.) was used for statistical analysis. Normality was assessed by Shapiro-Wilk test. For populations that did not follow a normal distribution significance between groups was assessed by a Mann–Whitney unpaired *t*-test. Comparative analysis between groups was performed by analysis of variance with Tukey's multiple comparison test for populations that followed a normal distribution. A *p*-value of ≤ 0.05 was accepted as the level of statistical significance.

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Abbreviations

CDI	<i>Clostridioides difficile</i> infection
IECs	Intestinal epithelial cells
BclA	<i>Bacillus</i> collagen-like protein of anthracis
NTD	N-terminal domain
CTD	C-terminal domain
BclA2 _{CTD}	C-terminal domain of BclA2
BclA3 _{CTD}	C-terminal domain of BclA3
APCs	Antigen-presenting cells
IEDB	Immune epitope database
MHC	Major Histocompatibility complex
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis
Sp	Spores of <i>B. subtilis</i>
PBS	Phosphate buffered saline

ANOVA	Analysis of variance
ELISA	Enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IgA	Immunoglobulin A
MALT	Mucosal-associated lymphoid tissue
I.P	Intraperitoneal
Wt	Wild type
PI	Pre-Immune
d13	Day 13
d27	Day 27
d42	Day 42
OD	Optical density
CFU	Colony forming unit
TTFC	C fragment of the tetanus toxin
TLR	Toll-Like Receptors
NLR	Nod-Like Receptors
FACS	Fluorescence-activated cell sorting
DMEM	Dulbecco's Modified Eagle Medium

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