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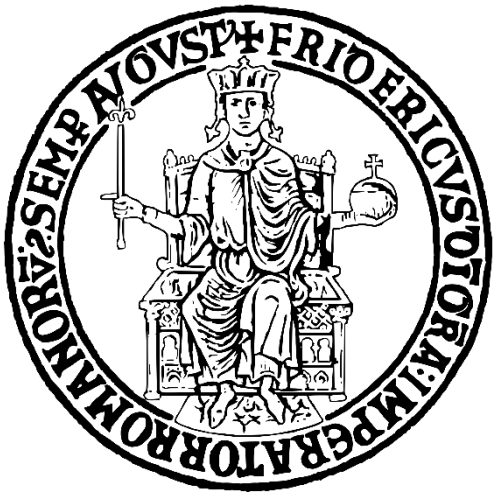

Ph.D. in Biology
Cycle XXXII

Development of a new vaccination strategy
against *Clostridium difficile* infection

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List of Abbreviations

CDI	*Clostridium difficile* infection
TTFC	Tetanus Toxin Fragment C
LTB	Labile Toxin B (B subunit of the heat-labile toxin of *Escherichia coli*)
PA	Protective Antigen of *Bacillus anthracis*
BclA	*Bacillus* collagen-like protein A
CdeC	Exosporium Cysteine rich protein
$BclA2_{CTD}$	C-terminal domain of BclA2
$BclA3_{CTD}$	C-terminal domain of BclA3
IgG	Immunoglobulin G
$CdeC_{CTD}$	C-terminal domain of CdeC
NTD	N-terminal domain
CTD	C-terminal domain
MHC-I	Major Histocompatibility complex I
MHC-II	Major Histocompatibility complex II
PBS	Phosphate buffered saline
ST	Standard
Sp	Spores of *B. subtilis*
Sp- $BclA2_{CTD}$	Spores of *B. subtilis* adsorbed with $BclA2_{CTD}$
ELISA	Enzyme-linked immunosorbent assay
I.P	Intraperitoneal
OD	Optical density
PI	Pre-Immune
d13	Day 13
d27	Day 27
Fl.I	Fluorescence intensity
CFU	Colony forming unit

ANOVA	Analysis of variance
DS	Difco Sporulation
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
BSA	Bovine serum albumine
CotB	Spore coat protein B
IEDB	Immune epitope database
PCR	Polymerase chain reaction
PBS-T	PBS-Tween 20
IECs	Intestinal epithelial cells
NHS	Normal human serum
ECM	Extracellular matrix
sIgA	Secreted immunoglobulin A
BoHV-5	Bovine herpesvirus type 5
IL	Interleukin
FITC	Fluorescein-5-isothiocyanate (FITC)
PCoA	Principal Coordinates Analysis
OUT	Operational Taxonomic Units
Sp- TTFC	Spores of *B. subtilis* adsorbed with TTFC
OPD	Ortho-Phenylenediamine

OUTLINE OF THE THESIS

This Thesis reports the results I obtained during the Ph.D. program in Biology at University Federico II of Naples, Italy. Two years and eight months were spent in the laboratory of Prof. Ezio Ricca at the University of Naples and four months in the Microbiota-Host Interactions and Clostridia laboratory at Universidad Andrès Bello, in Santiago, Chile, in the laboratory of Prof. Daniel Paredes-Sabja.

Aim of my Thesis was to develop a new **mucosal vaccination strategy** against the pathogen *Clostridium difficile* able to **target the spore** of this important pathogen. *C. difficile* is a Gram-positive, spore-forming and obligate anaerobic gastrointestinal bacterium, currently considered the most common cause of hospital-acquired infectious diarrhea in the developed world [1]. The *C. difficile* infection (CDI) is transmitted by *C. difficile* spores that, once in the gut, interact with the intestinal cells of the host and persist in the spore form. In the gastrointestinal environment, *C. difficile* spores can germinate and colonize the intestine when the conditions are favorable, i.e. when the number of other intestinal bacteria is reduced, for example, by an antibiotic treatment. Germination-derived vegetative cells of *C. difficile*, then produce virulence factors, such as the TcdA and TcdB toxins, that induce a strong immune response and cause CDI [2]. Therefore, a vaccine able to block the interaction of *C. difficile* spores with the intestinal cells by acting at the mucosal surfaces is a potentially effective therapeutic treatment against CDI. However, mucosal vaccines have some drawbacks that have so far limited their use: the low immunogenicity of most mucosal antigens, the lack of efficient mucosal adjuvants, and the lack of delivery systems, able to protect antigens from degradation at the mucosal surface [3]. For these reasons, in my Thesis work I decided to use the spore of ***Bacillus subtilis*** **as a mucosal delivery system**. The *B. subtilis* spore has been exploited as a vaccine vehicle in recent years and tested with several antigens and enzymes [4-6]. The C fragment of the tetanus toxin (TTFC) of *Clostridium tetani* [7-10], the binding subunit of the heat-labile toxin (LTB) of *Escherichia coli* [11, 12], the protective antigen (PA) of *B. anthracis* [10], the C terminus of toxin A of *Clostridium difficile* [13], the capsid proteins VP26 and VP28 of the White Spot

Syndrome virus [14, 15] and the MPT64 antigen of *Mycobaterium tuberculosis* [16] are examples of antigens displayed by *B. subtilis* spores and tested as mucosal vaccines.

The general idea to pursue the aim of my Thesis was to: i) identify potential antigens of *C. difficile* spores; ii) display the selected antigens on *B. subtilis* spores; iii) use *B. subtilis* spores carrying the antigens for the mucosal immunizations of model animals; and iv) assess the levels of immune response obtained.

C. difficile spores are structurally similar to those of other spore formers and are characterized by a dehydrated cytoplasm (core) surrounded by protective layers, the peptidoglycan-like cortex, the proteinaceous coat and the outermost exosporium, rich in glycoproteins [17]. The BclA family of collagen-like glycoproteins are homogeneously distributed in the exosporium of *C. difficile* spores. Two members of the family, BclA2 and BclA3, have been identified as responsible of the formation of hair-like projections on the spore surface of the hyper-virulent strain R20291 [18]. BclA2 and BclA3 are orthologous of the BclA proteins of *Bacillus anthracis*, are abundantly represented in the exosporium of *C. difficile* and, at least in the hyper-virulent strain R20291, mediate spore uptake by macrophages and epithelial cells [19-21]. The CdeC protein is also localized on the exosporium of *C. difficile* spores and has been shown available for antibody recognition and required for the morphogenesis of the coat and the exosporium [22]. For these reasons, I have selected BclA2, BclA3 and CdeC as potential antigens for a mucosal anti-spore vaccination strategy against CDI.

In **CHAPTER 1** of this Thesis, the C-terminal domain of *C. difficile* exosporium protein BclA2 ($BclA2_{CTD}$) was identified as a potential antigen by a bio-informatic approach. $BclA2_{CTD}$ was efficiently adsorbed on the surface of *B. subtilis* spores and shown to be more stable than the free antigen. Mice were nasally immunized with the adsorbed spores and shown to induce a $BclA2_{CTD}$–specific immune response. These results have been published on the journal *International Journal of Molecular Sciences (IJMS)*.

In **CHAPTER 2** of this Thesis the C-terminal domain of the exosporium protein BclA3 ($BclA3_{CTD}$) was selected as an antigen, displayed on the surface of *B. subtilis* by a recombinant approach and tested *in vivo* in a murine model. Mice immunized with recombinant spores or with the pure antigen were able to produce $BclA3_{CTD}$–specific IgG. The immunization with pure $BclA3_{CTD}$

impaired weight loss after a challenge with *C. difficile* spores and induced a decrease on *C. difficile* spore load in feces one day after the infection. A manuscript summarizing these results is in preparation.

In **CHAPTER 3** of this Thesis recombinant spores of *B. subtilis* displaying $BclA2_{CTD}$, $BclA3_{CTD}$ or $CdeC_{CTD}$ were tested for adherence to differentiated and undifferentiated Caco-2 cells, an *in vitro* model of intestinal epithelial cells. Spores displaying $CdeC_{CTD}$ were shown to adhere more efficiently to differentiated and undifferentiated Caco-2 than spores displaying the other antigens or wild type spores. The pre-incubation with human serum increased the adherence patterns in all the strains. Recombinant spores displaying $CdeC_{CTD}$ were also shown to recruit vitronectin, an extracellular matrix protein, at higher levels than spores displaying the other antigens. A manuscript summarizing these results is in preparation.

In **APPENDIX I** I report an unrelated study I contributed to during my stay in the laboratory of Prof. Ezio Ricca at the University of Naples. In this study, it was evaluated the efficacy of a probiotic treatment as an adjuvant of a mucosal vaccination with *B. subtilis* spores displaying the C fragment of the tetanus toxin (TTFC). Spore-adsorbed TTFC were shown able to induce an immune response in nasally immunized mice and the probiotic treatment with *Bacillus toyonensis* spores increased both humoral and cellular immune response. The effects of the probiotic treatment and of the immunization on the gut microbial composition were also evaluated. This work has been recently published on the journal *Microbial Cell Factories.* My contribution to this project relied on the steps of TTFC purification, adsorption to *B. subtilis* spores and following experiments to verify the correct adsorption reaction.

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CHAPTER 1

Induction of a specific humoral immune response by nasal delivery of $BclA2_{CTD}$ of *Clostridium difficile*

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Published on International Journal of Molecular Sciences

1.1. Abstract

Clostridium difficile is a spore-forming bacterium considered as the most common cause of nosocomial infections in developed countries. The spore of *C. difficile* is involved in the transmission of the pathogen and in its first interaction with the host, therefore, a therapeutic approach able to control *C. difficile* spores would improve the clearance of the infection. The C-terminal (CTD) end of BclA2, a spore surface protein of *C. difficile* responsible of the interaction with the host intestinal cells, was selected as a putative mucosal antigen. The BclA2 fragment, $BclA2_{CTD}$, was purified and used to nasally immunize mice both as a free protein and after adsorption to the spore of *Bacillus subtilis*, a well-established mucosal delivery vehicle. While the adsorption to spores increased the *in vitro* stability of $BclA2_{CTD}$, *in vivo* both, free and spore-adsorbed $BclA2_{CTD}$, were able to induce a similar, specific humoral immune response in a murine model. Although in the experimental conditions utilized, the immune response was not protective, the induction of specific IgG indicates free or spore-bound $BclA2_{CTD}$, as a promising mucosal antigen for a vaccine targeting *C. difficile* spores.

1.2. Introduction

Clostridium difficile is a Gram-positive, spore-forming and obligate anaerobe gastrointestinal bacterium, currently considered the most common cause of hospital-acquired infectious diarrhea in the developed world [1]. C. difficile spores are transmitted by the oro-fecal route and, once in the gut, interact with the intestinal cells of the host and persist in the spore form. In the gut, the spores can germinate and colonize the intestine when the conditions are favorable, i.e. when the number of other intestinal bacteria is reduced, for example, by an antibiotic treatment. Germination-derived vegetative cells of *C. difficile*, then produce virulence factors, such as the TcdA and TcdB toxins, that induce a strong immune response and cause the *C. difficile* infection (CDI) [2]. CDI produces a spectrum of clinical responses that can range from asymptomatic colonization to mild or severe diarrhea, pseudomembranous colitis, toxic megacolon, bowel perforation, sepsis, and possible death. In the last twenty years, the severity of CDI increased worldwide due to the emergence of new hyper-virulent strains such as R20291 [3]. These strains are resistant to broad-spectrum antibiotics and

contain mutations in a negative regulator of the expression of the TcdA and TcdB toxins, thus increasing toxin production [4] and host mortality rate [5].

The antibiotics vancomycin and metronidazole have been the first choices of treatment for CDI for about 30 years [6]. Nowadays, in some CDI cases the patients do not respond to the antibiotics while in some other cases they undergo recurrence of the infection after a first episode [7]. Recurrence of CDI can affect up to 35% of the patients and largely increases after a second and a third episode [8]. The reduced efficacy of antibiotics has stimulated the development of new potential therapeutic options. Vaccines, developed to target C. difficile toxins [9, 10], have not been successful so far, while fecal microbiota transplantation [11, 12] and probiotic therapies [13], although very promising, are still under investigation. Since *C. difficile* spores have an essential role in the transmission of the pathogen and in its first interaction with the intestinal cells, new anti-CDI treatments must focus on the spore and on its interaction with the host cells.

C. difficile spores are structurally similar to those of other spore formers and are characterized by a dehydrated cytoplasm (core) surrounded by protective layers, the peptidoglycan-like cortex, the proteinaceus coat and the outermost exosporium, rich in glycoproteins [14]. The BclA family of collagen-like glycoproteins are homogeneously distributed in the exosporium of *C. difficile* spores. Two members of the family, BclA2 and BclA3, have been identified as responsible of the formation of hair-like projections on the spore surface of the hyper-virulent strain R20291 [15]. Both BclA2 and BclA3 are involved in the interaction with intestinal epithelial cells [15] and therefore are potential targets of new anti-CDI treatments. BclA2 protein of *C. difficile* R20291 is a 47.8 kDa protein, organized into three domains: (i) an N-terminal domain (NTD) anchored to the exosporium; (ii) a collagen-like domain; (iii) and a C-terminal domain (CTD) exposed to the exterior [16].

In this study, the BclA2 protein of *C. difficile* R20291 was evaluated as a potential antigen to be used to develop a mucosal vaccination strategy against CDI. The 131 amino acids CTD domain of BclA2 ($BclA_{CTD}$) was selected as a candidate mucosal antigen. $BclA2_{CTD}$ was displayed on the surface of *B. subtilis* spores, a well characterized mucosal vaccine delivery system [17, 18], to increase its stability and favor its delivery. Free and spore-adsorbed $BclA2_{CTD}$ were nasally administered to mice and tested for specific anti-$BclA2_{CTD}$ immune response.

1.3. Results

1.3.1. Purified BclA2$_{CTD}$ has low stability

First, we used an *in silico* approach, based on the analysis of the physicochemical properties of amino acid residues and their frequency of occurrence in experimentally known segmental epitopes in the amino acid sequence of BclA2 (Kolaskar and Tongaonkar Antigenicity Method) [19]. The 131 amino acid residues corresponding to the C-terminal end of BclA2 (BclA2$_{CTD}$) was identified as a putative antigenic domain for the high score as B cell epitope [19] and as T cell MHC-I and MHC-II epitope [20, 21] (Figure 1.1A). Therefore, His-tagged BclA2$_{CTD}$ was overexpressed in *E. coli* BL21(DE3) and purified by affinity chromatography with Ni-sepharose columns as described in the Methods section. Next, to evaluate the stability of BclA2$_{CTD}$ 800 ng of the purified protein suspended in phosphate buffer (PBS) pH 7.0 were incubated for 48 hours either at -20°C, +4°C or +25°C and compared to the same amount of protein stored in the same buffer at -80°C by dot blotting experiments with anti-His antibody (Figure 1.2A). Results of the densitometric analysis of the dot blot (Suppl. Mat. Table S1.1) were plotted and showed that while storage at -20°C caused only a minimal decrease in the amount of protein detected by the antibody, storage at 4°C and 25°C caused over 30% and 80% decrease, respectively (Figure 1.2B).

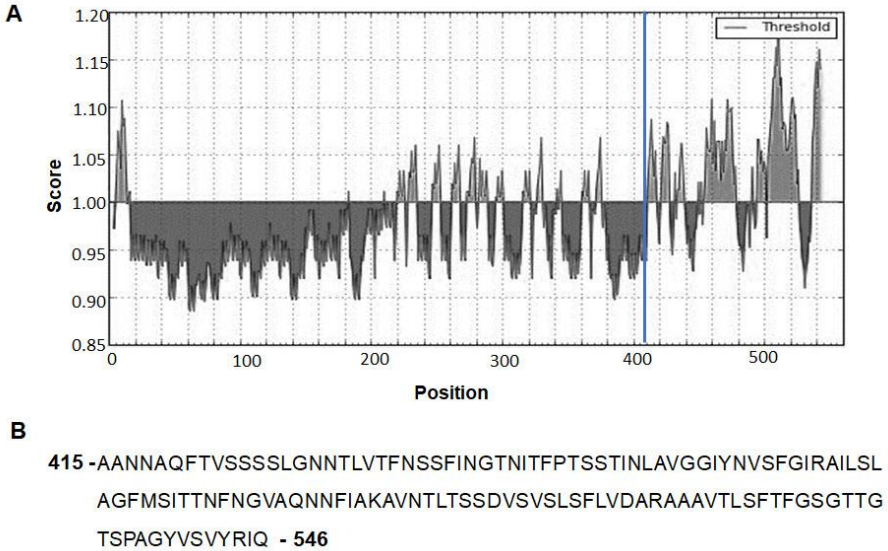

Figure 1. 1. *In silico* analysis of BclA2 from *C. difficile* R20291. (A) Analysis of the B cell epitope propensity score (Kolaskar & Tongaonkar Antigenicity Method from Immune Epitope Database) of BclA2. The X- and Y-axes represent the sequence position and antigenic propensity score, respectively. The threshold value was generated by default by Immune Epitope Database (http://tools.iedb.org/bcell/). The regions above the threshold are antigenic. **(B)** Amino acid sequence of the 131 residues at the C-terminal end of BclA2 identified as a potential antigenic region and indicated as BclA2$_{CTD}$.

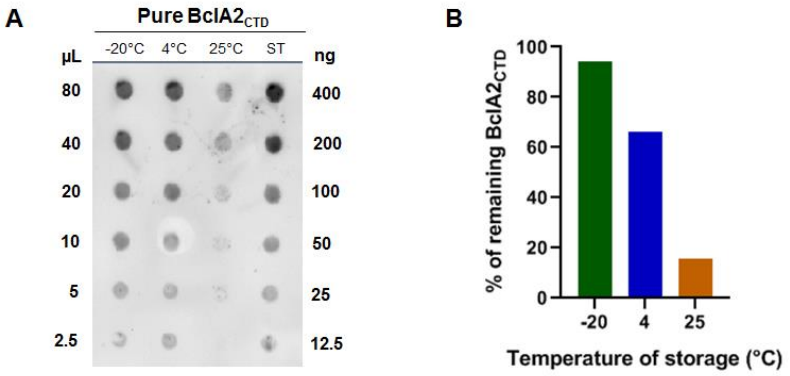

Figure 1. 2. Stability of purified BclA2$_{CTD}$. (**A**) Dot blotting of purified BclA2$_{CTD}$ stored for 2 days at -20°C, +4°C or at +25°C with anti-6xHis antibody. The protein stored at -80°C right after purification (ST) was considered as standard. Loaded volumes of purified protein (µl) and known amount of the standard (ng) are indicated. (**B**) Plot of the densitometric analysis of dot blot experiment of panel A, considering the amount of protein stored at -80°C as 100%.

1.3.2. BclA2$_{CTD}$ is efficiently displayed on B. subtilis spores

In order to increase the stability of BclA2$_{CTD}$, the antigen was adsorbed on *B. subtilis* spores, as previously reported for other antigens [22, 23]. Purified BclA2$_{CTD}$ (4 µg) was incubated with purified spores ($2x10^9$) of the *B. subtilis* strain PY79 [24] as schematically shown in Figure 1.3A.

The reaction mixture was then fractionated by centrifugation and pellet (containing spores and spore-bound BclA2$_{CTD}$) and supernatant (containing unbound, free BclA2$_{CTD}$) fractions analyzed. Spores in the pellet fraction were used to extract surface proteins that were then analyzed by western-blot with anti-6xHis antibody. As shown in Figure 1.3B, BclA2$_{CTD}$ was extracted from *B. subtilis* spores adsorbed with the purified protein, indicating that BclA2$_{CTD}$ was adsorbed to purified spores. The supernatant fraction of the adsorption reaction was then analyzed by dot blotting with anti-6xHis antibody to measure the amount of BclA2$_{CTD}$ left unbound and evaluate the efficiency of adsorption (Figure 1.3C), as previously reported [25]. In parallel, the same amount of purified BclA2$_{CTD}$ used in the adsorption reaction (4 µg) was incubated for 1 hour in PBS at pH 3.5 and analyzed by dot blotting with anti-6xHis antibody, showing no degradation under those conditions (Figure 1.3C). Results of the densitometric analysis of the dot blot (Suppl. Mat. Table S1.2) were plotted and showed that 0.4% of the BclA2$_{CTD}$ used in the adsorption reaction was left unbound, indicating that more than 99% (corresponding to 3.9 µg) of BclA2$_{CTD}$ was adsorbed to *B. subtilis* spores (Figure

1.3D). These results are consistent with previously reported efficiency of adsorption for other proteins and antigens [23].

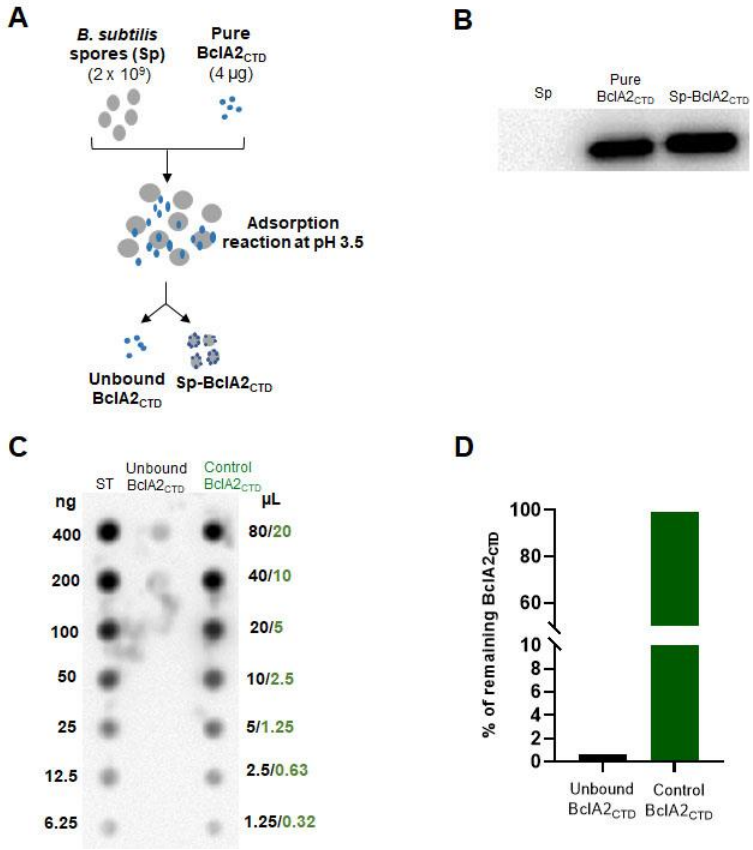

Figure 1. 3. Adsorption of BclA2$_{CTD}$ on *B. subtilis* spores. (**A**) Schematic representation of the adsorption reaction. (**B**) Western-blot of spore-extracted proteins after the adsorption reaction with anti-6xHis antibody. *B. subtilis* spores (Sp) and pure BclA2$_{CTD}$ were used as negative and positive controls, respectively. (**C**) Dot blot analysis of the supernatant of the adsorption reaction. Loaded volumes (µl) of the supernatant fraction (Unbound BclA2$_{CTD}$) and of the pure protein incubated in the same condition of adsorption (control BclA2$_{CTD}$) are indicated in black and green, respectively. Known amounts (ng) of the pure protein incubated at -80°C (ST) were used as standard. **(D**) Plot of the densitometric analysis of the dot blot experiment of panel C, considering the amount of protein stored at -80°C as 100%.

1.3.3. BclA2$_{CTD}$ is stabilized by the adsorption on B. subtilis spores

To evaluate the stability of spore-adsorbed BclA2$_{CTD}$, 2x10^9 spores displaying 4 µg of BclA2$_{CTD}$ were centrifuged and incubated as pellets for 48 hours either at -20°C, +4°C or +25°C. Pellets incubated at -80°C were used as control. The spore surface proteins were then extracted and analysed by dot blot experiments with anti-6xHis antibody and the amount of BclA2$_{CTD}$ compared to known amounts of pure protein stored at -80°C immediately after its purification as standard (ST)

(Figure 1.4A). Results of the densitometric analysis of the dot blot (Suppl. Mat. Table S1.3) were plotted and showed that the amount of $BclA2_{CTD}$ recognized by the antibody was not decreased after incubation at -20 or +4°C; however, a 50% decrease in $BclA2_{CTD}$ recognition was observed after incubation at +25°C (Figure 1.4B).

The comparison of the results of Figures 1.2 and 1.4 clearly indicate that upon adsorption to spores the heat stability of $BclA2_{CTD}$ was increased, suggesting that the binding to the spore protects the antigen from degradation.

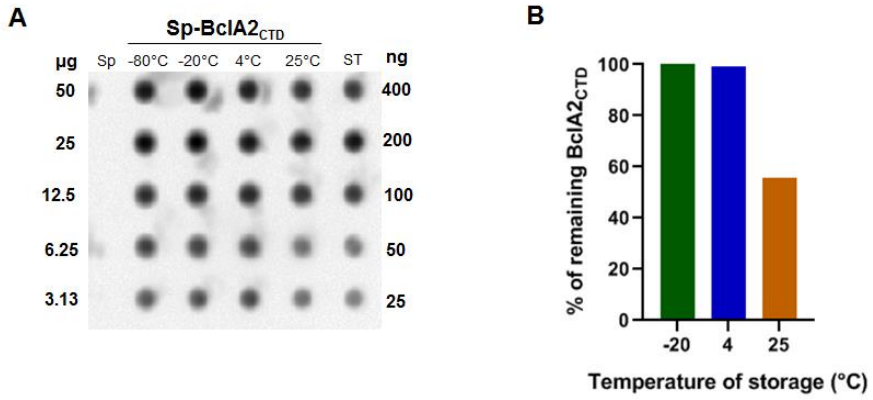

Figure 1. 4. Stability of spore-adsorbed $BclA2_{CTD}$. (**A**) Dot blot of the protein extracted from spores adsorbed with $BclA2_{CTD}$ stored for 2 days at -80°C, -20°C, +4°C or +25°C, using an anti-6xHis antibody. Proteins extracted from *B. subtilis* spores (sp) were used as negative control. Known amount of the pure protein stored at -80°C immediately after its purification (ST) were used as controls (right). (**B**) Plot of the densitometry analysis of dot blot experiment of panel A, considering the amount of protein extracted from spores stored at -80°C as 100%.

1.3.4. Adsorption of $BclA2_{CTD}$ increase spore adherence to Caco-2 cells

The adherence of *B. subtilis* spores adsorbed with $BclA2_{CTD}$ to differentiated Caco-2 cells was evaluated and compared to the adherence pattern of *C. difficile* R20291 and *B. subtilis* spores. While the adherence of R20291 spores reached 5 spores/cell, *B. subtilis* spores had an average adherence of less than 1 spore/cell (Figure 1.5). The adherence of *B. subtilis* spores to Caco-2 cells was significantly ($P < 0.0001$) increased upon adsorption of $BclA2_{CTD}$ (Figure 1.5), suggesting that $BclA2_{CTD}$ contributes to adherence of the spores to intestinal epithelial cells.

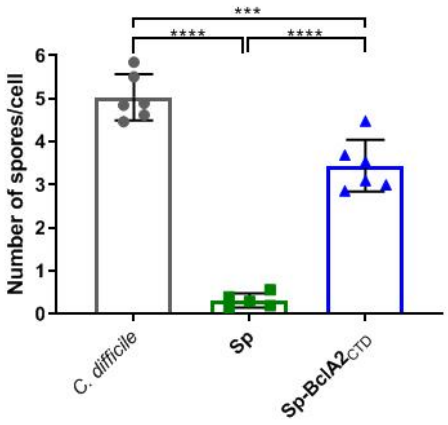

Figure 1. 5. Adherence of spores to human intestinal cells. Caco-2 cells were differentiated for 8 days and infected with spores of *C. difficile* R20291, *B. subtilis* (Sp) and *B. subtilis* spores adsorbed with $BclA2_{CTD}$ (Sp-$BclA2_{CTD}$). The number of adhered spores and cells was counted in 10 microscopic fields on phase-contrast microscopy. The data represent the mean of three independent experiments and the error bars are the Standard error of the mean. The groups were compared with One-way ANOVA Turkey's multiple comparison test and statistical differences ($P < 0.05$) are indicated by asterisks.

1.3.5. Intranasal immunization

B. subtilis spores presenting $BclA2_{CTD}$ on their surface, and the pure $BclA2_{CTD}$ peptide, were used for mucosal immunization experiments in mice. In particular, the animals were assigned into 4 experimental groups (n=11), according to the immunization regimen: i) PBS; ii) 2×10^9 *B. subtilis* PY79 spores (Sp); iii) 2×10^9 *B. subtilis* spores adsorbed with $BclA2_{CTD}$ (Sp-$BclA2_{CTD}$); iv) 4 μg of purified $BclA2_{CTD}$. The animals were nasally immunized 42, 28 and 14 days before the challenge with *C. difficile* R20291. Animal serum was collected one day before each immunization and on the time of sacrifice (Figure 1.6). The production of specific anti-$BclA2_{CTD}$ IgG was assessed by ELISA (Figure 1.7).

Animals treated with purified $BclA2_{CTD}$ showed a significant increase of IgG titers mainly after the second immunization ($P < 0.0001$). The same result was observed for animals immunized with Sp-$BclA2_{CTD}$ ($P < 0.0001$) suggesting that the spore-bound antigen is correctly assembled and able to induce a response. However, no differences were observed between the purified and the spore-bound antigen indicating that *Bacillus* spores did not have an adjuvant effect.

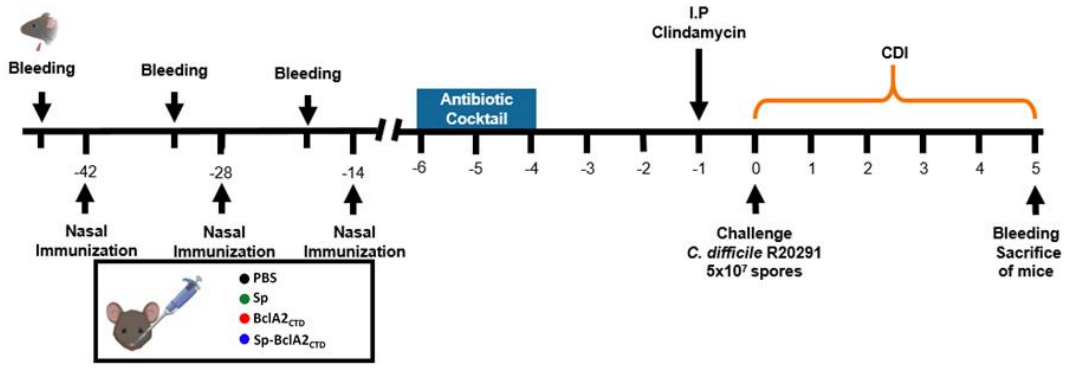

Figure 1. 6. Overview of the experimental design schematics for the prevention of *C. difficile* infection in a murine model. C57BL/6 mice were nasally immunized 3 times (42, 28 and 14 days before challenge with *C. difficile* R20291 spores) with PBS, spores of *B. subtilis* PY79 (Sp), pure $BclA2_{CTD}$ or spores of *B. subtilis* adsorbed with $BclA2_{CTD}$ (Sp-$BclA2_{CTD}$). Prior to the infection, the animals were submitted to an antibiotic cocktail (4 to 6 days before challenge) and clindamycin administration (1 day before challenge). On day 0 mice were infected with $5x10^7$ spores of *C. difficile* R20291 and were monitored for CDI symptoms from day 0 to day 5. Serum was collected one day before each immunization as well as on the day of sacrifice (days 0, 13, 27 and 47).

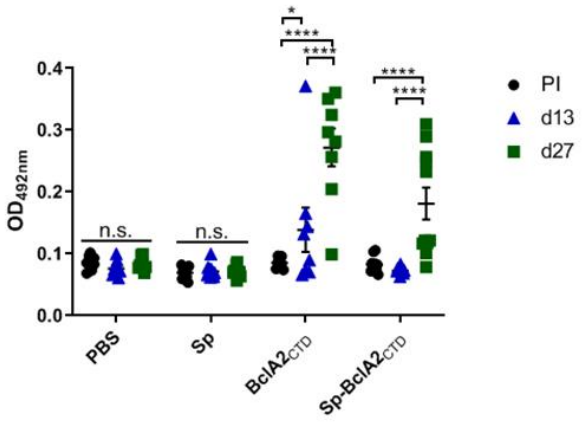

Figure 1. 7. Immunogenicity of $BclA2_{CTD}$ protein and Sp-$BclA2_{CTD}$ in mice after nasal immunizations. IgG anti-$BclA2_{CTD}$ levels were measured by ELISA in the serum of mice on days 0 (Pre-Immune serum, PI), 13 (d13) and 27 (d27) after the beginning of the experiment (one day before each immunization). Results are reported as optical density (OD) units at 492nm. The geometric mean (± standard error of the mean) for each group is shown. IgG titers of each group were compared between d0 (PI), d13 and d27 with One-way ANOVA Turkey's multiple comparison test and statistical differences ($P < 0.05$) are indicated by asterisks.

To explore the pattern of recognition of *C. difficile* spores by the raised antibodies, spores of *C. difficile* R20291 were incubated with the serum of mice immunized with purified $BclA2_{CTD}$, Sp-$BclA2_{CTD}$, or PBS and stained with secondary anti-mouse IgG Alexa 488 conjugate and then analysed by fluorescence microscopy (Figure 1.8A). No immunofluorescence reactivity was observed in

serum of mice immunized with PBS (control). By contrast, significant immunofluorescence was evidenced by antibodies produced by immunizing mice with purified BclA2$_{CTD}$ and spore-adsorbed BclA2$_{CTD}$ (average fluorescence intensity was 88- and 89-fold higher than the control, respectively) (Figure 1.8B). Altogether these results indicate that the nasal immunization with either purified free BclA2$_{CTD}$ or spore-bound BclA2$_{CTD}$ was capable of inducing BclA2$_{CTD}$-specific antibody production.

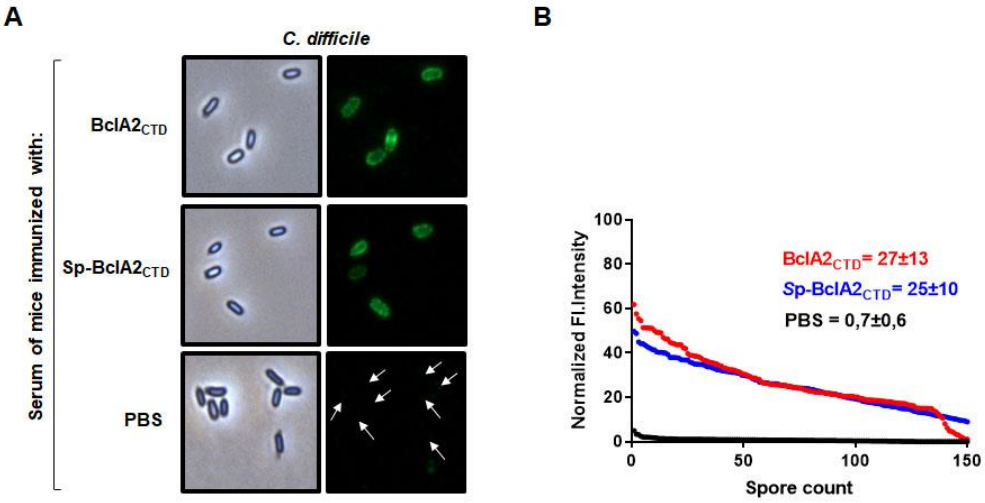

Figure 1. 8. Recognition of *C. difficile* spores by the serum of animals immunized with purified BclA2$_{CTD}$ and Sp-BclA2$_{CTD}$. (A) Spores of *C. difficile* R20291 were incubated 1 hour with serum (1:100) of mice immunized with BclA2$_{CTD}$ and Sp-BclA2$_{CTD}$ or PBS, as indicated. **(B)** The immunofluorescence micrographs are depicted in fluorescence intensity (FI.Intensity) profiles provided by fluorescence microscopy images using ImageJ. The values shown in the graphs are the average ± standard error of the fluorescence intensity from 150 spores. The secondary antibody is anti-mouse conjugated with Alexa 488.

1.3.6. Effect of nasal- BclA2$_{CTD}$ immunization against C. difficile R20291 infection

Next, we assessed whether nasal immunization of mice protected against a *C. difficile* challenge. Weight loss and presence of diarrhea after infection, characterized by high spore-load in feces and colonic tissues, are expected symptoms of CDI. We measured weight loss progression over the five days after challenge and assigned a score of diarrhea according to its severity. Results demonstrate that nasal immunizations did not halt the weight loss in the subsequent 5 days after infection (Figure 1.9A). However, despite not being statistically significant, the animals immunized with purified BclA2$_{CTD}$ showed a delayed onset of diarrhea, contrary to the other groups in which more than 50% of the animals already had diarrhea at day 1 after the infection (Figure 1.9B). Moreover, when comparing the score of diarrhea between groups (Figure 1.9C) it is possible to

observe that on day 1 after infection, the group of animals immunized with purified BclA2$_{CTD}$ had a reduced score of diarrhea even though not statistically significant. Interestingly, on the day 3 after infection the animals immunized with adsorbed spores showed a reduction in *C. difficile* spore load in feces (Figure 1.9D), which became significant on day 5 after infection (P = 0.0215 and P = 0.0306 when compared with Sp and purified free BclA2$_{CTD}$).

No significant differences between groups in *C. difficile* spore-load in the ileum or distal colon were observed (Figure 1.10A). However, animals immunized with Sp-BclA2$_{CTD}$ showed a slight decrease in spore-load in the proximal and middle colon in comparison with the other groups. This reduction became significant in comparison with the animals immunized with purified BclA2$_{CTD}$ (P = 0.0041 for proximal colon and P = 0.0324 for middle colon). However, there were no significant differences between groups on the cytotoxic titers of the cecal content (Figure 1.10B) meaning that the immunizations did not halt spore colonization inside the cecum.

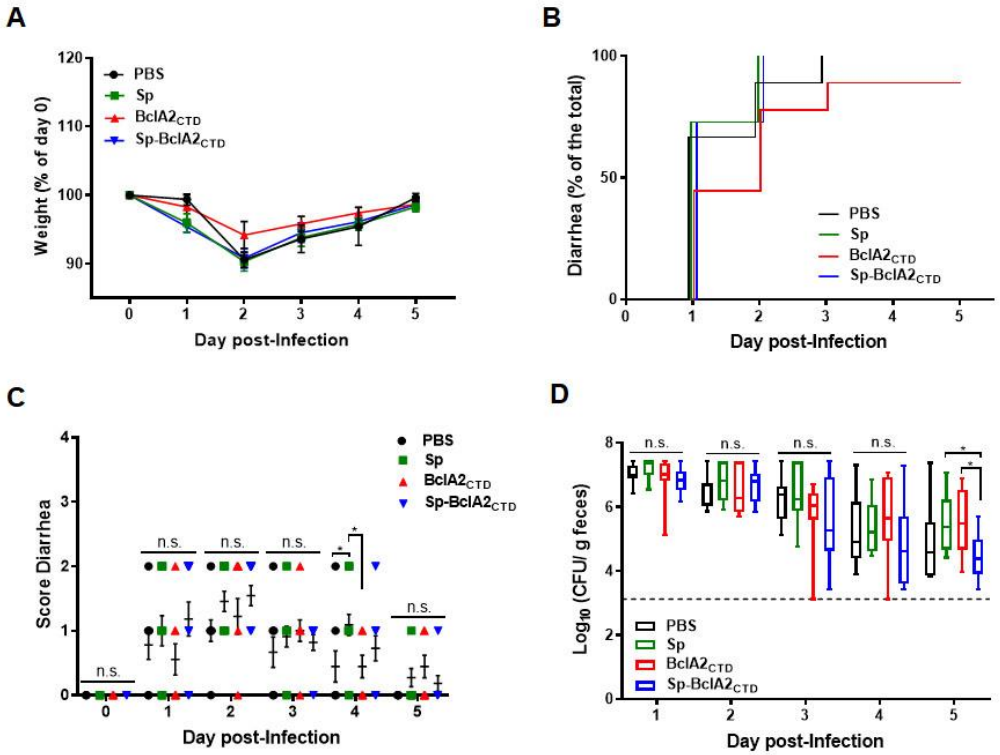

Figure 1. 9. Challenge experiments. C57BL/6 mice were nasally immunized with PBS, spores of *B. subtilis* (Sp), Sp-BclA2$_{CTD}$ and purified BclA2$_{CTD}$ (9 to 11 animals each group) and challenged with *C. difficile* R20291 spores. Mice were monitored in the following 5 days after infection for: (**A**) weight loss, presented as the relative % of the weight to the day of infection (day 0); (**B**) time of occurrence of diarrhea, presented as the relative % of diarrhea in a group to the total mice; (**C**) score of diarrhea per day and (**D**) number of *C. difficile* spores in feces (represented as $\log_{10}$ CFU/g of feces); Differences between groups were assessed by Two-way ANOVA Turkey's multiple comparison test and statistical differences ($P < 0.05$) are indicated by asterisks. The bars are the geometric mean ± standard error of the mean.

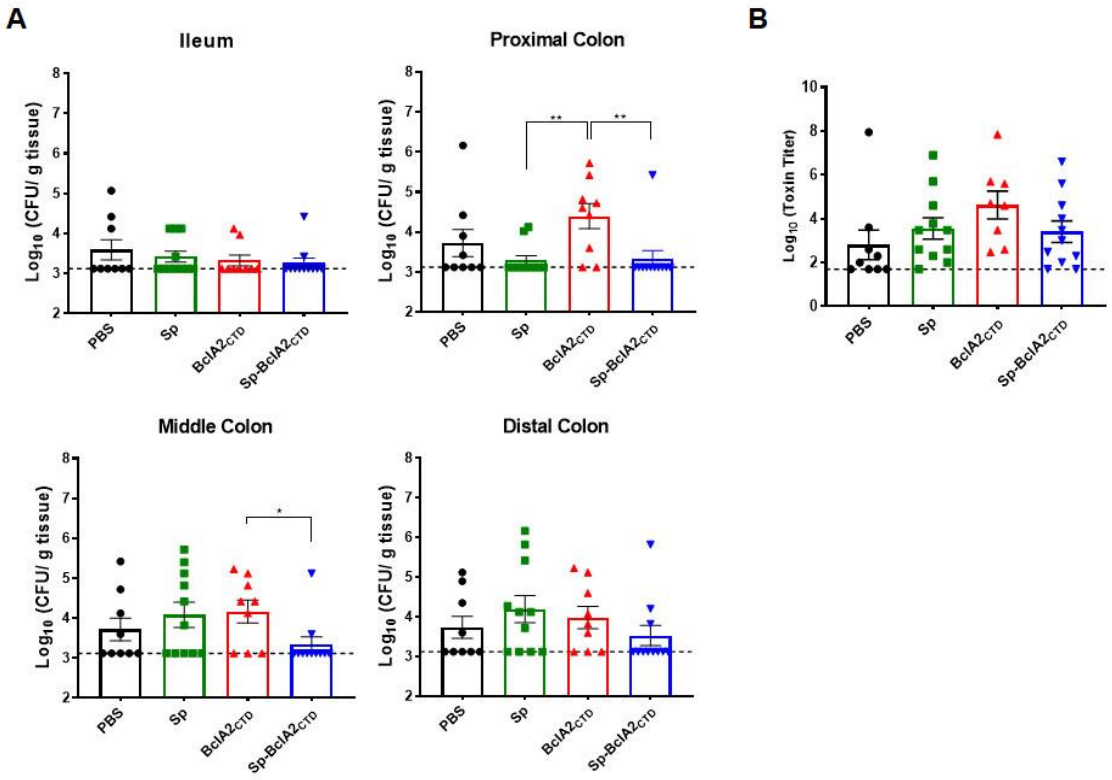

Figure 1. 10. Challenge experiments on colonic tissues. (A) The load of C. difficile spores in the Ileum, Proximal Colon, Middle Colon and Distal Colon was evaluated upon sacrifice as $\log_{10}$ CFU/g of tissue. **(B)** The cecum content toxicity was measured and represented as $\log_{10}$ toxin titer. The differences between groups are evaluated with Mann-Whitney between groups for each tissue and One-way ANOVA Turkey's multiple comparison test for Toxin titers. Statistical differences ($P < 0.05$) are indicated by asterisks The bars are the geometric mean mean ± standard error of the mean.

1.4. Discussion

In recent years the emergence of antibiotic-resistant, hypervirulent strains has urged the scientific community to develop new therapeutic strategies to fight *C. difficile* infections (CDI) [26, 27]. New treatments based on probiotics or fecal transplants, although very promising, are still at an experimental stage while vaccines targeting *C. difficile* cells [28] or toxins [29] have been so far unsuccessful. Since CDI is transmitted by spores that are also responsible of the initial interaction with the host, alternative vaccination strategies have to focus on the spore and on the surface proteins involved in the interaction with the intestinal epithelial cells. Most *C. difficile* genomes have a pseudogenized version of *bclA1*, leading to the expression of a 48 aa polypeptide sequence of the amino-terminal domain that localized to the exosporium layer of *C. difficile* spores [16]. In this context, recent work has shown that intraperitoneal immunization with the spore surface protein BclA1 induced a strong, specific immune response but failed to provide protective immunity [30].

Two additional collagen-like exosporium proteins are also encoded in *C. difficile* genomes, BclA2 and BclA3, which to date have not been evaluated as vaccine candidates. In this study, a fragment of the spore surface protein BclA2 ($BclA2_{CTD}$) was identified as a potential antigen and tested as a mucosal vaccine.

The mucosal surfaces of the host are the most common route of entry used by pathogens, including *C. difficile*, therefore it is important for a vaccine to elicit an immune response at the mucosal surfaces. However, only few mucosal vaccines are currently licensed, mostly because of the low immunogenicity of mucosal antigens and to antigen degradation during storage or at the mucosal surfaces [31]. One strategy that we used to avoid or reduce antigen degradation was to display $BclA2_{CTD}$ on *B. subtilis* spores, a mucosal vaccine delivery system previously tested with other antigens [22, 23]. Our data demonstrates that $BclA2_{CTD}$ was very efficiently displayed in *B. subtilis* spores and the interaction with the spore surface layers reduced $BclA2_{CTD}$ degradation during storage, as previously reported for other antigens and enzymes [32]. However, in spite of the low *in vitro* stability of the free $BclA2_{CTD}$ with respect to the spore-bound antigen, both forms of $BclA2_{CTD}$ induced a similarly strong humoral immune responses when administered to mice by the nasal route.

Upon immunizations mice were subjected to a challenge with the epidemically-relevant *C. difficile* strain R20291. Although no obvious protective effects were observed on mice, the *C. difficile* spore load in feces showed a tendency to decrease 3 days after infection in animals immunized with the spores displaying $BclA2_{CTD}$. In addition, these same group of animals also showed a reduction of spore load in the proximal and middle colon, possibly a consequence of *C. difficile* spore opsonization by anti-$BclA2_{CTD}$ antibodies. A similar lack of protection efficacy was previously observed upon immunization with the BclA1 and SleC, but not with the cysteine rich proteins CdeC and CdeM [30], suggesting that the collagen-like proteins BclA1 and SleC are not good vaccine candidates. A possible explanation of the lack of clear signs of protection observed in our results is that the dose of antigen might not have been high enough to reduce CDI symptoms and *C. difficile* spore germination inside the mice cecum. Indeed, works using *B. subtilis* spores adsorbed with heterologous antigens that induced a protective effect in nasally immunized mice applied a higher dose regimen and frequency than we did in this work. Plus, some works have also used the same

immunization regimen and induced both humoral and cellular immune responses. However, they aimed to have a stronger effect in the nasal mucosa and not in the gut, since they used *Mycobacterium tuberculosis* antigens or H5N1 virions [33, 34].

Additional *in vivo* experiments with higher doses of antigen and/or using the oral route of immunization would be needed to address this point. Even though no protection against a challenge was observed, the identification of an efficient antigen for *C. difficile* spores, active when administered by the mucosal route is a promising result that could open to a new vaccination strategy against CDI.

1.5. Materials and Methods

1.5.1. Bacterial strains and spore purification

B. subtilis PY79 [24] was used for the adsorption reaction. The hyper-virulent *C. difficile* strain R20291 was used for the challenge experiment. *E. coli* strain BL21 (DE3) (Invitrogen) was used for $BclA2_{CTD}$ overexpression.

Sporulation of *B. subtilis* was induced by the exhaustion method [35]. Briefly, after 35 hours 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 [36]. Spore suspensions were prepared by plating a 1:100 dilution of an overnight culture onto a 70:30 medium (63 g Bacto peptone (BD Difco), 3.5 g proteose peptone (BD Difco), 0.7 g ammonium sulfate $(NH_4)_2SO_4$, 1.06 g Tris base, 11.1 g brain heart infusion extract (BD Difco) and 1.5 g yeast extract (BD Difco) for 1 L) and incubating it for 7 days at 37°C under anaerobic conditions [37]. After incubation, plates were removed from the chamber and the surface was scraped up with ice-cold sterile water. Next, the spores were gently washed five times with ice-cold sterile water in micro centrifuge at 14 000 rpm for 5 minutes. Spores were loaded onto a 50% Nycodenz solution and centrifuged (14 000 rpm, 40 minutes). After centrifugation, the spore pellet was washed five times (14 000 rpm, 5 minutes) with ice-cold sterile water to remove Nycodenz remnants. The spores were counted in Neubauer chamber

and volume adjusted at $5x10^9$ 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.

1.5.2. BclA2$_{CTD}$ over-production and purification

The coding sequence of BclA2 C-terminal domain (CTD) was amplified using the chromosomal DNA of *C. difficile* R20291 (396 bp) as a template and oligonucleotides BclA2$_{CTD}$sense (ggtaccccatggggatccGCAGCAAACAATGCACAATTTACAG, in lower case is the recognition site for KpnI, NcoI and BamHI restriction enzymes) and BclA2$_{CTD}$anti (tctagactgcagCTATTGTATTCTATAAACTGATACATAC, in lower case are the recognition sites for XbaI and PstI restriction enzymes) to prime the reaction. Amplified DNA was cloned in pGEMT-easy (Promega). Subsequently, the gene of interest was cleaved with BamHI/PstI restriction enzymes and inserted in-frame to the sequence coding for an N-terminal poly-histidine tag in the expression vector pRSETA (Invitrogen) previously digested with the same enzymes. Expression of the recombinant gene was controlled by a T7 promoter inducible by lactose. The recombinant plasmid was used to transform competent cells of the *E. coli* strain BL21(DE3). The recombinant strain was grown for 16 hours at 37ºC in auto-induction medium [38] to express the heterologous protein. The His-tagged BclA2$_{CTD}$ protein was purified under native conditions using the His-Trap column (eluted with 500 mM of imidazole) as recommended by the manufacturer (GE Healthcare Life Science). The purified protein was desalted and concentrated with the Centricon cut-off 10 kDa (Merck Millipore). The purity of the protein was verified by SDS-page and Western-blot using Anti-His antibodies.

1.5.3. Adsorption reaction, stability and production of spores for animal immunization

Purified BclA2$_{CTD}$ (4 µg) was added to a suspension of $2x10^9$ *B. subtilis* spores in 0.15 M PBS pH 3.5 at 25°C in a final volume of 200 µL. After 1 hour of incubation free unbound BclA2$_{CTD}$ and spore-adsorbed BclA2$_{CTD}$ (Sp-BclA2$_{CTD}$) were separated by centrifugation and analysed by dot- and western-blot. The stability of free and spore-adsorbed BclA2$_{CTD}$ was assessed by exposing purified BclA2$_{CTD}$ (suspended in phosphate buffer pH 7) and the BclA2$_{CTD}$-adsorbed spores at different temperatures before analysis.

For mice immunization, 144 µg of purified $BclA2_{CTD}$ were incubated with a suspension of $7.4x10^{10}$ *B. subtilis* spores in 0.15 M PBS pH 3.5 at 25°C in a final volume of 8 mL. $2x10^9$ spores were used for western and dot blot analysis and the remaining spores were aliquoted and stored at -80°C for mice immunization.

1.5.4. Western and dot-blot analysis

$2x10^9$ *B. subtilis* spores or pure $BclA2_{CTD}$ were suspended in extraction buffer 2x [25] incubated at 100°C for 7 minutes and loaded onto a 15% SDS-PAGE gel. Proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) and used for Western-blot analysis. For the quantitative evaluation of the amount of $BclA2_{CTD}$, serial dilutions of purified $BclA2_{CTD}$, of the supernatant of the adsorption reaction or of protein extracted from spores displaying $BclA2_{CTD}$ were analysed by dot blot experiments. Protein extraction from the spore coat were obtained by incubating $2x10^9$ Sp-$BclA2_{CTD}$ in SDS 10%, DTT 1 M and Tris-HCl pH 6.8 1.5 M 1 hour at 65°C (200 µL final volume), 2 minutes at 4°C and posterior centrifugation. Filters were then visualized by the ECL-prime (Amersham Pharmacia Biotech) method and subjected to densitometry analysis by Quantity One 1-D Analysis Software (Bio-Rad). Antibodies anti-His were used both in western and dot blot assays (1:7000).

1.5.5. Adherence to Caco-2 cells

An existing stock of Caco-2 cells in the Microbiota-Host Interaction and Clostridia Research Group at the Universidad Andrés Bello was routinely grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's minimal essential medium (DMEM) (HyClone), supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone), penicillin (100 U/mL), and streptomycin (100 µg/ml). Spore adherence of *C. difficile* R20291, *B. subtilis* PY79 and *B. subtilis* adsorbed with $BclA2_{CTD}$ to Caco-2 cell line was measured as previously described [39]. Briefly, Caco-2 cells were seeded onto glass coverslips in 24-wells plates ($4x10^5$ cells per well), and to obtain differentiated Caco-2 cells, cells were cultured for 8-days post-confluence, changing the medium every other day, using previously described methods [40]. Monolayers were infected with $2.5x10^7$ spores in 200 µL of culture medium

without FBS. Spore-infected cells were incubated for 3 hours at 37°C under aerobic conditions, then unbound spores were removed from spore-infected Caco-2 cells by washing three times with DPBS. In order to count the number of cells the nuclei were stained with Hoechst stain (Sigma) 1:1000 in PBS 10 minutes. After several washes with PBS and dH_20 the coverslips were dried 15 minutes at 37°C, mounted using Dako Fluorescence Mounting medium (Dako, North America) and sealed with nail polish. Samples were analysed with an Olympus BX53 microscope. The number of spores and cells were counted and the adherence was represented as number of spores/cell. The experiment was made in triplicates.

1.5.6. Animals

Pathogen-free male or female C57BL/6 mice (age 8–12 weeks) were obtained from a breeding colony at Facultad de Ciencias Biologicas Universidad Andres Bello (Santiago, Chile), established with animals purchased from Jackson Laboratories. Water, bedding and cages were autoclaved, and mice had a 12-hour 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 (Protocol number 0035/2018).

1.5.7. Immunization regimen in mice

Mice were randomly assigned to four experimental groups (11 animals each group) according to the type of immunization received. The mice were intranasally immunized on days 0, 14 and 28 after the beginning of the experiment with 20 µL (10 µL per nostril) of PBS pH 7, $2x10^9$ spores of *B. subtilis* PY79, $2x10^9$ *B. subtilis* PY79 spores adsorbed with $BclA2_{CTD}$ (Sp- $BclA2_{CTD}$) or 4 µg of pure $BclA2_{CTD}$. The day before each immunization and on the day of the sacrifice (on 47^{th} day) the blood was collected.

1.5.8. Animal infection model

Prior to infection, mice were pre-treated with antibiotic cocktail of kanamycin (40 mg/kg body weight; Sigma-Aldrich, U.S.A.), 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. The antibiotic treatment was followed by intraperitoneal administration of a single dose of clindamycin (10 mg/kg) 1 day before *C. difficile* infection [41]. All animals were infected oro-gastrically with 100 µL of PBS containing $5x10^7$ spores of 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) colour 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 [42]. Other clinical symptoms as variations of weight, physical aspect (i.e., abnormal/hunched gait, piloerection), spontaneous behaviour (i.e., lethargy, inactivity or lack of mobility) and emaciation were monitored as described [43]. Moribund mice or mice displaying overt signs of disease were sacrificed. At the time of euthanasia, ileum, proximal, median and distal colon were collected as well as the caecum content.

1.5.9. Quantification of spores from feces and colon samples

Fecal samples were collected daily and stored at -20°C until spore quantification. 10 µL of PBS was added for each mg of stools, mixed and incubated for 30 minutes at room temperature. Then, 50 µL of absolute ethanol (Sigma-Aldrich) was added to 50 µL of faeces and incubated for 30 minutes at room temperature. Samples were serially diluted and plated onto selective medium supplemented with taurocholate (0.1% w/v), Cefoxitin (16 µg/mL) and L-cycloserine (250 µg/mL) (TCCFA plates). The plates were incubated anaerobically at 37ºC for 48 hours, the *C. difficile* colonies were counted and the results were expressed as the Log_{10} (CFU/g of faeces). Proximal, median and distal colon were collected from mice upon sacrifice and washed with PBS with a syringe. Then, they were resuspended and homogenized with 2.5 µl of PBS for each mg of tissue. Upon incubation at room temperature with absolute ethanol and serially diluted they were plated onto

TCCFA plates. The plates were incubated anaerobically at 37ºC for 48 hours. Finally, the colony count was expressed as the Log_{10} (CFU/g of tissue).

1.5.10. Evaluation of BclA2$_{CTD}$-specific IgG levels in mice serum

The blood collected the day before each immunization and at the time of sacrifice was incubated at 37°C for 30 minutes and posteriorly centrifuged at 5 000 rpm for 20 minutes at 4°C. The supernatant, containing the serum fraction was stored at −20°C until use. To assess the production of IgG against BclA2$_{CTD}$, an Enzyme-linked immunosorbent assay (ELISA) was performed. Pure BclA2$_{CTD}$ was coated onto 96-wells plates at 100 ng/well overnight at 4°C. Plates were blocked with PBS-0.05% Tween-20 (PBS-T) containing 2% BSA for 1 hour at 37ºC. 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 hours at 37°C. After the removal of non-adherent IgG by several washes, the plates were incubated with secondary antibody anti-mouse HRP, for 1 hour at 37°C. Finally, the colorimetric reaction was initiated upon the addition of 50 µL of reaction buffer containing 0.05 M citric acid, 0.1 M disodiumhydrogen phosphate, 2 mg/mL of o-phenlyendiamine (Sigma-Aldrich, U.S.A.) and 0.015% of H_2O_2 (Merck, Germany). The reaction was stopped after 20 minutes with 25 µL of 4,5 N of H_2SO_4 and absorbance was measured at 492 nm. The experiment was performed in duplicate.

1.5.11. Immunofluorescence analysis

Spores of *C. difficile* R20291 were fixed on poly-L-lysine-coated glass cover slides with 3% paraformaldehyde (pH 7.4) for 20 minutes. Next, fixed spores were rinsed three times with PBS, blocked with 1% BSA for 1 hour and further incubated for 1 hour at room temperature with 1:100 of serum from an immunized mouse with PBS, Sp-BclA2$_{CTD}$, or purified BclA2$_{CTD}$ (in PBS BSA 1%). The coverslips were rinsed three times with PBS and incubated 1 hour at room temperature with 1:400 anti-mouse conjugated with Alexa 488 (Invitrogen) in PBS BSA 1% and washed three times with PBS and once with sterile water. Samples were then dried at room temperature for 30 minutes, mounted using Dako Fluorescence Mounting medium (Dako, North America) and sealed with nail

polish. Samples were analysed with an Olympus BX53 fluorescence microscope. The fluorescence images were obtained with 30 ms of exposition. ImageJ software was used to quantify the fluorescence signal, as previously reported [25].

1.5.12. Cytotoxicity assay

Vero cell cytotoxicity was performed as described previously [44]. Briefly, 96-well flat-bottom microtiter plates were seeded with Vero cells at a density of 10^5 cells/well. Mice caecum contents were kept at -20ºC prior use. At the time of the experiment caecum contents were suspended in PBS (10 µL of PBS per mg of caecum content), vortexed and centrifuged (14 000 rpm, 5 minutes). Filter-sterilized supernatant was serially diluted in DMEM supplemented with 10% FBS and 1% penicillium streptomycin; 100 µL of each dilution was added to wells containing Vero cells. Plates were screened for cell rounding 16 hours 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 X200 magnification.

1.5.13. 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 Mann-Whitney unpaired t-test. Comparative analysis between groups was performed by analysis of variance with Turkey'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.

1.6. References

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Supplementary Material

Table S1. 1. Densitometric analysis of dot blot experiments with pure free $BclA2_{CTD}$ incubated at -20°C, +4°C and +25°C for 48 hours.

$BclA2_{CTD}$ source	Amount of sample used	Density (OD/mm^2)	Amount of $BclA2_{CTD}$ (ng) in 200 µL
Standard (Pure $BclA2_{CTD}$)	200 ng	823 143	NA
	100 ng	347 614	NA
	50 ng	189 283	NA
Pure $BclA2_{CTD}$ stored at -20°C	40 µL	653 320	769±79
	20 µL	320 474	
	5 µL	140 431	
Pure $BclA2_{CTD}$ stored at +4°C	80 µL	799 310	529±92
	40 µL	490 148	
	10 µL	152 129	
Pure $BclA2_{CTD}$ stored at +25°C	40 µL	255 412	127±126
	20 µL	115 186	
	10 µL	82 294	

Table S1. 2. Densitometric analysis of dot blot experiments with the supernatant of the adsorption reaction and pure $BclA2_{CTD}$ incubated 1 hour at +25°C.

$BclA2_{CTD}$ source	Amount of sample used	Density (OD/mm^2)	Amount of $BclA2_{CTD}$ (ng)	Average of $BclA2_{CTD}$ in 200 µL (ng) (% of total)
Standard (Pure $BclA2_{CTD}$)	100 ng	1 328 352	NA	NA
	50 ng	766 719	NA	NA
	25 ng	304 895	NA	NA
	12.5 ng	170 229	NA	NA
	6.25 ng	55 361	NA	NA
Unbound $BclA2_{CTD}$	80 µL	101 740	0.10	24±6 (0,6)
	40 µL	71 450	0.14	
Control $BclA2_{CTD}$ (1 hour at +25°C)	5 µL	1 274 764	90	3978±336 (99)
	2.5 µL	669 612	48	
	1.25 µL	377 034	27	
	0.63 µL	175 257	13	

Table S1. 3. Densitometric analysis of dot blot experiments with *B. subtilis* spores adsorbed with $BclA2_{CTD}$ incubated at -20ºC, +4ºC and +25ºC for 48 hours.

$BclA2_{CTD}$ source	Amount of sample used	Density (OD/mm^2)	Amount of $BclA2_{CTD}$ (ng) in 200 µL
Standard (Pure $BclA2_{CTD}$)	200 ng	1 700 071	NA
	100 ng	928 229	NA
	50 ng	416 608	NA
Protein extract from Sp-$BclA2_{CTD}$ at -80°C	6.25 mg	875 027	4013±29
	3.13 mg	519 456	
	1.56 mg	297 202	
Protein extract from Sp-$BclA2_{CTD}$ at -20°C	12.50 mg	1 490 629	4043±665
	6.25 mg	795 663	
	3.13 mg	520 585	
Protein extract from Sp-$BclA2_{CTD}$ at +4°C	6.25 mg	980 878	3970±331
	3.13 mg	533 091	
	1.56 mg	309 536	
Protein extract from Sp-$BclA2_{CTD}$ at +25°C	12.50 mg	1 245 880	2225±368
	6.25 mg	563 470	
	3.13 mg	398 974	

CHAPTER 2

The C-terminal domain of BclA3 of *Clostridium difficile* induces a humoral immune response in nasally immunized mice

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Manuscript in preparation

2.1. Abstract

Clostridium 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 nasal or oral route, interact with intestinal epithelial cells and persist in the gut. Spore germination in the gut causes a diffuse intestinal colonization and perpetuates the disease due to toxins produced by the growing bacteria. Therefore, spores are major players of the infection and ideal targets of new therapeutic treatments. In this context, spore surface proteins of *C. difficile*, involved in the interaction with epithelial cells, are potential antigens for the development of vaccines targeting *C. difficile* spores. Here we report that a fragment of the spore surface protein BclA3, $BclA3_{CTD}$, was identified as an epitope, over-produced in *Escherichia coli* and tested to immunize mice. To increase antigen stability and efficiency, $BclA3_{CTD}$ was also exposed on the surface of *B. subtilis* spores, a well-established mucosal vaccine delivery system. However, while free $BclA3_{CTD}$ induced antibody production in mice and attenuated some CDI symptoms after a challenge with the pathogen, the spore-displayed antigen resulted less effective. Although dose regimen and immunization route need to be optimized, our results indicate $BclA3_{CTD}$ as a potentially effective antigen to develop a new vaccination strategy targeting *C. difficile* spores.

2.2. Introduction

Clostridium 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. 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% [2-4]. Nowadays, CDI is a major concern and an economic burden. Recent data indicated that the CDI is more common than methicillin-resistant *Staphylococcus aureus* infections [5] and estimated a cost per CDI episode ranging from 5.000 to 12.000 euros [6].

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 low numbers in the animal gut. When the gut conditions are favorable, i.e. when the number of other members of the gut microbiota is severely reduced, 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. Being infection vehicles, mediators of the initial interaction with intestinal cells and responsible of the persistence of the pathogen in the animal gut, *C. difficile* spores are key players of CDI and ideal targets of anti-*C. difficile* therapeutic treatments.

The outermost layer of *C. difficile* spore is the exosporium, a protective layer mainly composed of proteins and glycoproteins [7, 8]. BclA1, BclA2 and BclA3 are collagen-like glycoproteins homogeneously distributed in the *C. difficile* exosporium and able to form, at least in the hyper-virulent strain R20291, hair-like projections [9, 10]. The morphology of these structures and their surface localization suggest a possible role for the BclA proteins in the interaction with the host epithelial cells and propose them as potential antigens [11]. While the antigenicity of BclA1 and BclA2 has been recently tested [7, 12, 13], BclA3 has never been directly evaluated. However, an interesting study has already showed that mutant spores lacking BclA3 have shown a reduced colonization efficiency in a mice infection model [13].

BclA3 is produced in the mother cell compartment of the *C. difficile* sporangium during the late stages of sporulation [14]. The protein is modular and is composed by three domains: (i) an N-terminal domain possibly oriented to the inside; (ii) a collagen-like domain formed by GXX repeats; (iii) and a C-terminal domain that is faced outwards of the exosporium [7].

In this study, the BclA3 amino acid sequence was analysed *in silico* and the C-terminal domain, $BclA3_{CTD}$, identified as a potential epitope. $BclA3_{CTD}$ antigenicity was then tested *in vivo* in a murine model both as a free protein or displayed on *Bacillus subtilis* spores, a well-established antigen delivery system [15]. To this aim, DNA coding for $BclA3_{CTD}$ was fused in frame to DNA coding for CotB, a *B. subtilis* spore surface protein previously tested as an anchor motif to display heterologous proteins on the spore surface [16].

2.3. Results

2.3.1. In silico analysis of BclA3 and construction of the recombinant strain expressing the chimera protein CotBΔ- BclA3$_{CTD}$

We used the Immune Epitope Database (IEDB) to analyse BclA3 amino acid sequence and predict continuous linear B and T cells MHC-I and MHC-II epitopes. As demonstrated in Figure 2.1A, 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 (data not shown). Based on that and on a previous report showing that the C-terminus of BclA3 is faced outwards of the exosporium of *C. difficile* spores [7], we selected the C-terminal part of BclA3 (BclA3$_{CTD}$) (Figure 2.1B) as a putative antigen to be tested *in vivo* as a free protein and upon display on *B. subtilis* spores.

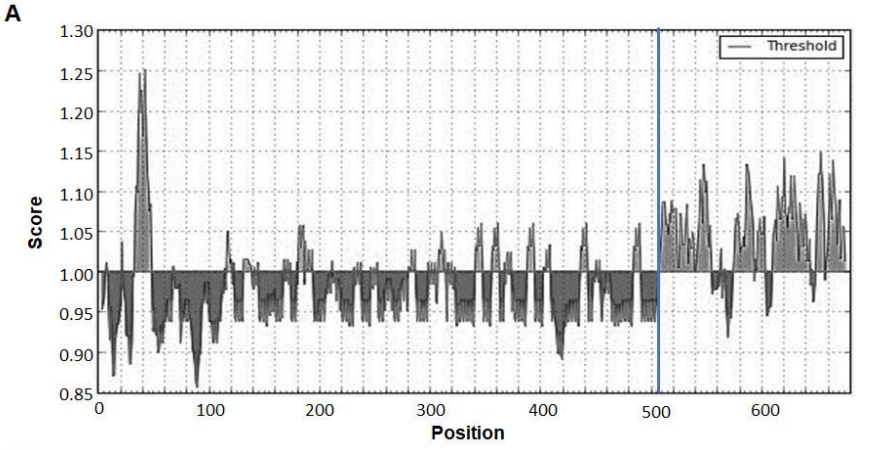

Figure 2. 1. *In sillico* analysis of the glycoprotein BclA3 from *C. difficile* R20291. (A) The C-terminal domain (CTD) of BclA3 (last 170 amino acid residues) shows higher B cell epitope propensity score compared with the rest of the protein (Kolaskar & Tongaonkar Antigenicity Method from Immune epitope 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.

To these aims, DNA coding for BclA3$_{CTD}$ was used to: i) over-express and purify the protein fragment (Methods), and ii) construct a gene fusion with DNA coding for the *B. subtilis* spore surface protein CotB. In particular, we used a truncated version of CotB, CotBΔ, that does not contain a

region with repeated sequences and is more genetically stable [16]. As previously reported [16], the gene fusion was cloned into an integrative vector adjacent to a chloramphenicol-resistance gene cassette (Cm^R) and used to transform competent cells of the *B. subtilis* strain PY79 [17]. Chloramphenicol-resistant clones were the result of a double cross-over integration event, schematically indicated in Figure 2.2A. Chloramphenicol-resistant clones were tested for the site of chromosomal integration by PCR (not shown) and one clone, AZ703, selected for further analysis.

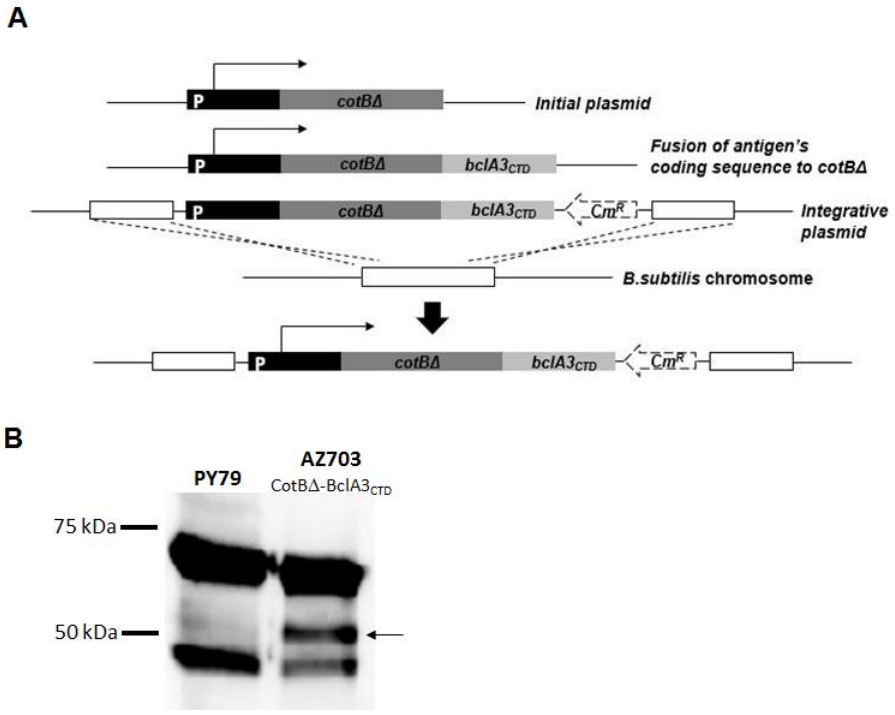

Figure 2. 2. Construction of *B. subtilis* recombinant strain AZ703. (A) Schematic representation of the strategy for the integration of the gene fusion *cotBΔ*::*bcla3*$_{CTD}$ in the chromosomal DNA of *B. subtilis*. The western-blot analysis of proteins extracted from spores of *B. subtilis* laboratory 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 53 kDa which corresponds to the chimera protein CotBΔ-BclA3$_{CTD}$. $5x10^8$ of spores were resuspended in 100 µL of loading buffer and 20 µg 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 SDS-DTT procedure [18] and extracted proteins analyzed by Western-blot with anti-CotB antibody. As shown in Figure 2.2B, a protein, not extracted from spores of the isogenic parental strain PY79, was observed in the extract of strain AZ703.

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 [19]. The truncated form of CotB has instead a predicted size of 36 kDa. The additional protein extracted from AZ703 spores and recognized by the anti-CotB antibody migrated on SDS-PAGE slightly above the 50 kDa marker, matching well with the expected size for the fusion protein (36 kDa for CotBΔ plus 16.7 kDa for $BclA3_{CTD}$ = 52.7 kDa).

2.3.2. 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 3 times either with PBS pH 7 (n=11), $2x10^9$ spores of *B. subtilis* PY79 (n=11) (Sp), $2x10^9$ spores of AZ703 (n=11), or 4 µg 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 $5x10^7$ spores of the *C. difficile* strain R20291, mice were treated with Clindamycin as previously reported [20] and schematically shown in Figure 2.3.

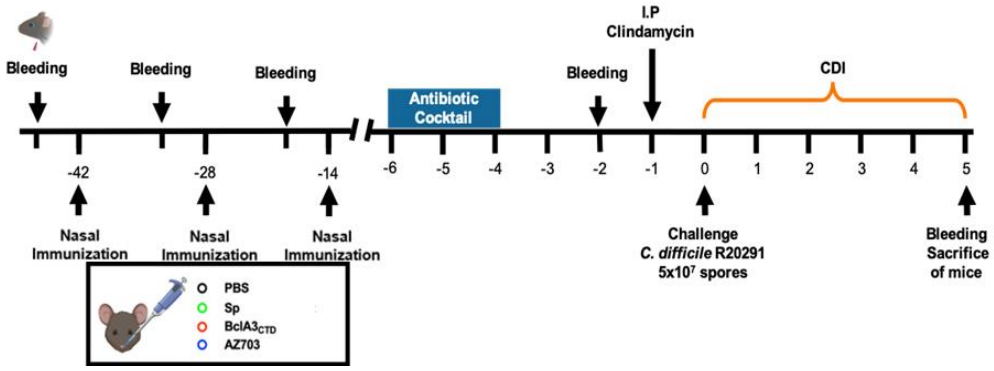

Figure 2. 3. Overview of the experimental design schematics for the prevention of *C. difficile* infection in a murine model. C57BL/6 mice were nasally immunized 3 times (42, 28 and 14 days before challenge with *C. difficile* R20291 spores) with PBS, spores of *B. subtilis* PY79, purified $BclA3_{CTD}$ 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 $5x10^7$ 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.

BclA3$_{CTD}$ immunogenicity was measured by ELISA analysing the presence of anti-BclA3$_{CTD}$ IgG in serum throughout the experiment. As shown in Figure 2.4A 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.

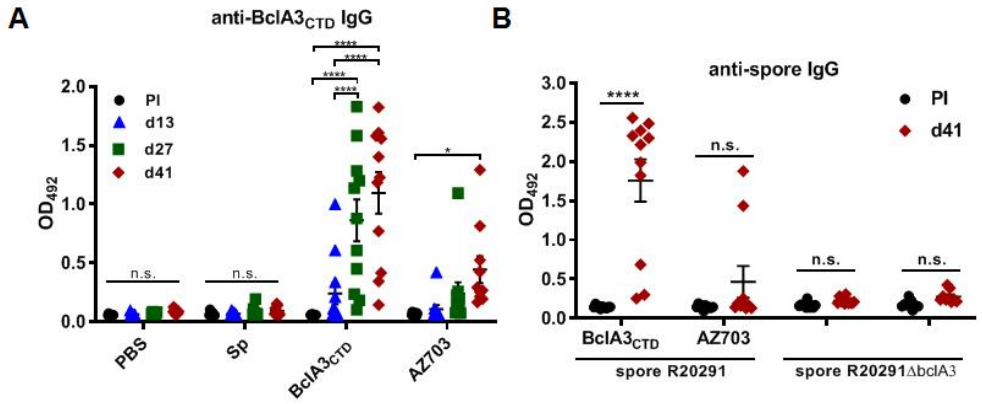

Figure 2. 4. Immunogenicity of pure BclA3$_{CTD}$ protein and AZ703 spores in mice nasal immunization. (A) IgG anti-BclA3$_{CTD}$ was measured in mice serum on days 0 (Pre-Immune serum, PI), 13 (d13), 27 (d27) and 41 (d41). **(B)** Anti-*C. difficile* R20291 spores or anti-*C. difficile* R20291Δ*bclA3* spores IgG levels were measured in the serum of mice on day 0 and 41. Serum collection on day 0, 13 and 27 was made one day before each immunization and on day 41 two days before infection. Results were determined by ELISA and are reported as optical density (OD) units at 492nm. 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 Turkey's multiple comparison test and statistical significance ($P < 0.05$) is indicated by asterisks. n.s means no significance.

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 2.4B, 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 2.4A), only two out of eleven

mice immunized with spore-displayed BclA3$_{CTD}$ produced IgG able to specifically recognize R20291.

In conclusion, results of Figure 2.4 show that BclA3$_{CTD}$ is a mucosal antigen able to induce the production BclA3$_{CTD}$-specific IgG in a murine model. When displayed on *B. subtilis* spores BclA3$_{CTD}$ is still able to induce the production of BclA3-specific IgG, even if at a low level.

2.3.3 Effect of nasal-BclA3$_{CTD}$ immunization against C. difficile R20291 infection

Figure 2.5A shows that intranasal immunization with purified BclA3$_{CTD}$ prevented weight loss after the challenge with 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 2.5A). No statistically significant differences were observed concerning the appearance of diarrhea caused by the 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 2.5B). Plus, the severity of diarrhea, associated with a high score (0 meaning normal stool and 3 liquid stool), did not vary significantly between groups in the same day (Figure 2.5C).

In order to evaluate if the immunization influenced *C. difficile* sporulation inside the host and spore clearance, we compared the spore levels present in the 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 2.6A) 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 2.6A). 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* spore germination inside the cecum. As shown in Figure 2.6B, no statistically significant differences

were observed indicating that in mice of all experimental groups *C. difficile* spores germinated inside the animal cecum.

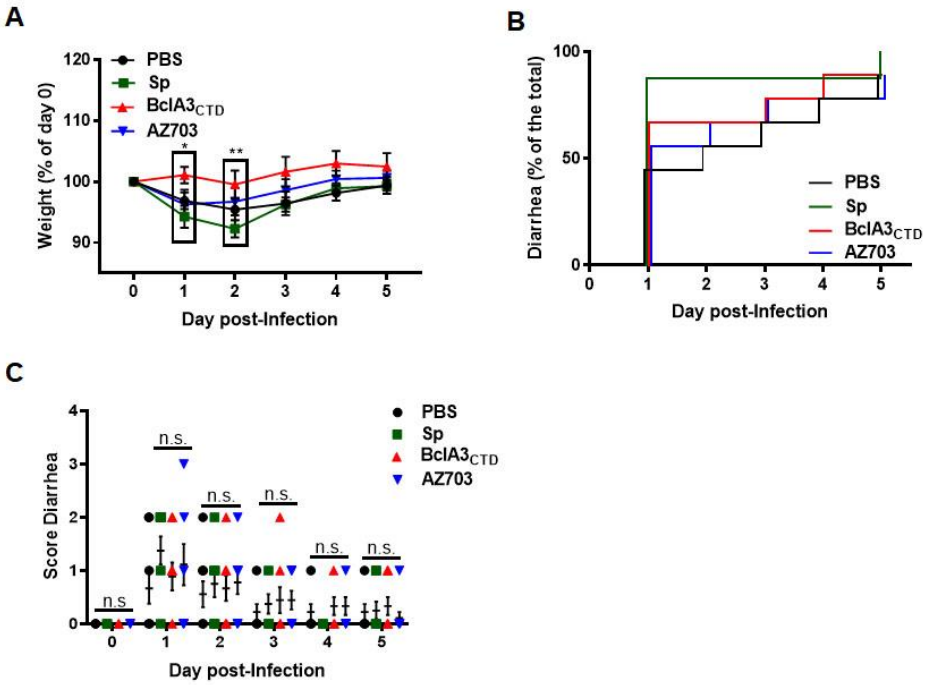

Figure 2. 5. Protective effect of BclA3$_{CTD}$ and AZ703 intranasally administered against CDI in murine model. C57BL/6 mice were nasally immunized with PBS, *B. subtilis* PY79 spores, purified BclA3$_{CTD}$ or AZ703 spores and challenged with *C. difficile* R20291 spores. Mice were monitored in the 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. n.s means no significance.

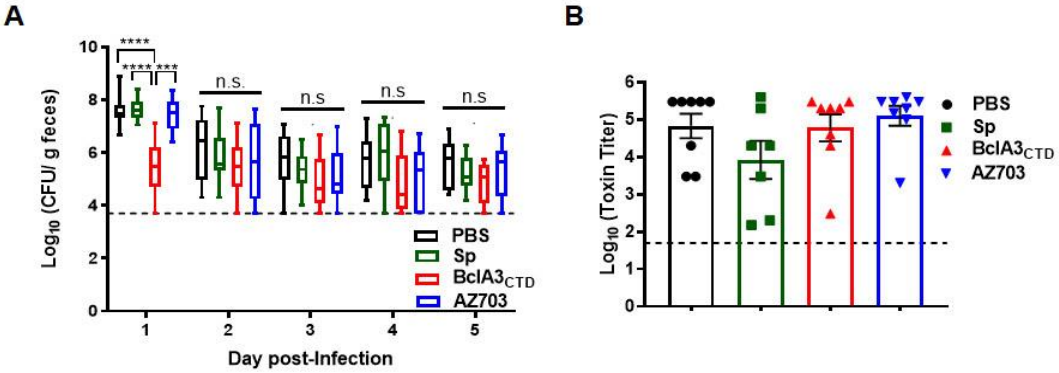

Figure 2. 6. Analysis of spore load in feces and cecal toxin titers. (A) The load of *C. difficile* spores in the feces was evaluated upon sacrifice as $\log_{10}$ CFU/g of feces. **(B)** The cecum content toxicity was measured and represented as $\log_{10}$ toxin titer. One-way ANOVA Tukey's multiple comparisons test in A and Mann-Whitney non-parametric test in B were used; Statistical differences ($P < 0.05$) are indicated by asterisks. The bars are the geometric mean ± standard error of the mean. n.s means no significance.

2.4. Discussion

Current treatment options for CDI rely on the use of antibiotics, fecal microbiota transplantation, probiotic administration or monoclonal antibodies against *C. difficile* toxins [21]. 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. To address this issue, 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*. Mice nasally immunized with pure $BclA3_{CTD}$ were able to produce BclA3-specific IgG, indicating that the antigen was able to elicit a specific immune response. On the other hand, the same antigen displayed on the surface of *B. subtilis* spores induced low levels of anti-BclA3 IgG, indicating a low immune response. This is most likely due to the low amount of antigen displayed on each spore and, as a consequence, to a low dose of antigen administered to mice. To confirm this, experiments aimed at quantifying the amount of the chimera protein CotBΔ-$BclA3_{CTD}$ displayed on each *B. subtilis* spore are still in progress. However, we consider promising the weak immune response observed in the experiments reported here and plan to explore the possibility of using higher doses of spore-delivered antigen and of using the oral route of immunization to increase the efficacy of spore-displayed BclA3.

Upon the challenge with spores of the hyper-virulent strain R20291, immunized animals were expected to activate the complement signaling cascade leading to the opsonization of *C. difficile* spores, to their phagocytic elimination and to the attenuation of the CDI symptoms [22, 23]. We observed, in mice immunized with pure $BclA3_{CTD}$, a reduction of some CDI symptoms, i.e. no body weight loss and a statistically significant decrease in *C. difficile* spore load in feces 1 day after challenge, suggesting the induction of a systemic immune response and the consequent clearance of *C. difficile* spores from mice. However, the immunization was not able to halt neither the occurrence and intensity of diarrhea, to reduce the spore load in colonic tissues or to avoid *C. difficile* spore germination in mice cecum, indicating that the systemic immune response was not strong enough to prompt local immunity and a protective immune response. The change of route and/or dose of regimen will possibly overcome these limitations.

In spite of this, the induction of serum IgG 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 with different doses and route of administration.

2.5. Materials and Methods

2.5.1. Bacterial strains and spore purification

E. coli strains DH5α and BL21 (DE3) (Invitrogen) were used for cloning and $BclA3_{CTD}$ overexpression, respectively. *B. subtilis* PY79 [17] was used as a 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 R20291Δ*bclA3* knock-out mutant was used.

Sporulation of *B. subtilis* PY79 and AZ703 was induced by the exhaustion method [24]. Briefly, after 35 hours of growth in Difco Sporulation (DS) medium at 37ºC 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 [25]. Spore suspensions were prepared by plating a 1:100 dilution of an overnight culture onto a 70:30 medium (63 g Bacto peptone (BD Difco), 3.5 g proteose peptone (BD Difco), 0.7 g ammonium sulfate $(NH_4)_2SO_4$, 1.06 g Tris base, 11.1 g brain heart infusion extract (BD Difco) and 1.5 g yeast extract (BD Difco) for 1 L) and incubating it for 5 days at 37°C under anaerobic conditions [26]. 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 minutes. Spores were loaded onto a 45% Nycodenz solution, centrifuged (14 000 rpm, 40 minutes). After centrifugation, the spore pellet was washed five times (14 000 rpm, 5 minutes) with ice-cold sterile water to remove Nycodenz remnants.

The spores were counted in Neubauer chamber and volume adjust at $5x10^9$ 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.

2.5.2. 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$_{CTDsense}$ (ggtaccggatccGCAATAATACCTTTTGCATCAGG, in lower case is the recognition site for KpnI, NcoI and BamHI restriction enzymes) and BclA3$_{CTDanti}$ (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 µg/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 hours at 37ºC 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). The purified protein was desalted and concentrated with the Centricon cutoff 10 kDa (Merck Millipore). The purity of the protein was analyzed by SDS-page and Western-blot using Anti-His antibodies.

2.5.3. 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 *cotBΔ* 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::*cotBΔ*). 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 *cotBΔ* gene carried by plasmid pGEMT-easy::*cotBΔ* previously diggested with BglII/PstI,

yielding plasmid pGEMT-easy::*cotBΔ*::*bclA3*$_{CTD}$. The fusion *cotBΔ::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 hours incubation at 37°C, spores were collected, washed and purified as described before. The coat proteins from $5x10^8$ 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.

2.5.4. Western-blot analysis

The coat proteins from $5x10^8$ spores of *B. subtilis* PY79 and AZ703 were extracted by SDS-DTT treatment [18] and quantified by Bradford assay (BioRad). 20 µg of protein extract or 500 ng of pure BclA3$_{CTD}$ with protein sample buffer 2x [27] were incubated at 100ºC for 7 minutes and loaded onto a 12% SDS-Page gel. Proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) 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.

2.5.5. Animals

Mice 8-12 weeks old C57BL/6 (male or female) were obtained from a breeding colony at Facultad de Ciencias Biologicas Universidad Andres Bello (Santiago, Chile), established using animals purchased from Jackson Laboratories. Water, bedding and cages were previously autoclaved and mice had a 12-hour 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.

2.5.6. 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, $2x10^9$ spores of *B. subtilis* PY79, $2x10^9$ 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.

2.5.7. Animal infection model

Prior to infection, mice were pre-treated with antibiotic cocktail of kanamycin (40 mg/kg body weight; Sigma-Aldrich, U.S.A.), 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 [20]. Two days after the antibiotic treatment, mice were intraperitoneally administrated with a single dose of clindamycin (10 mg/kg) and on the next day were infected orogastrically with 100 µL of PBS containing $5x10^7$ 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 [28]. 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 [29]. 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.

2.5.8. Evaluation of BclA3$_{CTD}$-specific IgG levels in mice serum

The blood collected the day before each immunization, two days before and at the time of sacrifice was incubated at 37ºC for 30 minutes and then centrifuged at 5 000 rpm for 10 minutes at 4ºC. The supernatant, containing the serum fraction was stored at −20ºC 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 ($1.6x10^7$ spores/well) or *C. difficile* R20291Δ*bcla3* ($1.6x10^7$ 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 hour at 37ºC. 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 hours at 37ºC. After the removal of non-adherent IgG by several washes, the plates were incubated with 1:5 000 secondary antibody anti-IgG mouse HRP, for 1 hour at 37ºC. Finally, the colorimetric reaction was initiated upon the addition of 50 µL of reaction buffer containing 0.05 M citric acid, 0.1 M disodiumhydrogen phosphate, 2 mg/mL of o-phenlyendiamine (Sigma-Aldrich, U.S.A.) and 0.015% of H_2O_2 (Merck, Germany). The reaction was stopped after 20 minutes with 25 µL of 4,5 N of H_2SO_4 and absorbance was measured at 492 nm. The experiment was performed in duplicate.

2.5.9. 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 µL of PBS was added for each mg of stools, mixed and incubated for 30 minutes at room temperature. Then, 50 µL of absolute ethanol (Sigma-Aldrich) was added to 50 µL of feces which were incubated for 30 minutes at room temperature. Samples were serially diluted and plated onto selective medium supplemented with Taurocholate (0.1% w/v), Cefoxitin (16 µg/mL) and L-cycloserine (250 µg/mL) (TCCFA plates).

The plates were incubated anaerobically at 37ºC for 48 hours, the *C. difficile* colonies were counted, and the results were expressed as the Log_{10} of CFU/g of feces.

Proximal, median and distal colon were collected from mice upon sacrifice and washed with PBS with a syringe. They were posteriorly resuspended and homogenized with 2.5 µl of PBS for each mg of tissue. Upon incubation at room temperature with absolute ethanol and serially diluted they were plated onto TCCFA plates. The plates were incubated anaerobically at 37ºC for 48 hours. Finally, the colony count was expressed as the Log_{10} of CFU/g of tissue.

2.5.10. Cytotoxicity assay

Vero cell cytotoxicity was performed as described previously [30]. Briefly, 96-well flat-bottom microtiter plates were seeded with Vero cells at a density of 10^5 cells/well. Mice caecum contents were kept at -20ºC prior use. At the time of the experiment caecum contents were suspended in PBS (10 µL of PBS per mg of cecal content), vortexed and centrifuged (14 000 rpm, 5 minutes). Filter-sterilized supernatant was serially diluted in DMEM supplemented with 10% Fetal bovine serum (FBS) and 1% penicillium streptomycin. 100 µL of each dilution was added to wells containing Vero cells. Plates were screened for cell rounding 16 hours 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 X200 magnification.

2.5.11. 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 Mann-Whitney unpaired t-test. Comparative analysis between groups was performed by analysis of variance with Turkey'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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CHAPTER 3

Display of CdeC of *Clostridium difficile* on *Bacillus subtilis* spores increases *in vitro* adhesion to Caco-2 cells and vitronectin recruitment

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Manuscript in preparation

3.1. Abstract

Bacillus subtilis spores have become extremely important biotechnology tools and find applications in a variety of fields, from cell factories for the production of bioactive molecules, to probiotics for human and animal use, to mucosal vaccine delivery systems and to plant-growth promoters. In this work, we used *B. subtilis* spores as a carrier to display three spore surface proteins of *Clostridium difficile*, BclA2, BclA3 and CdeC, and evaluate their interaction with Caco-2 cells. The analyzed proteins are components of the exosporium of *C. difficile* and have been proposed as potential mediators of the interaction of the spore with intestinal cells. Recombinant spores of *B. subtilis* displaying each of the *C. difficile* proteins were analyzed *in vitro* for their ability to adhere to Caco-2 cells, a model of intestinal epithelial cells, and to vitronectin, a glycoprotein common in the animal extracellular matrix that is recruited by several pathogens, including *C. difficile*, to cross the epithelial barrier. CdeC but not BclA2 or BclA3, when displayed on *B. subtilis* spores, increased the spore adhesion to Caco-2 cells and vitronectin confirming a role of CdeC in the interaction of *C. difficile* spores with the host and therefore in the initial steps of the *C. difficile* infection.

3.2. Introduction

C. difficile is an anaerobic spore-forming bacterium transmitted by the oro-fecal route. In response to the gradual absence of oxygen and presence of bile salts, taurocholate and cholate in the gastrointestinal tract, *C. difficile* spores germinate, switching from a dormant state to a metabolically active vegetative cell. In the intestine of healthy individuals, commensal bacteria degrade residual bile components and, in this way, contribute to prevent *C. difficile* spore germination. As a consequence, in healthy individuals *C. difficile* persist asymptomatically in the spore form [1, 2]. When the normal intestinal microflora is strongly altered or reduced, for example as a consequence of an antibiotic treatment, *C. difficile* spores germinate and rapidly colonize the colonic tract [1, 3]. Germination-derived *C. difficile* cells produce the enterotoxins TcdA and TcdB, that are the main virulence factors responsible of the development of *C. difficile* infection (CDI). In patients with CDI, the high number of *C. difficile* spores in the feces indicates that *C. difficile* cells present in the intestine, sporulate in the lower intestinal tract [4-6].

The outermost layer of *C. difficile* spores is the exosporium, a protective structure composed of proteins and glyco-proteins, thought to mediate spore-host interaction and spore persistence in the host. Major components of the exosporium are collagen-like glycoproteins of the BclA family: BclA1, BclA2 and BclA3, all proposed to assemble in the exosporium with their C-terminal domains oriented outward [7]. At least in the hyper-virulent strain R20291, these proteins are homogeneously distributed in the exosporium and form hair-like projections. It has been shown that these structures interact closely with the microvilli of intestinal epithelial cells (IECs), suggesting that BclA proteins have a role in the modulation of the immune system during CDI, possibly influencing *C. difficile* spore internalization by IECs and consequently contributing to perpetuate the recurrence of the disease [8]. A recent study has demonstrated that BclA1 is important for animal susceptibility to colonization and infection by the laboratory strain of *C. difficile* 630 [9]. However, the observation that the hyper-virulent and highly epidemic strain R20291 has a truncated and most likely not functional form BclA1 of approximately 6 kDa, induced the hypothesis that, BclA2 and BclA3 might have a special role in the pathogenicity of this strain.

CdeC is a cysteine-rich protein, able to self-assemble into thermally stable structures and essential for the proper assembly of coat and exosporium [10-12]. A recent study suggested that CdeC monomers are localized between the coat and the exosporium of *C. difficile* spores while CdeC homo-multimers are found in the exosporium [13].

In order to study the role of BclA2, BclA3 and CdeC of *C. difficile* in CDI and in particular, in the early steps of the infection, in this study we exposed fragments of these three proteins on *Bacillus subtilis* spores and measured the adhesion of spores of wild type and recombinant strains to differentiated and undifferentiated Caco-2 cells. *B. subtilis* spores, widely used as probiotics [14], have a low efficiency of adhesion to Caco-2 cells. Therefore, we used *B. subtilis* spores to evaluate whether the display of *C. difficile* proteins increases their efficiency to *in vitro* adhesion to model intestinal cells as an indirect measurement of the adhesion properties of the *C. difficile* proteins.

3.3. Results

3.3.1. The C-terminal domain of BclA2, BclA3 and CdeC of C. difficile are immunogenic for B and T cells

The exosporal proteins BclA2, BclA3 and CdeC from the strain R20291 of *C. difficile* have a molecular weight of 47.9 kDa, 59.9 kDa and 44.8 kDa, respectively. The first two are glyco-proteins composed by an N-terminal domain of variable size (NTD); a central domain with collagen-like repeats (GXX) and a C-terminal domain (CTD) exposed to the exterior [7]. CdeC is a cysteine-rich protein with six NPC repeats in the central region and a CTD rich in cysteines.

In Chapter 1 and 2 of this Thesis, it is reported that the CTD of BclA2 and BclA3 have high antigenic score for B and T cells. The *in silico* analysis for B cells were obtained using the Kolaskar and Tongaonkar Antigenicity Method which is based on the analysis of the physicochemical properties of amino acid residues and their frequency of occurrence in experimentally known segmental epitopes and predicts the most antigenic part of a protein. We have applied the same method for CdeC (Figure 3.1) and found that the C-terminal 285 amino acid residues show continuously higher antigenicity score. The same part of the protein was also found the most antigenic regarding T cells epitopes. The orientation of CdeC on *C. difficile* spore exosporium remains unknown, however it is likely that the CTD is exposed outwards since possible disulphide bonds promoted by the abundant cysteines, enhance cross binding between the exosporium and exterior proteins [13]. For these reasons, we have selected the CTD of BclA2, BclA3 and CdeC for being displayed on the surface of *B. subtilis* spores.

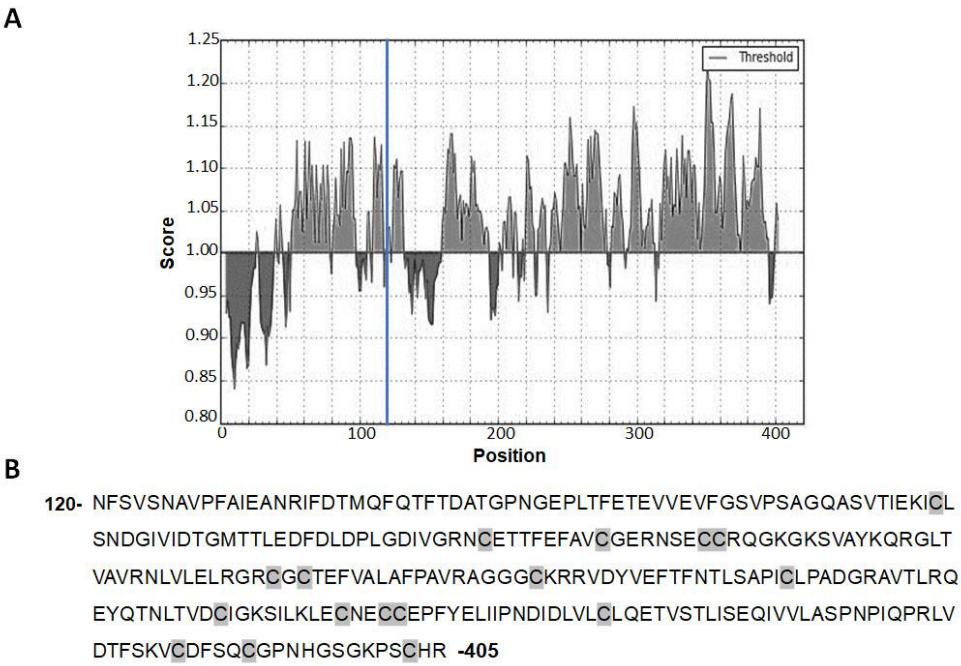

Figure 3. 1. ***In silico*** **analysis of CdeC from** ***C. difficile*** **R20291. (A)** Analysis of the B cell epitope propensity score (Kolaskar & Tongaonkar Antigenicity Method from Immune Epitope Database) of CdeC. The X- and Y-axes represent the sequence position and antigenic propensity score, 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)** Amino acid sequence of the 285 residues at the C-terminal end of CdeC ($CdeC_{CTD}$) was identified as a potential antigenic region. Cysteines are highlighted in grey.

3.3.2. Construction of the B. subtilis recombinant strains displaying $BclA2_{CTD}$ or $CdeC_{CTD}$

The coat protein CotB of *B. subtilis* has been widely used as a carrier for the successful display of several heterologous proteins [15]. CotB has a strongly hydrophilic C-terminal half formed by three serine-rich repeats. As previously suggested [15], we used as a carrier protein a truncated form of CotB (CotBΔ), with the C-terminal 105 residues deleted. The construction of the recombinant strain displaying CotBΔ-$BclA3_{CTD}$ has been reported previously (see Chapter 2 of this Thesis). The same strategy (schematically reported in Figure 3.2) was followed for the construction of the recombinant strains displaying CotBΔ-$BclA2_{CTD}$ or CotBΔ-$CdeC_{CTD}$. In brief, the gene fusion was constructed in *E. coli* and transferred to an integrative plasmid between two homology regions to allow the chromosomal integration in *B. subtilis* of the gene fusion as previously described [18].

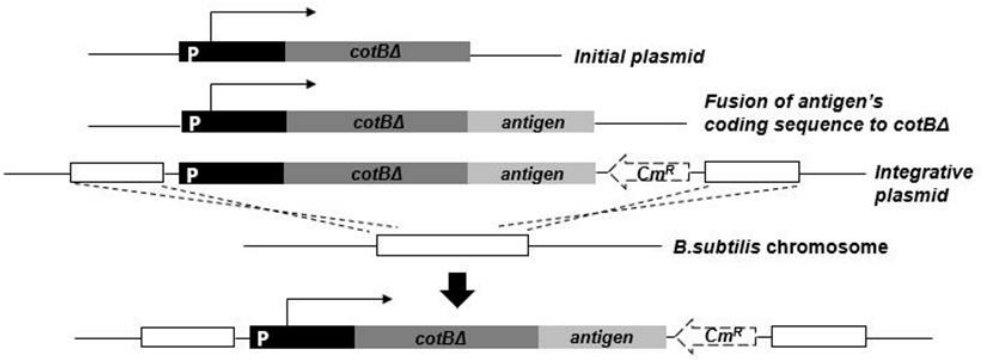

Figure 3. 2. Schematic representation of the construction of *B. subtilis* recombinant strains. A gene fusion between *cotBΔ* (carrier) and the DNA sequence of $bcla2_{CTD}$, $bcla3_{CTD}$ or $cdec_{CTD}$ (heterologous antigens), with a chloramphenicol resistance cassete adjacent, was integrated in the chromosomal DNA of *B. subtilis* by double cross-over with the non-essential *amyE* gene. Chloramphenicol-resistant clones were tested for the site of chromosomal integration by PCR and one clone for each antigen was selected for further analysis.

Spores of the three recombinant strains were purified and the coat proteins extracted by SDS-DTT treatment, as previously described [19]. A western-blot approach with anti-CotB antibodies was used to evaluate the production of the recombinant proteins (Figure 3.3).

The wild-type copy of CotB has a deduced molecular mass of 46 kDa but it is known to migrate on SDS-PAGE as a less abundant protein of 46 kDa and a more abundant protein of 66 kDa, presumably a dimer [20]. Since the recombinant strain carry a wild type copy of *cotB*, proteins of approximately 46 and 66 kDa were produced by all strains but the *cotB* deletion mutant (RH201). Each recombinant strain also produced an additional protein recognized by the anti-CotB antibody (indicated by arrows in Figure 3.3). The apparent size of the recombinant proteins was about 50, about 55 and more than 66 kDa for CotBΔ-$BclA3_{CTD}$, CotBΔ-$BclA2_{CTD}$ or CotBΔ-$CdeC_{CTD}$, respectively, and was in good agreement with the predicted sizes calculated considering the size of CotBΔ (36 kDa) and the size of $BclA2_{CTD}$ (13.5 kDa), $BclA3_{CTD}$ (16.6 kDa) and $CdeC_{CTD}$ (31.1 kDa).

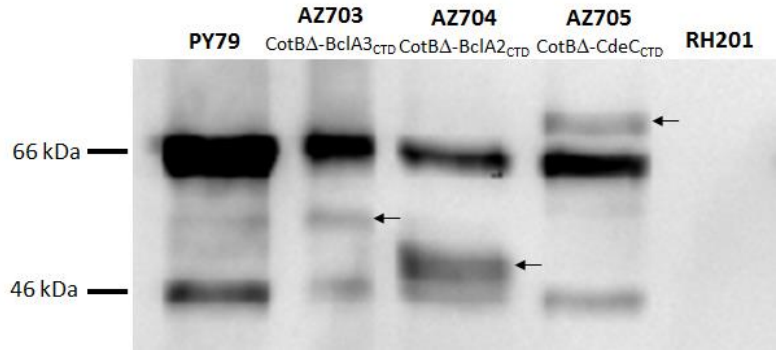

Figure 3. 3. Construction of *B. subtilis* recombinant strains AZ703, AZ704 and AZ705. The western-blot analysis of proteins extracted from spores of *B. subtilis* laboratory strain PY79 and from the recombinant strains AZ703, AZ704 and AZ705 show bands of 66 kDa and 46 kDa, which correspond to the endogenous CotB protein. The lanes corresponding to the recombinant strains also display bands of more than 50 kDa, about 50 kDa and more than 66 kDa for CotBΔ-BclA3$_{CTD}$ (AZ703), CotBΔ-BclA2$_{CTD}$ (AZ704) or CotBΔ-CdeC$_{CTD}$ (AZ705), respectively. 20 µg of protein extract was loaded in each well. The immunoreaction was performed with anti-CotB antibodies and anti-rabbit secondary antibody conjugated with horseradish peroxidase.

3.3.3. Recombinant spores displaying CdeC$_{CTD}$ strongly adhere to differentiated and undifferentiated Caco-2 cells

Differentiated and undifferentiated Caco-2 cells were infected with spores of *C. difficile* R20291, *B. subtilis* PY79 and with the isogenic strains of *B. subtilis* displaying CotBΔ-BclA2$_{CTD}$, CotBΔ-BclA3$_{CTD}$ or CotBΔ-CdeC$_{CTD}$ (AZ704, AZ703 and AZ705, respectively) as described in Methods section. Considering the efficiency of adhesion of *C. difficile* spores as 100%, the relative adherence of all the strains to Caco-2 monolayers is shown in Figure 3.4.

While spores displaying BclA2$_{CTD}$ or BclA3$_{CTD}$, only showed a minimal increase of adhesion with respect to the laboratory strain of *B. subtilis* PY79, recombinant spores displaying CdeC$_{CTD}$ showed a statistically significant ($P < 0.0001$) increase of adhesion with respect to PY79 (Figure 3.4).

It is also noteworthy that all strains showed higher adhesion to differentiated than undifferentiated Caco-2. Since differentiated Caco-2 monolayers have been found to express several cell-receptors that are absent in undifferentiated cells, results of Figure 3.4 then suggest that the observed adhesion is mediated by still unknown cell-receptors.

Altogether, these results suggest that the C-terminal domain of the *C. difficile* exosporium protein CdeC might have a role in adherence to IECs and this adherence is possibly mediated by a host-cell receptor.

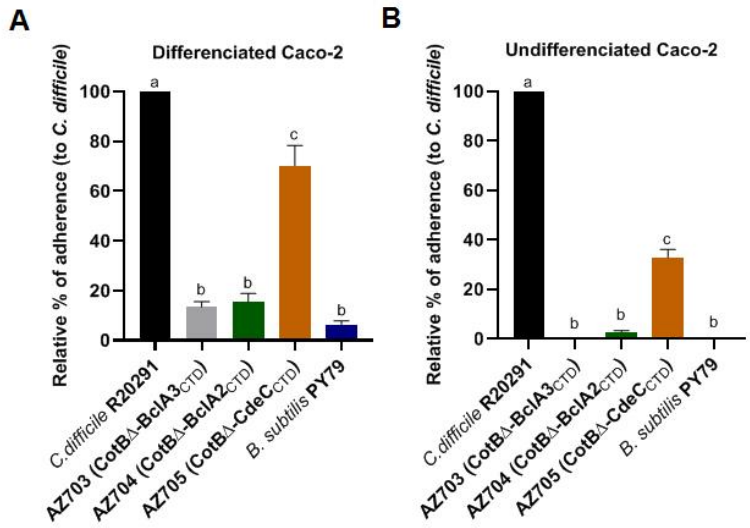

Figure 3. 4. Adherence of spores from *C. difficile* epidemic R20291 strain, *B. subtilis* PY79 and recombinant spores to Caco-2 cell line. (A) Monolayers of differentiated and **(B)** Undifferentiated Caco-2 were infected with spores for 3 hours at 37°C and upon washes and coloration of cell nuclei, adhered spores and cells were counted in the Olympus BX53 microscope. Differences between groups were found using One-way ANOVA Turkey's multiple comparison test. Different letters above the plot mean statistical difference ($P < 0.05$).

3.3.4. Pre-blocking of spores with serum increases spore adherence

Treating the purified spores with Normal Human Serum (NHS) before the infection of Caco-2 cells increased the efficiency of adhesion to both differentiated and undifferentiated cells of all spores (Figure 3.5). In particular, recombinant spores displaying $CdeC_{CTD}$ showed almost the same efficiency of adhesion of *C. difficile* spores to differentiated cells, suggesting that proteins present in NHS specifically mediate spore adhesion to a receptor present on the Caco-2 cells.

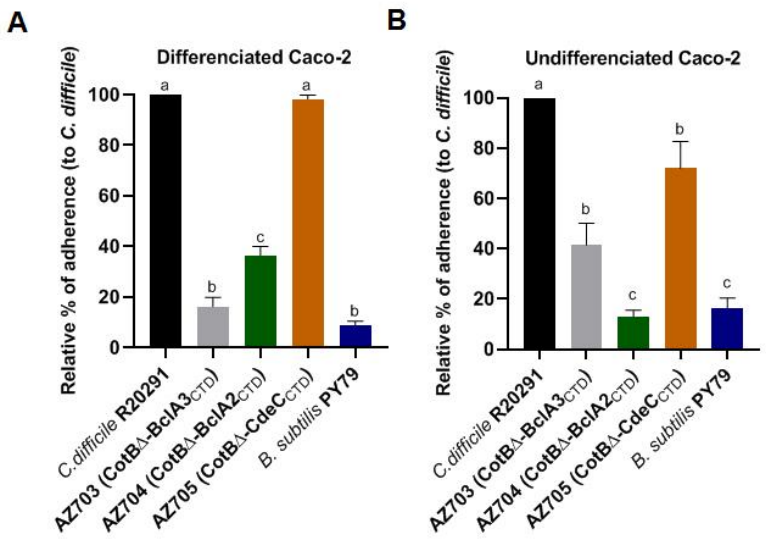

Figure 3. 5. Effect of Normal Human Serum (NHS) on the adherence of *C. difficile* R20291 spores, recombinant spores and *B. subtilis* PY79 spores, to monolayers of Caco-2 cells. (A) Differentiated and **(B)** Undifferentiated Caco-2 cells were infected 3 hours at 37ºC with spores previously incubated with NHS. Upon washes and coloration of cell nuclei, adhered spores and cells were counted in the Olympus BX53 microscope. Differences between groups were found using One-way ANOVA Turkey's multiple comparison test for A and Mann-Whitney for B. Different letters above the plot mean statistical difference ($P < 0.05$).

3.3.5. B. subtilis spores displaying CdeC$_{CTD}$ recruit vitronectin at the same levels as C. difficile spores

Vitronectin is a protein present in the extracellular matrix and is broadly used by bacterial pathogens to gain entry into host surfaces [18]. To investigate whether BclA3$_{CTD}$, BclA2$_{CTD}$ or CdeC$_{CTD}$ have a role in spore-binding to vitronectin we used an immunofluorescence approach to analyse infected differentiated Caco-2 cells with the recombinant strains (description in Methods section). To this aim, rabbit monoclonal anti-vitronectin antibody and anti-rabbit IgG Alexa Fluor 568 secondary antibody were used. A fluorescence signal around spores was observed and interpreted as an indication of vitronectin recruitment by the spores. The fluorescence intensity around *C. difficile* spores was considered 100% and vitronectin recruitment for all other strains represented a % (Figure 3.6).

The laboratory strain PY79 of *B. subtilis* exhibited vitronectin recruitment of approximately 55% however, the presence of the *C. difficile* proteins differently affected vitronectin recruitment. While spores displaying BclA2$_{CTD}$ or BclA3$_{CTD}$ showed a relative vitronectin recruitment of 60 and

75%, respectively, spores displaying $CdeC_{CTD}$ showed approximately the same levels of vitronectin recruitment of *C. difficile* spores.

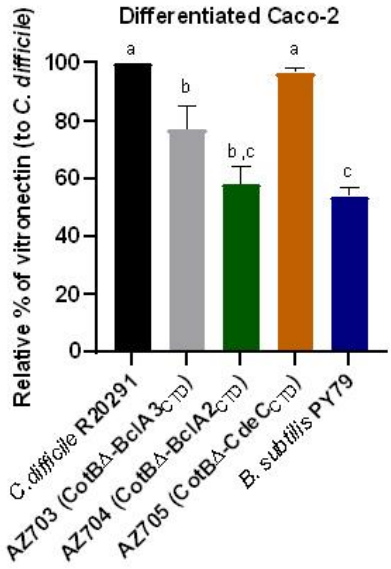

Figure 3. 6. Vitronectin recruitment by *C. difficile* R20291, *B. subtilis* recombinant and PY79 spores. Differentiated cells were infected 3 hours at 37ºC with spores not previously blocked with NHS. The coverslips with the monolayers and the spores were incubated with anti-vitronectin antibodies and anti-rabbit IgG Alexa Fluor 568 conjugate as secondary antibody. Upon washes and coloration of cell nuclei, adhered spores and cells were counted in the Olympus BX53 microscope. Fluorescent signal around the spores, quantified by ImageJ, was interpreted as recruited vitronectin by the spores. Differences between groups were found using One-way ANOVA Turkey's multiple comparison test. Different letters above the plot mean statistical difference ($P < 0.05$).

3.4. Discussion

The role of *C. difficile* spores in persistence and interaction with the host is still poorly described. It is known that the hydrophobicity of the spores, due to the proteinaceous exosporium, contributes to adhesion to hospital surfaces and to IECs [21, 22] and members of the BclA family of collagen-like glyco-proteins 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* [23], known to be highly immunogenic and to act as spore surface ligand for α2β1 integrin present in IECs driving spore entry into the epithelial barrier [24]. CdeC, a morphogenetic factor of the exosporium of *C. difficile*, has been localized on the spore surface [25] and is, therefore, a potential candidate as an additional mediator of the spore-host interaction. To evaluate *in vitro* the role of these *C. difficile* proteins in such interaction, we used *B. subtilis* spores

as carriers to display the three proteins of *C. difficile* and easily measure the efficiency of the interaction to Caco-2 cells. To this aim, we constructed recombinant *B. subtilis* spores displaying the C-terminal domain of BclA2, BclA3 or CdeC, verified the display by western-blot and measured both the efficiency of adhesion to Caco-2 cells and vitronectin recruitment.

The recombinant strain displaying $CdeC_{CTD}$ showed the highest efficiency of adhesion to Caco-2 cells and the effect was higher with differentiated than undifferentiated cells, proposing this $CdeC_{CTD}$ as one of the proteins responsible for the interaction with a still-uncharacterized receptor present of the surface of Caco-2 cells. This conclusion is also supported by the observation that *B. subtilis* spores displaying $CdeC_{CTD}$ recruit vitronectin with an efficiency higher than wild type spores of *B. subtilis* and similar to that of *C. difficile* spores.

Overall, our results suggest that CdeC have a role in interaction with IECs through a still-unknown, specific receptor. In addition, these results highlight a new application for the *B. subtilis* spore, as a tool to study the binding properties of a spore-displayed protein and a target. To this aim, the proposed spore-display approach has advantages over the well-established phage-display system: the low binding activity of wild type spores that can be increased by the displayed heterologous protein and the simple evaluation of the interaction, that can be performed by microscope count, as in this work, or by plate count. A possible future extension of the spore-display system for the study of protein-protein interactions will be the use of spores to screen libraries of proteins/peptides to search interaction partners.

3.5. Materials and Methods

3.5.1. Bacterial strains and spore purification

The *B. subtilis* laboratory strain PY79 [29] was used as negative control for spore adherence to Caco-2 cells assays and as a parental strain of the recombinant strains AZ703, AZ704 and AZ705. The hyper-virulent strain R20291 of *C. difficile* was used as positive control in Caco-2 adherence experiments. A *cotB* null mutant (strain RH201), described in [18], was used as negative control in Western-blot. The used strains and relevant phenotype are depicted in Table 3.1.

Table 3. 1. *B. subtilis* strains.

Strain	Relevant phenotype	Source
PY79	Parental strain	[29]
RH201	*cotB::spc*	[18]
AZ703	*amyE::cotBΔ::bclA3$_{CTD}$*	This study
AZ704	*amyE::cotBΔ::bclA2$_{CTD}$*	This study
AZ705	*amyE::cotBΔ::cdeC$_{CTD}$*	This study

Sporulation of *B. subtilis* strains was induced by the exhaustion method [30]. Briefly, after 35 hours of growth in Difco Sporulation (DS) medium at 37ºC 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 [13]. Spore suspensions were prepared by plating a 1:100 dilution of an overnight culture onto a 70:30 medium (63 g Bacto peptone (BD Difco), 3.5 g proteose peptone (BD Difco), 0.7 g ammonium sulfate $(NH_4)_2SO_4$, 1.06 g Tris base, 11.1 g brain heart infusion extract (BD Difco) and 1.5 g yeast extract (BD Difco) for 1L) and incubating it for 5 days at 37°C under anaerobic conditions [31]. 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 minutes. Spores were loaded onto a 45% Nycodenz solution, centrifuged (14 000 rpm, 40 minutes). After centrifugation, the spore pellet was washed five times (14 000 rpm, 5 minutes) with ice-cold sterile water to remove Nycodenz remnants.

The spores were counted in Neubauer chamber and volume adjust at $5x10^9$ 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.

3.5.2. Construction of the recombinant strains AZ703, AZ704 and AZ705

DNA coding for $BclA3_{CTD}$, $BclA2_{CTD}$ and $CdeC_{CTD}$ (antigens) were PCR amplified using the *C. difficile* R20291 chromosome as template. *cotBΔ*, gene coding for the N-terminal 275 amino acids of CotB (carrier), was primed from *B. subtilis* PY79 chromosomal DNA. The oligonucleotides used are depicted on Table 3.2..

The obtained amplification products were cloned in pGEMT-easy (yielding pGEMT-easy::$bclA3_{CTD}$, pGEMT-easy::$bclA2_{CTD}$, pGEMT-easy::$cdeC_{CTD}$ and pGEMT-easy::*cotBΔ*). The antigens' genes were digested from pGEMT-easy with BamHI/PstI restriction enzymes and cloned in-frame to the 3′ end of the *cotBΔ* gene carried by plasmid pGEMT-easy::*cotBΔ* previously diggested with BglII/PstI, yielding plasmid pGEMT-easy::*cotBΔ*::$bclA3_{CTD}$, pGEMT-easy::*cotBΔ*::$bclA2_{CTD}$ and pGEMT-easy::*cotBΔ*::$cdeC_{CTD}$. The fusion genes *antigen::carrier* were 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.

3.5.3. Western-blot analysis

Sporulation of RH201, PY79, AZ703, AZ704 and AZ705 was induced by the exhaustion method as described above. The coat proteins from $5x10^8$ spores were extracted by SDS-DTT treatment [19] and quantified by Bradford assay (BioRad). 20 µg of protein extract were resuspended in protein sample buffer 2x [32], incubated at 100ºC for 7 minutes and loaded onto a 12% SDS-Page gel. Proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) and used for Western-blot analysis by standard procedures. The identification of the recombinant proteins was made using anti-CotB 1:7000 as primary antibody and anti-rabbit secondary antibody conjugated with horseradish peroxidase 1:7000.

Table 3. 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')[a]	Restriction site
BclA2$_{CTD}$senso	ggtaccccatggggatccGCAGCAAACAATGCACAA TTTACAG	KpnI/NcoI/BamHI
BclA2$_{CTD}$anti	*tctagactgcagCTATTGTATTCTATAAACTGATACA TAC*	XbaI/PstI
BclA3$_{CTD}$senso	*ggtaccggatccGCAATAATACCTTTTGCATCAGG*	KpnI/BamHI
BclA3$_{CTD}$anti	*tctagactgcagCTAATTTATTGCAATTCCTGCAC*	XbaI/PstI
CdeC$_{CTD}$senso	*ggtaccggatccAACTTCTCTGTATCAAATGCAGTG*	KpnI/BamHI
CdeC$_{CTD}$anti	*tctagactgcagCTATCTGTGGCAACTTGGCTTTC*	XbaI/PstI
B1	*acatgcatgcACGGATTAGGCCGTTTGTCC*	SphI
B3	*gaaagatctGGATGATTGATCATCTGAAG*	BglII

[a]Capital and lowercase letters indicate bases of DNA coding for proteins and of an unpaired tail carrying a restriction site, respectively.

3.5.4. Adherence to Caco-2 cells

An existing stock of Caco-2 cells in the Microbiota-Host Interaction and Clostridia Research Group at the Universidad Andrès Bello was routinely grown at 37ºC with 5% CO_2 in Dulbecco's modified Eagle's minimal essential medium (DMEM) (HyClone), supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone), penicillin (100 U/mL), and streptomycin (100 µg/mL). Caco-2 cells were seeded onto glass coverslips in 24-wells plates ($4x10^5$ cells per well) and incubated until confluence. Caco-2 cells were further incubated for 2 and 8 days to obtain undifferentiated and differentiated monolayers, respectively, using previously described methods [33]. Monolayers were infected with $2.5x10^7$ spores of *C. difficile* R20291, *B. subtilis* PY79, AZ703, AZ704 and AZ705 in 200 µL of culture medium with or without previous incubation with Normal Human Serum (NHS). Spore-infected cells were incubated for 3 hours at 37ºC under aerobic conditions, then unbound

spores were removed from spore-infected Caco-2 cells by washing three times with DPBS. In order to count the number of cells the nuclei were stained with Hoechst stain (Sigma) 1:1 000 in PBS 10 minutes. After several washes with PBS and dH_20 the coverslips were dried 15 minutes at 37ºC, mounted using Dako Fluorescence Mounting medium (Dako, North America) and sealed with nail polish. Samples were analyzed with an Olympus BX53 microscope. The number of adhered spores and cells were counted and the adherence was represented as number of spores/cell. The experiment was made in triplicates.

3.5.5. Immunofluorescence of vitronectin bound to the spores

Differentiated cells ($4x10^5$ per well) fixed onto glass coverslips were infected with $2.5x10^7$ spores of *C. difficile* R20291, *B. subtilis* PY79, AZ703, AZ704 and AZ705 without being previously pre-blocked in NHS. After 3 hours at 37ºC under aerobic conditions, the unbound spores were removed from spore-infected Caco-2 cells by washing three times with DPBS. Next, the spores were fixed to the coverslips in 3% paraformaldehyde (pH 7.4), rinsed three times with PBS, blocked with 1% bovine serum albumin (BSA) for 30 minutes, and further incubated over night at 4ºC with 1:200 rabbit anti-vitronectin antibodies (Santa Cruz Biotechnologies, USA). Then, coverslips were incubated for 1 hour at room temperature with 1:400 anti-rabbit IgG Alexa Fluor 568 conjugate (Invitrogen) in PBS with 1% BSA and washed three times with PBS and once with sterile distilled water. Samples were then dried at room temperature for 30 minutes, and then coverslips were mounted using Dako Fluorescence Mounting medium (Dako, North America) and sealed with nail polish. Samples were analyzed with an Olympus BX53 microscope. The experiment was made in triplicates. Using ImageJ, an outline was drawn around 150 spores for each strain and the area, the integrated density and the mean fluorescence measured, along with several adjacent background readings. The fluorescence intensity observed for *C. difficile* spores was considered 100% and the calculation of vitronectin recruitment for all the other strains was depicted as the % of fluorescence intensity relative to *C. difficile* spores.

3.5.6. 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 Mann-Whitney unpaired t-test. Comparative analysis between groups was performed by analysis of variance with Turkey'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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APPENDIX I

A probiotic treatment increases the immune response induced by the nasal delivery of spore-adsorbed TTFC

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Published on Microbial Cell Factories

1.1. Abstract

Background - Spore-forming bacteria of the *Bacillus* genus are widely used probiotics known to exert their beneficial effects also through the stimulation of the host immune response. The oral delivery of *Bacillus toyonensis* spores has been shown to improve the immune response to a parenterally administered viral antigen in mice, suggesting that probiotics may increase the efficiency of systemic vaccines. We used the C fragment of the tetanus toxin (TTFC) as a model antigen to evaluate whether a treatment with *B. toyonensis* spores affected the immune response to a mucosally administered antigen.

Results - Purified TTFC was given to mice by the nasal route either as a free protein or adsorbed to *B. subtilis* spores, a mucosal vaccine delivery system proved effective with several antigens, including TTFC. Spore adsorption was extremely efficient and TTFC was shown to be exposed on the spore surface. Spore-adsorbed TTFC was more efficient than the free antigen in inducing an immune response and the probiotic treatment improved the response, increasing the production of TTFC-specific sIgA and causing a faster production of serum IgG. The analysis of the induced cytokines indicated that also the cellular immune response was increased by the probiotic treatment. A 16S RNA-based analysis of the gut microbial composition did not show dramatic differences due to the probiotic treatment. However, the abundance of members of the *Ruminiclostridium* 6 genus was found to correlate with the increased immune response of animals immunized with the spore-adsorbed antigen and treated with the probiotic.

Conclusion - Our results indicate that *B. toyonensis* spores significantly contribute to the humoral and cellular responses elicited by a mucosal immunization with spore-adsorbed TTFC, pointing to the probiotic treatment as an alternative to the use of adjuvants for mucosal vaccinations.

1.2. Introduction

Mucosal surfaces are the most common route used by pathogens to enter the human and animal body. For this reason, it is extremely important for a vaccine to induce sIgA antibody production and elicit immune protection at the mucosal surfaces [1]. While injected vaccines induce specific T cell responses in the bloodstream and serum IgG production but generally fail to induce sIgA, mucosal

vaccines administered via the oral or nasal routes induce humoral and cellular immune responses at both the systemic and mucosal sites [2, 3]. Therefore, mucosal, needle-free vaccines are potentially preferable over parenteral vaccinations [4]. However, only few mucosal vaccines are currently licensed for vaccination against viral (Rotavirus, Poliovirus, Influenza type A virus) or bacterial (*Salmonella typhi*, *Vibrio cholerae*) pathogens [3]. This is mostly due to the low immunogenicity of most mucosal antigens and to the lack of efficient adjuvants and delivery systems [4]. Indeed, adjuvants commonly used in injected vaccines fail to induce sIgA and therefore are not efficient with mucosal antigens, while the lack of appropriate delivery systems does not prevent antigen degradation by enzymes present in the mucosal tissues [3]. Major efforts have been devoted to the development of new mucosal vaccination strategies based on adjuvants able to induce sIgA or on novel delivery systems based on synthetic nanoparticles, viral particles, microbial cells or bacterial spores [5-8].

Increasing interest is also receiving the use of probiotics before and/or during the vaccination period to modulate the immune response [9] and increase the effectiveness of vaccines against bacterial [9, 10] or viral [11, 12] infections. In a recent study, spores of *Bacillus toyonensis* were shown able to increase the immune response to a parenteral vaccine against bovine herpesvirus type 5 (BoHV-5) in mice [13]. *B. toyonensis*, originally defined as *B. cereus* var. toyoi and then identified as a new species by genomic analysis [14], has long been used in animal nutrition for swine, poultry, cattle, rabbits and aquaculture. In 1994 its use has been authorized by the European Community as a feed additive for use in poultry, cattle and rabbits [15]. Animals parenterally immunized with BoHV-5 and orally supplemented with *B. toyonensis* spores had higher serum IgG, IL-4 and IL-12 levels than immunized animals that did not receive the probiotic [13], suggesting this probiotic treatment as a potential alternative to the use of adjuvants.

Aim of this work was to investigate whether the oral treatment with spores of *B. toyonensis* was also effective in inducing the production of specific sIgA thus improving the immune response induced by a mucosal antigen. As a model antigen the C fragment of the tetanus toxin (TTFC), the protective antigen used in evaluations of vaccines against tetanus, was selected [16]. TTFC administered by the oral or nasal route was shown to induce a protective immune response in mice

when delivered by *B. subtilis* spores either as a fusion protein exposed on the spore surface [17-19] or as a pure protein adsorbed on the spore surface [20].

The use of *B. subtilis* spores as a mucosal delivery system has been exploited in recent years and tested with several antigens and enzymes [6, 21, 22]. In addition to TTFC, the binding subunit of the heat-labile toxin (LTB) of *Escerichia coli* [23, 24], the protective antigen (PA) of *B. anthracis* [20], the C terminus of toxin A of *Clostridium difficile* [25], the capsid proteins VP26 and VP28 of the White Spot Syndrome virus [26, 27] and the MPT64 antigen of *Mycobaterium tuberculosis* [28] are examples of antigens displayed by *B. subtilis* spores and tested as mucosal vaccines.

1.3. Results

1.3.1. Spore adsorption of the C fragment of the tetanus toxin (TTFC)

Aliquots (2 μg) of TTFC, over-expressed in *E. coli* and purified by affinity chromatography columns (Methods), were incubated in 200 μl of 50 mM sodium citrate buffer at pH 4 with $2x10^9$ spores of the *B. subtilis* strain PY79 [29], purified as previously described [30]. After 1 hour of incubation at 25°C spores were collected by centrifugation and surface proteins extracted by SDS-DTT treatment [31]. Proteins were then analyzed by western-blot with anti-TTFC antibody [17] and, as previously reported [20], TTFC was found among the proteins extracted from the spore surface (Figure I.1A). To assess the stability of spore-TTFC interaction, spores adsorbed with TTFC were re-suspended in 200 μl of 50 mM sodium citrate buffer at pH 4 and stored one week at 4°C. Upon centrifugation, spores were used to extract surface proteins as described above while the supernatant was five-fold concentrated by ultra-filtration (3 kDa cut-off) and analyzed by western-blot. As shown in Figure I.1A, TTFC was still extracted from one-week-old spores (lane 3) and was not present in the supernatant (lane 4), indicating that TTFC was not degraded and or released during the storage at 4°C.

To indirectly quantify the amount of TTFC adsorbed on the spore, the adsorption reaction mixture was fractionated by centrifugation and the supernatant, containing the unbound, free TTFC was analyzed by dot blotting with anti-TTFC antibody (Figure I.1B). The intensity of the various spots was then quantified by a densitometry analysis as previously described [22] and indicated that

in our experimental conditions less than 3% of TTFC was left free in the supernatant. Such a high efficiency of adsorption was not surprising since previous reports have shown that in similar experimental conditions over 90% of reacted proteins were adsorbed to *B. subtilis* spores [22, 24].

A flow cytometric approach was used to evaluate the exposure of TTFC on the spore surface. Spores adsorbed with TTFC were reacted with anti-TTFC specific antibody, then with FITC-conjugated secondary antibody and analyzed by flow cytometry (Figure I.1C). In parallel, free spores incubated or not with antibodies (primary and secondary) were analyzed to take into consideration the unspecific fluorescence of spores (Figure I.1C, brown and red histogram, respectively). These controls, overlaid and used as a reference guide in the measurement of the TTFC-specific fluorescence, indicated that when adsorbed with TTFC the majority of the spore population (64% of the 100 000 counted spores) were specifically fluorescent and, therefore, displayed the antigen (Supplem. Mat. Figure SI.1).

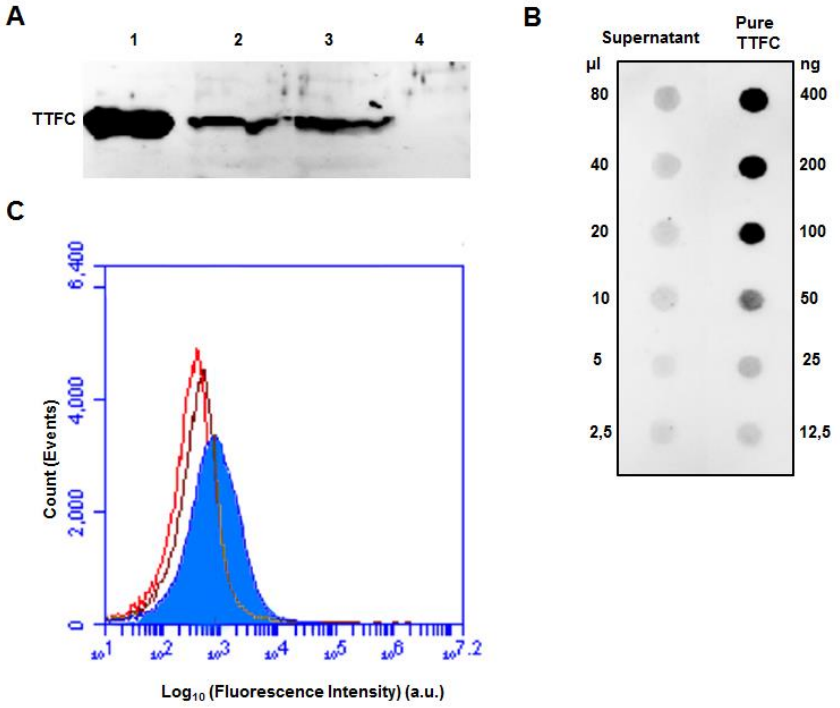

Figure I. 1. TTFC adsorption on *B. subtilis* spores. (A) Western-blot of spore surface proteins after adsorption with 2 µg of purified TTFC. Lane 1: purified TTFC; 2: proteins extracted from adsorbed spores; 3: proteins extracted from adsorbed spores after one week storage at 4°C; 4: five-fold concentrated supernatant after one week storage at 4°C. **(B)** Dot blotting experiment performed with the serial dilutions of the supernatant (unbound TTFC) fraction of the adsorption reaction. Serial dilutions of purified TTFC were used as a standard. **(C)** Flow cytometric analysis of: free spores incubated (brown histogram) or not (red histogram) with specific antibodies and TTFC-adsorbed spores incubated with specific antibodies (filled blue histogram). The analysis was performed on the entire spore population (ungated). Immune-reactions were performed with polyclonal anti-TTFC and anti-rabbit HRP conjugate (panels A and B) or with FITC-conjugated secondary antibodies (C).

1.3.2. A probiotic treatment increases sIgA production induced by a nasal administration of spore-adsorbed TTFC

In a previous study [20], *B. subtilis* spore-adsorbed TTFC was administered by the nasal route to mice and shown able to induce an antigen-specific mucosal response. We used the same dosage and administration route used before [20] to assess whether a probiotic treatment with *B. toyonensis* was able to influence the mucosal immune response elicited by spore-adsorbed TTFC. To evaluate the effect of the probiotic also on the immune response induced by the pure antigen, parallel groups of animals were also immunized with 2 μg of purified TTFC. Figure I.2 schematically shows the experimental plan: three groups of animals received the oral probiotic treatment ($1x10^6$ spores/gram of food from day -7 to day 35), two groups were immunized with 2 μg of purified TTFC by the nasal route on day 0, 14 and 28 (blue arrows in Figure I.2) and two groups received $2x10^9$ spores adsorbed with TTFC by the nasal route on day 0, 14 and 28 (red arrows in Figure I.2). A naive group that did not receive either probiotics or the antigen was also included. Blood samples were collected from all animals at days 14 and 21 and at day 35 all animals were sacrificed for analysis.

As calculated in the previous paragraph, $2x10^9$ spores adsorbed with 2 μg of TTFC displayed about 1.9 μg of TTFC (over 90% of the total TTFC), therefore, three doses of spores delivered a total 5.7 μg of TTFC, slightly less than the amount of antigen received by the animals immunized with the purified antigen (6 μg).

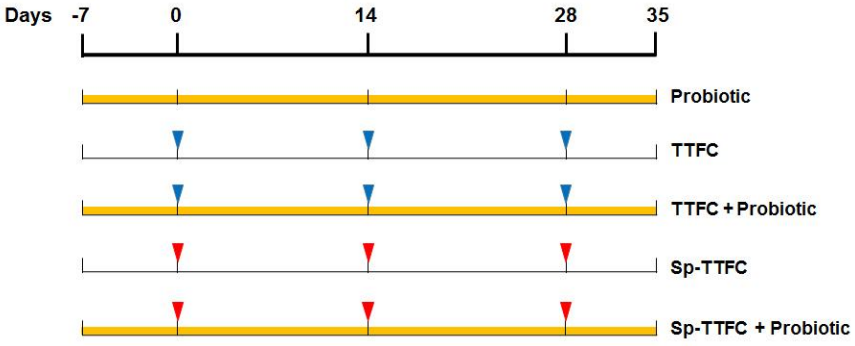

Figure I. 2. Experimental plan. Three experimental groups received the oral probiotic treatment (yellow lines) with $1x10^6$ spores/ gram from day -7 to day 35. One of these groups was immunized with 2 μg of purified TTFC (blue arrows) and another one with $2x10^9$ spores adsorbed with TTFC (Sp-TTFC) (red arrows) on day 0, 14 and 28. All immunizations were performed by the nasal route. Two groups were immunized only with purified TTFC or Sp-TTFC without probiotics. A naive group that did not receive either probiotics or the antigen was also included. Blood samples were collected from all animals at days 0, 14 and 21 and 35, at day 35 all animals were sacrificed for analysis.

High anti-TTFC fecal sIgA levels, indicative of a mucosal immune response, were induced by spore-adsorbed TTFC in animals treated with the probiotic (Figure I.3A). The response was maximal after 14 days and slightly decreased at days 21 and 35. As expected, the free antigen did not induce high levels of sIgA and the treatment with the probiotic cause only a minimal increase (Figure I.3A). The analysis of serum antibodies showed a positive effect of the probiotic on the immune response induced by spore-adsorbed TTFC at day 14 (Figure I.3B). A days 21 and 35 similar levels of IgG were induced by spore-adsorbed TTFC with or without the probiotic treatment (Figure I.3B). Low levels of TTFC-specific IgG were induced by the purified antigen after 14 days, those levels were slightly increased after 21 and 35 days and were not affected by the treatment with the probiotic (white and light grey bars in Figure 3B, respectively). The ability of nasally administered spore-adsorbed TTFC to induce a stronger immune response than purified TTFC, at days 21 and 35 (compare white and dark grey bars in Figure I.3B), could be due to an increased antigen uptake by immune cells or, alternatively, to a reduced antigen degradation, as previously suggested for another antigen [24]. Additional experiments are required to fully address this issue. For the aim of this work, it is noteworthy that the probiotic increased the mucosal (sIgA) immune response and accelerated the production of serum IgG induced to spore-adsorbed TTFC.

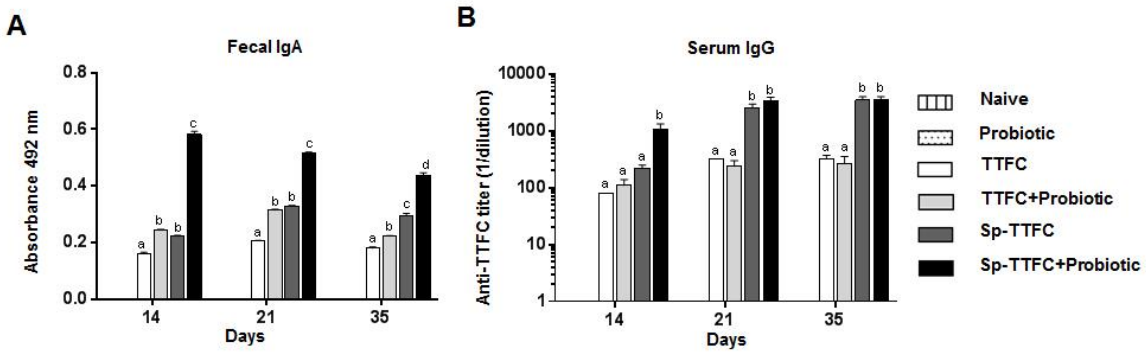

Figure I. 3. Antibody production. (A) Anti-TTFC specific fecal sIgA detected on days 14, 21 and 35. Data were expressed as the mean (± standard error) of absorbance values at 492nm. **(B)** Anti-TTFC specific serum IgG detected on days 14, 21, and 35. The data represent the mean (± standard error) of reciprocal endpoint titers. Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

The phenotype of the induced humoral immune response was then examined analyzing IgG subclasses. High levels of IgG1, IgG2b, IgG2c or IgG3 subtypes were induced at all time-points in animals immunized with Sp-TTFC, independently from the probiotic treatment (Figure I.4).

Only at day 14 IgG2c was higher in probiotic-treated animals than in those that did not receive *B. toyonensis* (Figure I.4B). Since in mice, the IgG1 isotype is associated with a Th2 response, whereas IgG2c (analogous to IgG2a in other mouse strains) and IgG2b sometimes associated with IgG3 reflect a Th1 response [32, 33], results of Figure 4 suggest the induction of potent and mixed Th1/Th2-type immune responses elicited by spore-adsorbed TTFC independently of the probiotic treatment.

Altogether, the results of Figure I.3 and I.4, indicate that the treatment with *B. toyonensis* spores increases fecal sIgA production in animals nasally immunized with TTFC carried by *B. subtilis* spores while does not affect the level and the phenotype of the serum IgG response.

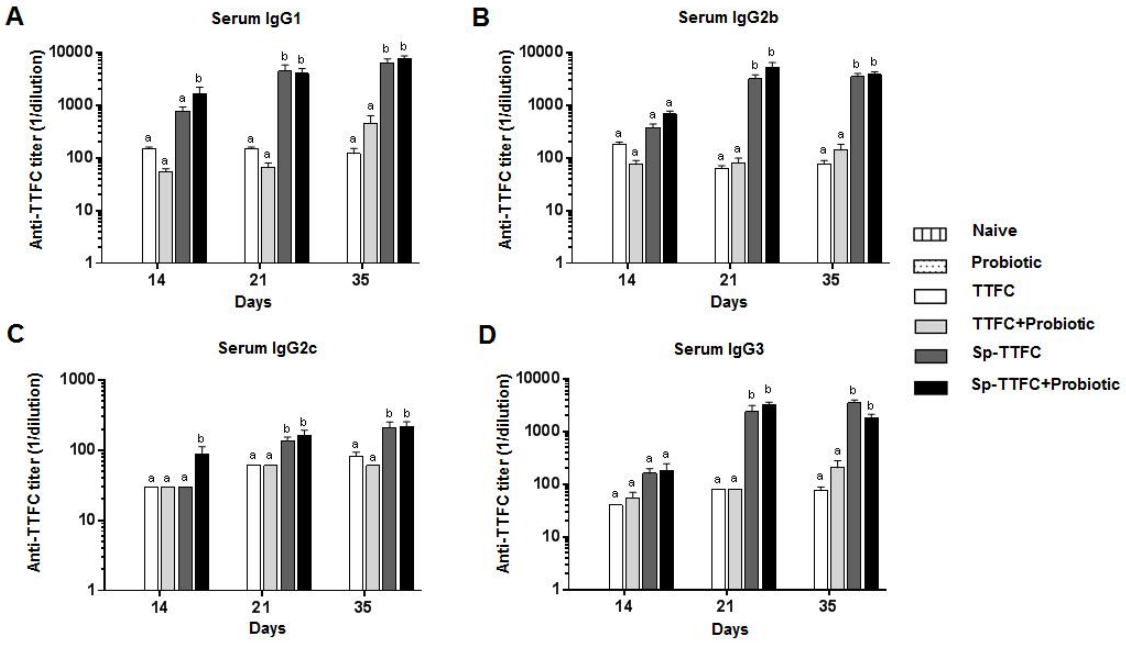

Figure I. 4. IgG subclasses analysis. The phenotype of the induced humoral immune response. Anti-TTFC **(A)** IgG1, **(B)** IgG2b, **(C)** IgG2c, and (**D)** IgG3 levels detected in mice serum on days 14, 21, and 35. The data represent the mean (± standard error) of reciprocal endpoint titers. Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

1.3.3. A probiotic treatment increases the cellular immune response elicited by a nasal administration of spore-adsorbed TTFC

The spleen of all vaccinated animals was analyzed for TTFC-specific production of cytokines IL-4, IL-6, IL-10, IL-12 and IFN-γ. While IL-4 was not produced at detectable levels (not shown), all other analyzed cytokines were detected in the culture supernatants. High levels of IL-6 were produced by splenocytes from mice that received spore-adsorbed TTFC treated and not treated with the probiotic, however, in probiotic-treated animals IL-6 levels were statistically higher (Figure I.5A).

The IL-6 is pro-inflammatory cytokine that plays a central role during the transition from innate to adaptive immunity [34]. Recent studies showed that IL-6 induces the maturation of B cells into antibody-secreting cells and promotes the survival and maintenance of long-lived plasma cells [35].

IL-10 was detected only in the spleen of mice immunized with either pure TTFC or spore-bound TTFC that were treated with the probiotic (Figure I.5B). Animals treated with the probiotic but not immunized only showed basal levels of IL-10. Results on IL-10 are consistent with recent reports showing an increase in IL-10 expression in splenocytes of animals supplemented with *B. toyonensis* spores and vaccinated with a parenteral vaccine against bovine herpesvirus type 5 [12, 36]. IL-10 is a cytokine that can be produced by a number of cell types including T cells, B cells and macrophages and acts controlling the intensity of the immune response [37], increasing the survival of B cells, increasing the production of immunoglobulins, and mediating the immune stimulatory effects on T cells [38].

The probiotic treatment did not affect the production of IL-12 that was low in the spleen of mice immunized with TTFC and high in mice immunized with Sp-TTFC, independently from the probiotic treatment (Figure I.5C). Instead, *B. toyonensis* spores were able to increase the IFN-γ levels produced by spleen cells of mice vaccinated with Sp-TTFC (Figure I.5D). IFN-γ directs the differentiation of naïve T lymphocytes into Th1 cells [39], and the induction of a Th1 type of immune response by spores is in agreement with previous reports on spores displaying antigens [20, 24].

Overall, the results of Figure I.5 indicate that the probiotic treatment increases the cellular response to nasally administered TTFC carried by *B. subtilis* spores.

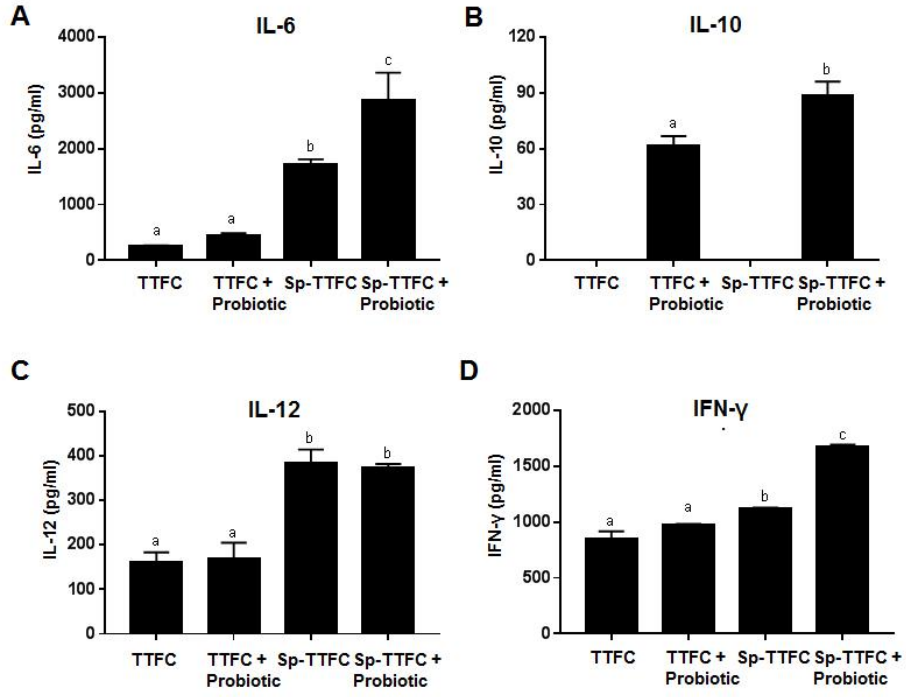

Figure I. 5. Cytokine induction. The cellular immune response elicited by TTFC and spore-adsorbed TTFC treated with probiotic. IL-6 **(A)**, IL-10 **(B)**, IL-12 **(C)**, and IFN-γ **(D)** levels secreted *in vitro* from spleen cells. The results were expressed as pg/mL of the mean values (± standard error). Data are reported after subtracting the cytokine values detected in control groups (naive and not immunized mice that received the probiotic). Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

1.3.4. The probiotic treatment did not strongly alter the microbial composition of the animal gut

A 16S DNA-sequencing approach was used to investigate the effect of the probiotic treatment on the gut microbial composition. As reported below, the analysis performed on samples of animals of the control group was in agreement with previous data for mice, with Firmicutes much more abundant than Bacteroidetes [40].

PCoA based on Bray-Curtis distance showed that the gut microbiota of mice of the various groups did not form clear separate clusters, suggesting that the immunizations and/or probiotic treatments did not dramatically alter the microbial composition of the animal gut (Figure I.6).

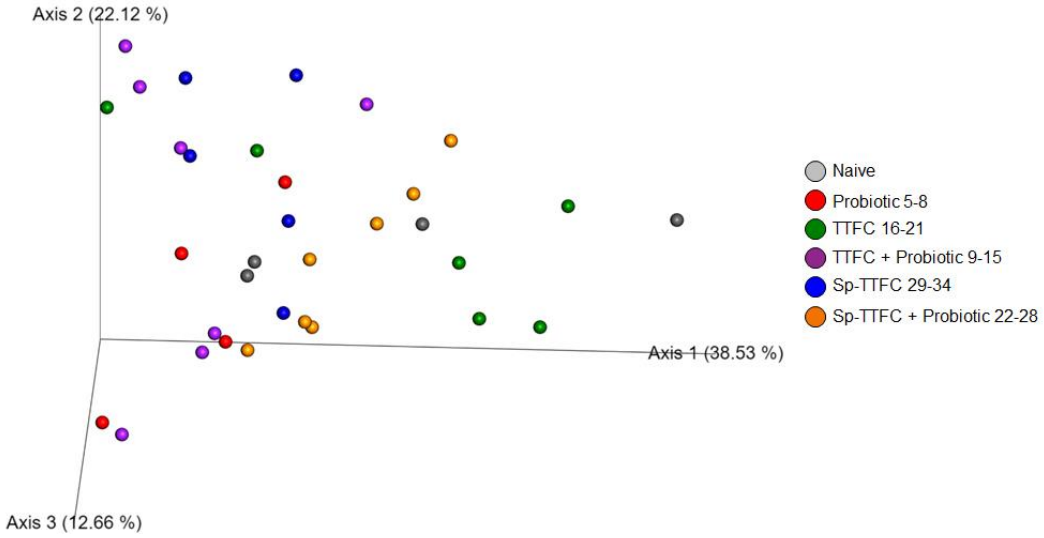

Figure I. 6. Principal Coordinate Analysis (PCoA). Plots were generated using weighted UniFrac distance matrix.

The OTU representation curves indicated that the microbial diversity of the samples was completely covered while the alpha-diversity analysis showed a higher number of species in two animals of the control (naive) group than in all other groups that did not differ significantly among each other (Suppl. Mat. Figure SI.2.). The analysis of the bacterial composition, reported as the average of the relative abundance of bacterial taxa at the Phylum, Family and Genus level, did not show dramatic differences among the experimental groups. The identified phylotypes showed that Firmicutes were the most abundant bacteria in all groups (54–70%) while Bacteroidetes and Proteobacteria were always less represented (18-33% and 2–14%, respectively) with the latter Phylum that was less represented in all experimental groups with respect to the naive group (Figure I.7).

The analysis at the family and genus level (Suppl. Mat. Figure SI.3.) was focused on the bacterial taxa of the various groups that showed a statistically significant variation ($p < 0.05$) in their representation with respect to the naive group. By this approach three bacterial genera were found to have a statistically different representation between the probiotic-supplemented and naive groups: *Eubacterium* (Figure I.8A), *Fusobacterium* (Figure I.8B) and *Ruminococcaceae* UCG-014 (Figure I.8C). In addition, also the *Bacillus* genus which includes species used here as the probiotic and the antigen delivery vehicle, was differently represented between probiotic-supplemented and naive

groups (Figure I.8D). However, in this case the difference was statistically significant only for two of the three groups (Figure I.8D).

Altogether, the results of Figure I.8 indicate that the probiotic treatment did not drastically affect the gut microbial composition but instead altered the abundance of few genera.

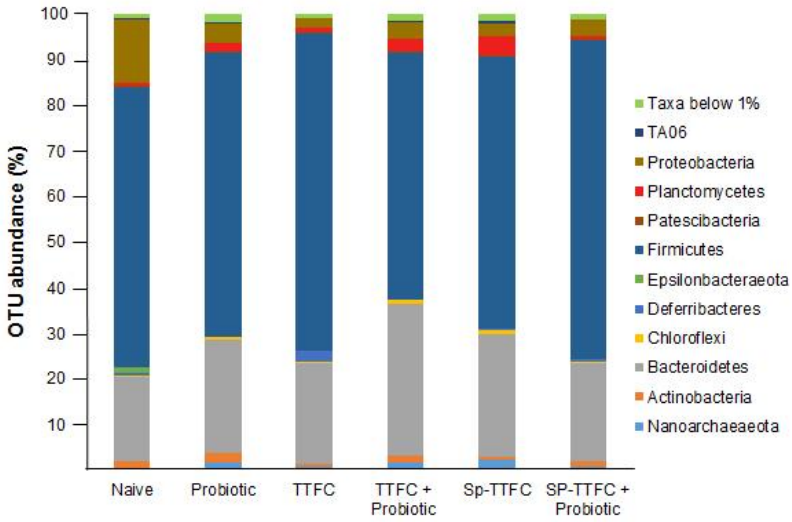

Figure I. 7. Fecal bacterial composition. Relative Operational Taxonomic Units (OTUs) abundance at the Phylum level in the six experimental groups, reported as mean values within each group. Only Taxa represented by OTUs abundance >1% have been considered for the analysis.

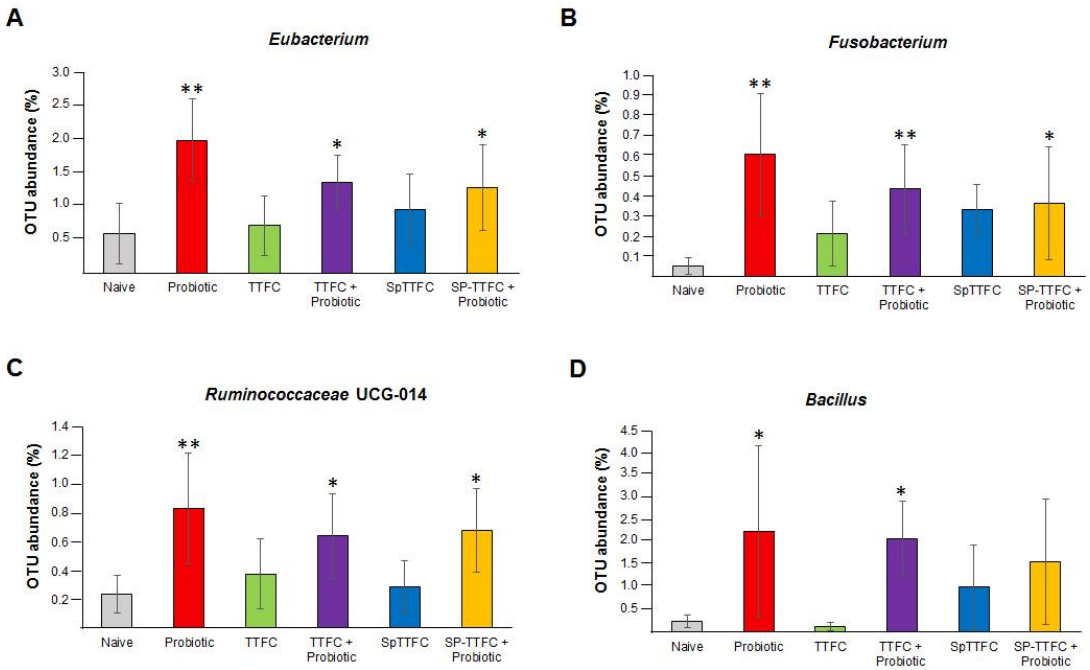

Figure I. 8. Representativeness of four bacterial genera. The different abundance of four genera between the probiotic treated groups and the control is reported. Statistically significant differences are indicated by asterisks (* $P < 0.05$; ** $P < 0.005$).

An additional analysis was performed looking at the statistically relevant differences between genera of the two groups that gave better immune responses (Sp-TTFC and Sp-TTFC+Probiotic) and all other groups. By this approach it was found that members of *Ruminiclostridium* 6 genus were abundant in the gut of animals immunized with spore-displayed TTFC that received the probiotic (Figure I.9). The same genus was also abundant in the gut of animals of the Sp-TTFC group, however, the differences were statistically significant with the naive, probiotic, TTFC groups, and slightly above the threshold ($p<.05$) with the other groups (Figure I.9).

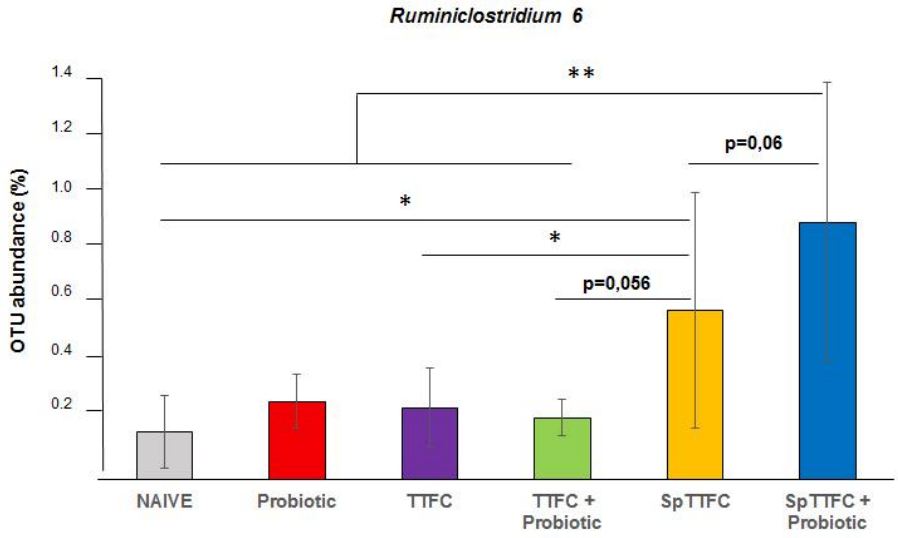

Figure I. 9. Representativeness of the *Ruminiclostridium* 6 genus. The different abundance of *Ruminiclostridium* 6 between groups immunized with Sp-TTFC and the other groups is reported. Statistically significant differences are indicated by asterisks (* $P < 0.05$; ** $P < 0.005$). Differences with P-value slightly above the threshold are also shown.

1.4. Discussion

Main conclusion of this chapter is that a probiotic treatment with *B. toyonensis* spores positively affects a nasal immunization with the C fragment of the tetanus toxin (TTFC) displayed by *B. subtilis* spores. While it was already known that *B. toyonensis* spores increased the immune response to a systemic vaccination [13], their efficacy as adjuvant of a mucosal vaccination was never tested before. The observed increased production of fecal sIgA and of IL-6, IL-10 and IFN-γ in the spleen of immunized animals in response to the probiotic treatment clearly points to the *B. toyonensis* spore as a potential mucosal adjuvant.

B. toyonensis spores also increased the serum IgG production in animals immunized with spore-adsorbed TTFC. However, this effect was only observed at early, day 14, and not at late, day 21 or 35, time points suggesting that the probiotic cause a faster serum IgG response, probably driven by the IgG2c subclass.

The analysis of the gut microbiota did not show dramatic changes in the various experimental groups. Three genera, *Eubacterium, Fusobacterium and Ruminococcaceae* UCG-014, were found to have statistically significant differences in their representation between the naive group and the groups that received the probiotic treatment. Members of the *Eubacterium* genus belong to the *Lachnospiraceae* family and are anaerobic, Gram-positive, non-spore-forming rods, previously associated with dietary fiber-induced modulation of the human gut microbiota [41]. Bacteria of the *Fusobacterium* are obligate anaerobe, Gram-negative rods commonly found as components of the normal flora of the human oropharynx. Some species of the *Fusobacterium* genus are considered as pathogenic, have been associated with colon cancer or found to increase in response to other infections [42]. The *Ruminococcaceae* UCG-014 genus groups obligate anaerobes belonging to the *Ruminococcaceae* family, the same family of *Faecalibacterium prausnitzii*, a commensal bacterium of the human gut recently proposed as a probiotic [43]. This analysis then indicates that although the probiotic treatment did not drastically affect the gut microbial composition, it altered the relative abundance of few genera. However, those differences did not correlate with the different immune responses observed.

By comparing the gut microbiota of the two experimental groups that gave better immune responses (Sp-TTFC and Sp-TTFC+Probiotic) vs. all other groups, *Ruminiclostridium* 6 was found statistically more abundant in the Sp-TTFC+Probiotic group. This observation points to a correlation between the abundance of the *Ruminiclostridium* 6 genus and the induction of a strong immune response. Such conclusion is also supported by a recent study [44] in which *Ruminiclostridium* 6 abundance was shown to correlate with high levels of IL-6 inflamed mice.

1.5. Materials and Methods

1.5.1. Bacterial Strains, Spore and TTFC production

The *B. subtilis* strain PY79 [29] was used in this study and sporulation was induced by the exhaustion method [45]. After 30 hours of growth in Difco Sporulation (DS) medium at 37ºC with vigorous shaking, spores were collected, washed three times with distilled water and purified as described before [30]. Spore counts were determined by serial dilution and plating counting.

The TTFC (tetanus toxin fragment C) from *C. tetani* was expressed from recombinant plasmid (pET-28b) in the *E. coli* strain BL21. The plasmid pET-28b-TTFC expressed *C. tetani* TTFC as a 52.6 kDa polypeptide and has been described elsewhere [17]. The expressed protein carried a poly-histidine tag at its 3'-end and following expression was purified using His-Trap column as recommended by the manufacturer (GE Healthcare Life Science).

B. toyonensis BCT-7112T used in this study was obtained from the collection of microorganisms of the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (Brazil). Bacteria were grown in DS medium at 37ºC for 96 hours as previously reported [13] and analyzed under the optical microscope for the presence of cells and spores. The cultures containing over 95% of free spores were centrifuged at 5 000 g for 20 minutes at 4°C and the pellet suspended in phosphate buffer to a concentration of spores of approximately $2x10^7$ CFU/mL.

1.5.2. Adsorption reaction, western- and dot-blotting analysis

The adsorption reaction was performed by mixing purified TTFC (2 µg) and $2x10^9$ spores in 50 mM Sodium Citrate pH 4 at 25ºC in a final volume of 200 µl. After 1 hour of incubation, the binding mixture was centrifuged (10 minutes at 13 000 g) to fractionate pellet and supernatant and stored at 4ºC [31]. The pellet fraction, containing TTFC-adsorbed spores ($2x10^9$) was suspended in 20 µl of spore coat extraction buffer [31], incubated at 68°C for 1 hour to solubilize spore coat proteins and loaded onto a 12% SDS-PAGE gel. The proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) and used for Western-blot analysis as previously reported [24] using anti-TTFC specific rabbit polyclonal antibodies [17] and Goat Anti-Rabbit (H+L)-HRP Conjugate (Bio-rad). A quantitative determination of the amount of TTFC was obtained by dot blotting experiments analyzing serial dilutions of purified TTFC, and binding assay

supernatant. Filters were then visualized by the ECL-prime (Amersham Pharmacia Biotech) method and subjected to densitometric analysis by Quantity One 1-D Analysis Software (Bio-Rad).

1.5.3. Flow cytometry

A total of $5x10^5$ TTFC-adsorbed spores were blocked with 1x PBS containing 3% of fetal bovine serum for 30 minutes at 25ºC and subsequently incubated with anti-TTFC specific rabbit polyclonal antibodies diluted starting at 1:20 for 1 hour at 25ºC. After three washes with PBS, fluorescein isothiscyanate (FITC)-conjugated anti-rabbit IgG (1:50; Invitrogen) was added and incubated for 30 minutes at 25ºC, followed three washes with PBS. To evaluate the non-specific fluorescence, free spores stained or not with primary and secondary antibodies were analyzed. Samples were then resuspended in 400 µl of PBS and analyzed using by BD AccuriTM C6 Cytometer and BD AccuriTM C6 Software (BD Biosciences, Inc., Milan, Italy) collecting 100 000 events.

1.5.4. Animals, probiotic supplementation and vaccination

Male C57BL/6 mice (Charles River, Italy) 8 weeks old were singularly caged in a temperature-controlled room (23 ± 1°C) with a 12-h light/dark cycle (6.30 am – 6.30 pm).

Treatment,housing, and euthanasia of animals met the guidelines set by the Italian Health Ministry. All experimental procedures were approved by the "Comitato Etico-Scientifico per la Sperimentazione Animale" of the Federico II University of Naples (Italy). We used 40 mice that were divided in 6 groups named Naïve (n=4), Probiotic (n=4), TTFC (n=8), Probiotic + TTFC (n=8), Sp-TTFC (n=8), and Probiotic + Sp-TTFC (n=8). The Naïve, TTFC, and Sp-TTFC were fed with a commercial feed (Standard chow, Mucedola 4RF21, Italy), free of chemotherapeutic agents; whereas, the Probiotic, Probiotic +TTFC, and Probiotic + Sp-TTFC groups received the same commercial feed but supplemented with 1×10^6 spores of *B. toyonensis* per gram of food from 7 days before the first vaccination for diet adaptation.

Mice were vaccinated by the intranasal route on day 0 and received a booster on days 14 and 28 of the experiment. TTFC and Probiotic + TTFC groups were vaccinated with 2 µg of purified TTFC suspended in 50 mM Sodium Citrate buffer. The Sp-TTFC and Probiotic + Sp-TTFC groups

were vaccinated with $2x10^9$ spore-adsorbed with 2 µg of TTFC in a volume of 20 µl of 50 mM Sodium Citrate buffer. The naïve and probiotic groups were not vaccinated. Blood samples were collected by the submandibular puncture on days 0, 14, 21 and 35. After collection, serum was separated, labelled and stored -20°C until analysis. Fecal pellets were collected on day 0, 14, 21 and 35 to monitor the induction of the TTFC-specific IgA.

1.5.5. Antibody analysis

Indirect ELISA was performed to evaluated serum levels of total IgG and IgG1, IgG2b, IgG2c, and IgG3 specific against TTFC. Microtitre plates (96 well, Corning, Lowell, MA, USA) were coated overnight at 4°C with 0.2 µg of TTFC per well and subsequently washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Plates were blocked with PBS containing 5% of Milk. Samples of individual serum samples were serially two-fold diluted starting at 1:2 to 20 480 and added to the plates in triplicate. After incubation at 37°C for 1 hour, the plates were washed with PBS-T, followed by addition of horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG whole molecule antibodies (1:4 000 dilution, Sigma-Aldrich, St. Louis, MO, USA). Following a further incubation at 37°C 1 hour, the plates were promptly washed again with PBS-T and added developing solution containing 10 ml of substrate buffer, 0.004 g of Ortho-Phenylenediamine (OPD) (Sigma-Aldrich) and 15 µl of H_2O_2 were added, and incubated in the dark at room temperature for 15 minutes and then stopped by adding 2 N sulphuric acid. Absorbance values were measured in a microplate reader (Thermo Fischer Scientific, Waltham, MA, USA) with a 492-nm filter. IgG isotype analysis performed according to the instruction manual of the Mouse Monoclonal Antibody Isotyping Reagents kit (Sigma-Aldrich), following the same protocol above describe. For ELISA analysis of fecal IgA, we followed the procedure described by [46], using approximately 0.1 g of fecal pellets that had been suspended in 1% of PBS and 1 mM of phenylmethylsulfoyl fluoride (Sigma-Aldrich), incubated at 4ºC overnight, and stored at -20ºC prior to ELISA. The fecal extracts were tested by indirect ELISA for the presence of TTFC-specific IgA using a similar method to that shown above. Secretery IgAs were detected using Goat Anti-Mouse IgA alpha chain (HRP) (1:1,000 dilution, Abcam, Cambridge, UK).

1.5.6. Spleen cell cultures and cytokine production

Mice were sacrificed on day 35 and their spleen collected and macerated. Spleen cells ($2x10^6$) were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (Gibco) and antibiotic and antifungal agents (penicillin 10 000 IU/mL, streptomycin 10 mg/mL and amphotericin B 25 mg/mL) (Gibco) in 24-well plates (Corning) and incubated for 24 hours at 37°C in 5% CO_2 atmosphere. Culture medium was replaced after 24 hours and the cells were stimulated with 10 µg of TTFC, 10 µg of concanavalin A (ConA; Sigma-Aldrich), and with RPMI 1640, and incubated for 72 hours under the same conditions. ConA and RPMI were used as positive and negative control, respectively, for cell stimuli. Supernatants were harvested from cultures and analysed by Murine ELISA kit to detected production of followed cytokines IL-4 (Elabscience, USA), IL-6 (Diaclone, France), IL-10 (Diaclone), IL-12 (Elabscience), and IFN-γ (Diaclone). The assays were performed according to the manufacturers' instructions.

1.5.7. Microbiota identification by 16S rRNA sequencing

Total genomic DNA was extracted from 220 mg of mice fecal samples from all experimental groups using the QIAamp DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions.

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and Probio_Rev, which target the V3 region of the 16S rRNA gene sequence [47]. 16S rRNA gene amplification and amplicon checks were carried out as previously described [47]. 16S rRNA gene sequencing was performed using a MiSeq (Illumina) at the DNA sequencing facility of GenProbio srl (www.genprobio.com) according to the protocol previously reported [47].

Following sequencing and demultiplexing, the obtained reads of each sample were filtered to remove low quality and polyclonal sequences. All quality-approved, trimmed and filtered data were exported as .fastq files. The .fastq files were processed using a script based on the QIIME software suite [48]. Paired-end reads pairs were assembled to reconstruct the complete Probio_Uni / Probio_Rev amplicons. Quality control retained those sequences with a length between 140 and 400

bp and mean sequence quality score >20. Sequences with homopolymers >7 bp and mismatched primers were omitted.

In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 100 % sequence homology using DADA2 (Callahan et al., 2016) and OTUs not encompassing at least 2 sequences of the same sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 [48, 49] and the SILVA database v. 132 as reference dataset [50]. Biodiversity of the samples (alpha-diversity) was calculated with Chao1 and Shannon indexes. Similarities between samples (beta-diversity) were calculated by weighted uniFrac [51]. The range of similarities is calculated between the values 0 and 1. PCoA representations of beta-diversity were performed using QIIME2 [48, 49].

1.5.8. Statistical analysis

The data were analyzed using GraphPad Prism version 7 (USA). Differences among the various experimental groups were determined by the one-way ANOVA or two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. The analysis of the fecal microbial composition was performed with SPSS software v. 25 (www.ibm.com/software/it/analytics/spss/). Analysis of Variance (ANOVA) was performed to compare differential abundance of bacterial genera. For multiple comparison the post hoc analysis LSD (least significant difference) was calculated and differences with a p-value < 0.05 were considered significant.

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Supplementary Material

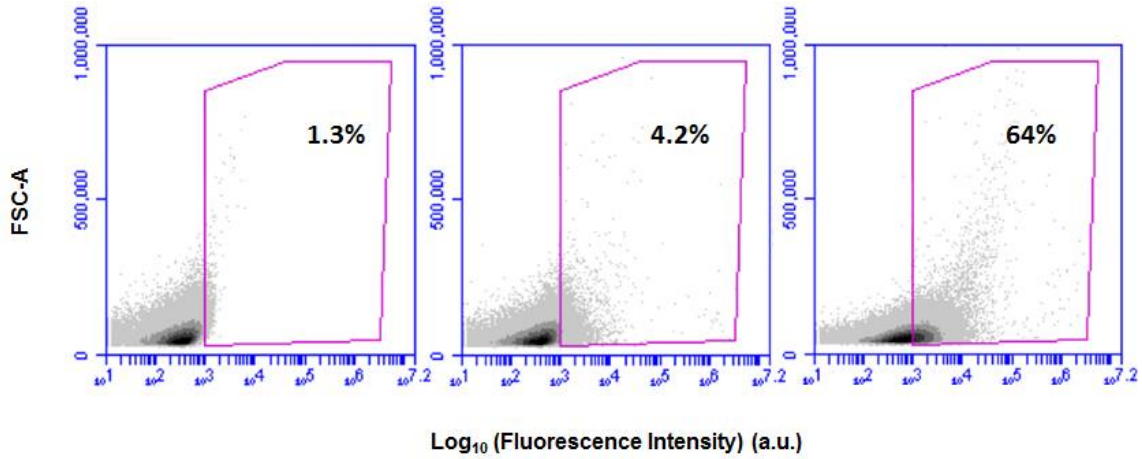

Figure SI. 1. Dot plots of the cytofluorimeter analysis. The dot plots show the forward scatter (FSC-A) vs Fluorescence intensity distribution of free spores without antibodies (Sp), free (Sp (Ab1/Ab2)) and TTFC-adsorbed (Sp-TTFC (Ab1/Ab2)) spores incubated with polyclonal anti-TTFC and FITC-conjugated secondary antibodies. In all cases 100 000 spores were analyzed. Regions boxed in pink contain events (spores) with a fluorescence intensity higher than 1000 a.u. The percentage of positive events is reported for each graph.

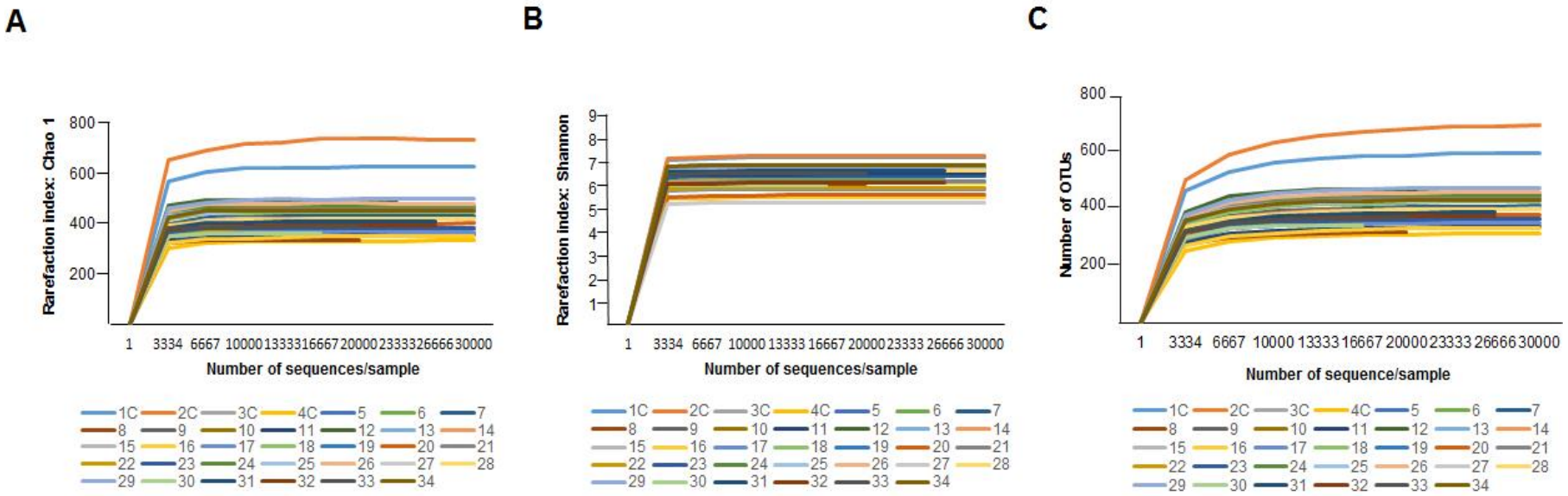

Figure SI. 2. Alpha diversity rarefaction plots. (A) Estimation of the microbial taxa richness and diversity in fecal samples, based on Chao 1 and **(B)** Shannon indexes. **(C)** The number of observed OTUs in each sample is also reported.

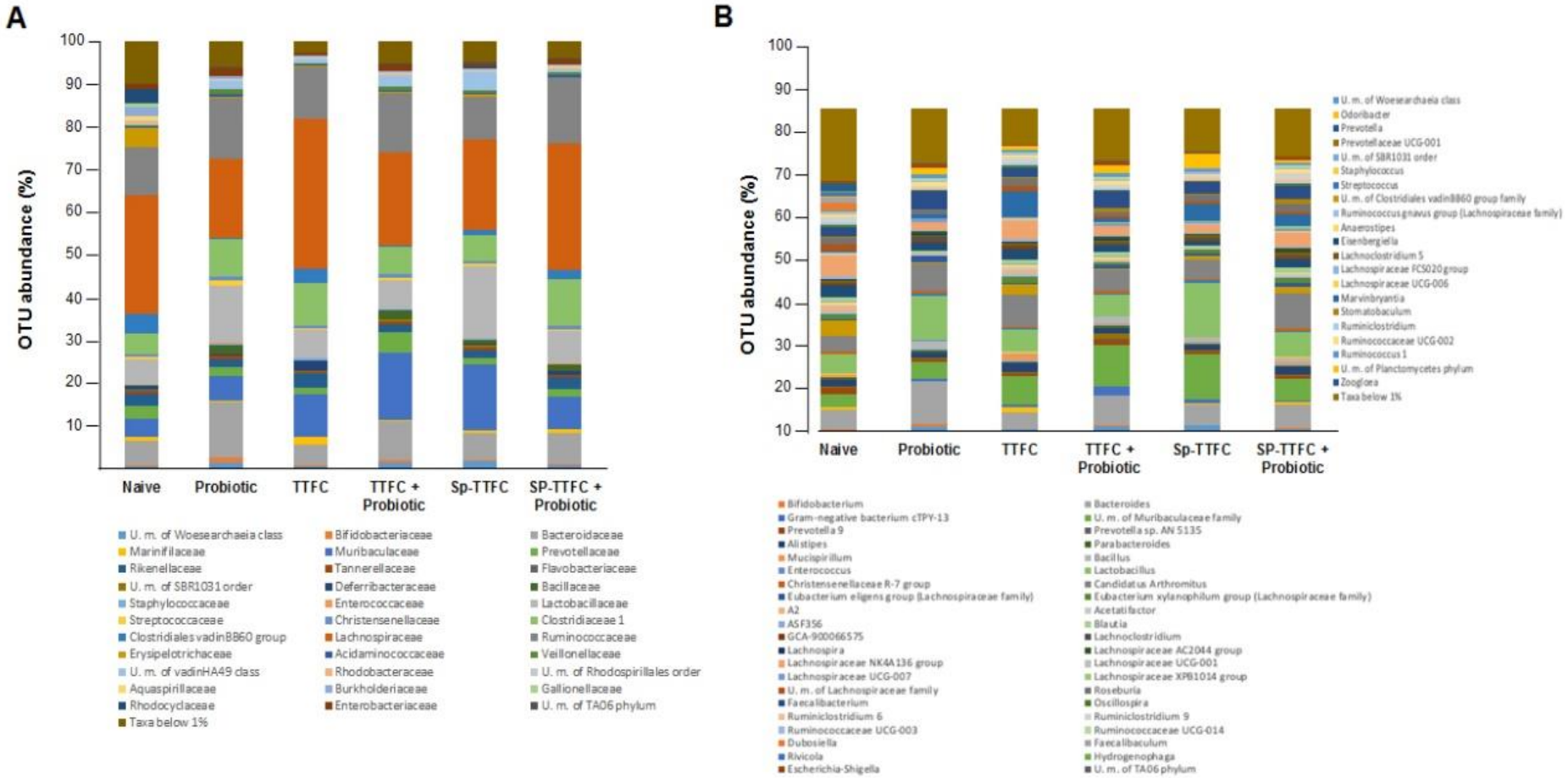

Figure SI. 3. Fecal bacterial composition. (A) Relative Operational Taxonomic Units (OTUs) abundance at the **(B)** Family and Genus level in the six experimental groups, reported as mean values within each group. Only Taxa represented by OTUs abundance >1% have been considered for the analysis.

FINAL CONCLUSIONS

Despite the emergence of new anti-CDI therapies and improvement of health care systems around the world, the incidence of CDI has increased over 200% since 2000 and it is now one of the leading causes of healthcare-associated infections [1]. Effective eradication measures should focus not only on the reduction of massive antibiotic consumption, which damage healthy gut microbiota and enhances CDI, but also the promotion of an optimal hand hygiene by nurses and other healthcare personnel in contact with patients with CDI [2]. Alongside these efforts, a complete therapy able to control the first steps of the disease is also imperative. In this context, the main aim of this project was to develop a mucosal vaccine against *C. difficile* spores, the vehicle of transmission, infection and perpetuation of the disease. The C-terminal domain of the exosporium proteins BclA2 and BclA3 form *C. difficile* spores were chosen as potential antigens for the nasal immunization of mice. Both antigens where used as free proteins and bound to *B. subtilis* spores used as display system and the results are described in Chapters 1 and 2, respectively. The main result of this work is that both antigens where able to induce a specific immune response suggesting $BclA2_{CTD}$ and $BclA3_{CTD}$ as promising mucosal antigens for a vaccine targeting *C. difficile* spores.

When used in challenge experiments using a murine model of CDI infection, we did not observe clear signs of protection in mice immunized with both antigens, used as free or spore-bound forms. Other experiments with higher doses regimen and frequency and/or using the oral route of immunization would be needed to address this point.

Chapter 3 of this thesis reports in vitro adhesion experiments of *Bacillus* spores displaying the *C. difficile* exosporium proteins $BclA2_{CTD}$, $BclA3_{CTD}$ and $CdeC_{CTD}$ on model intestinal Caco-2 cells. Our results indicate that when displayed on *B. subtilis* spores $CdeC_{CTD}$, but not $BclA2_{CTD}$ or $BclA3_{CTD}$, increased the spore adhesion to Caco-2 cells supporting its role in the interaction of *C. difficile* spores with the host and therefore in the initial steps of the *C. difficile* infection. Further studies are needed to address how CdeC interacts with the colonic mucosa.

The study reported in Appendix 1 shows that the oral administration of the probiotic *B. toyonensis* increases the immune response induced by the nasal delivery of spore-adsorbed TTFC, increasing the production of TTFC-specific sIgA and causing a faster production of serum IgG. These data point to the probiotic treatment as an alternative to the use of adjuvants for mucosal vaccinations.

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