Immunogenicity and safety in rabbits of a Clostridioides difficile vaccine combining novel toxoids and a novel adjuvant

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ABSTRACT

Clostridioides difficile infection (CDI) is a serious healthcare-associated disease, causing symptoms such as diarrhea and pseudomembranous colitis. The major virulence factors responsible for the disease symptoms are two secreted cytotoxic proteins, TcdA and TcdB. A parenteral vaccine based on formaldehyde-inactivated TcdA and TcdB supplemented with alum adjuvant, has previously been investigated in humans but resulted in an insufficient immune response. In search for an improved response, we investigated a novel toxin inactivation method and a novel, potent adjuvant. Inactivation of toxins by metal-catalyzed oxidation (MCO) was previously shown to preserve neutralizing epitopes and to annihilate reversion to toxicity. The immunogenicity and safety of TcdA and TcdB inactivated by MCO and combined with a novel carbohydrate fatty acid monosulphate ester-based (CMS) adjuvant were investigated in rabbits. Two or three intramuscular immunizations generated high serum IgG and neutralizing antibody titers against both toxins. The CMS adjuvant increased antibody responses to both toxins while an alum adjuvant control was effective only against TcdA. Systemic safety was evaluated by monitoring body weight, body temperature, and analysis of red and white blood cell counts shortly after immunization. Local safety was assessed by histopathologic examination of the injection site at the end of the study. Body weight gain was constant in all groups. Body temperature increased up to 1 ℃ one day after the first immunization but less after the second or third immunization. White blood cell counts, and percentage of neutrophils increased one day after immunization with CMS-adjuvanted vaccines, but not with alum. Histopathology of the injection sites 42 days after the last injection did not reveal any abnormal tissue reactions. From this study, we conclude that TcdA and TcdB inactivated by MCO and combined with CMS adjuvant demonstrated promising immunogenicity and safety in rabbits and could be a candidate for a vaccine against CDI.

1. Introduction

Clostridioides difficile (formerly *Clostridium difficile*) is a spore-forming bacterium known to be the leading cause of healthcare-associated infectious diarrhoea and it is listed as one of the top three urgent threats by the Center for Disease Control and Prevention [1,2]. *C. difficile* infection (CDI) causes almost half a million disease incidences and close to 30,000 deaths annually in the United States alone. Almost two thirds of these CDI incidences required hospitalization, and more than 80% of these deaths occurred in elderly patients above 65 years [1]. Likewise, it also remains a challenge to control the incidence rates of CDI in other regions of the world such as Europe [3–5] and Asia [6]. CDI gives rise to a spectrum of disease symptoms, ranging from milder symptoms like fever, nausea, and diarrhea, to pseudomembranous colitis, toxic megacolon, and death [1,7]. Although the global health care costs associated with CDI are not precisely known, prolonged hospital stays, expensive treatments and strict guidelines for preventing spread of infection in the hospitals, make CDI a substantial financial burden to health systems with estimated annual costs of US$ 6.3 billion in the United States [8,9].

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Disease caused by *C. difficile* is primarily attributed to two large homologous exotoxins, toxin A (TcdA) and toxin B (TcdB), with molecular weights of 308 and 270 kDa, respectively, and sharing 68% similarity [10]. After being secreted by the bacteria both toxins bind to specific receptors on the epithelial cells in the colon and are transported into the cells by endocytosis. In the endosome the toxins undergo a pH-induced conformational change, which results in a catalytically active fragment of the toxins being translocated across the endosomal membrane and released into the cytosol of the epithelial cells [10–12]. Once in the cytosol, the fragment inactivates GTPases of the Rho family [13–15], causing disruption of the actin cytoskeleton and leading to apoptotic cell death of the colonic epithelium. This causes a loosening of the epithelial tight junctions resulting in increased permeability and fluid accumulation in the intestinal lumen [10,16,17]. Whilst TcdA and TcdB have the same mode of action, contradicting results have been presented regarding the individual roles of each toxin during disease progression *in vivo*. Earlier studies highlighted TcdA as the main virulence factor during disease and ignored the role of TcdB [18–21]. Recently, it has become clear that both toxins are important for fulminant disease in a hamster model [22–24] and are also found in many strains causing clinical disease in humans [25–28]. However, TcdB has been shown to be 10 times more potent than TcdA at disrupting epithelial integrity and causing tissue damage in human colon explants [29]. Furthermore, a licensed monoclonal antibody specific to TcdB, Bezlotoxumab, has been shown to reduce the risk of CDI recurrence in humans and is approved as antibody therapy for patients with recurrent CDI [30]. The development of a corresponding monoclonal antibody towards TcdA, Actoxumab, was abandoned since this was shown to have no effect on treating recurrent infections. Nevertheless, studies indicate that TcdA and TcdB both play essential roles at different stages of *C. difficile* pathogenesis, but that TcdB is responsible for advanced and severe disease [12] which may explain why Bezlotoxumab is useful for treatment of recurrent CDI.

Standard-of-care treatment of CDI consists of antibiotics such as metronidazole and vancomycin, and the recently approved, narrow-spectrum antibiotic, fidaxomicin [31]. Despite a positive initial response in more than 90% of patients, recurrence of infection occurs in 20–30% of these patients, usually in the first few weeks after discontinuation of the antibiotic treatment [32,33]. Furthermore, 40–60% of patients recovered from the first recurrent infection will experience a second recurrent infection. Recurrence is likely the consequence of reinfection by resilient spores compounded by further disruption of the healthy colonic microbiota due to the antibiotic treatment [33,34]. The importance of a healthy microbiota as a natural defense against CDI is supported by successful cases of fecal microbiota transplantations, with reports of up to 90% disease resolution [35,36]. However, microbiota transplantation is complicated by rigorous donor screening, lack of standardized material, and risks of long-term adverse effects [37].

In vaccination studies, detoxified TcdA and TcdB (deTcdA and deTcdB) have been shown to be efficient in preventing CDI in animals [38–42] and humans [43–45]. Toxin-specific neutralizing antibodies (nAbs) in serum elicited by parenteral vaccination with deTcdA and deTcdB can prevent disease symptoms [42,46,47]. The decisive role of toxin-specific antibodies in treating recurrent CDI is further substantiated by the FDA approval of Bezlotoxumab for this purpose [30]. Conventional toxoid-based vaccines often exploit the cross-linking reagent formaldehyde (FA) to inactivate the toxins [48–50] and examples include licensed tetanus and diphtheria toxoid-based vaccines [51]. However, FA-based detoxification of toxins comes with several disadvantages including a time-consuming process [48], risk of reversion to toxicity [52,53], inherent carcinogenicity of FA [54,55], and a delicate balance between detoxification and loss of immunogenicity [56–58]. Recently, a vaccine candidate based on FA detoxified TcdA and TcdB, failed to prevent initial episodes of CDI in a Phase III clinical trial [59] presumably due to low toxoid immunogenicity. Previously, we have described a novel method for detoxification based on metal-catalyzed oxidation (MCO) that generates immunogenic toxoids [42]. The produced toxoids fully protected mice against a challenge with *C. difficile*. The principle of detoxification by MCO (also known as a Fenton reaction), has previously been applied to pertussis toxin [60,61] where it resulted in higher epitope conservation compared to FA and showed no reversion to toxicity [57].

Here, we describe the immunogenicity and safety in rabbits of a toxoid-based vaccine against CDI comprising of deTcdA and deTcdB detoxified by MCO and combined with a novel carbohydrate fatty acid monosulphate ester (CMS)-based adjuvant [53]. This adjuvant is a new class of synthetic, adjuvant-active carbohydrate esters formulated in a submicron emulsion of squalane-in-water with extreme physical and chemical stability. It has been shown previously to be a potent adjuvant for poor immunogens [62] and is in the stage of clinical evaluation. Hence, the goal of this study was primarily to determine if CMS is a safe and suitable adjuvant for a toxoid-based vaccine against CDI. Rabbits were selected as a practical, nonrodent species that is susceptible to bacterial products (including toxins and endotoxins), they show similarity to humans in pathogenesis of bacterial infections [63,64] and are the standard model for toxicity assessment.

2. Results

2.1. Detoxification of TcdA and TcdB

Native TcdA and TcdB were purified from the culture supernatant of *C. difficile* strain R20291 as described previously [65]. The purified toxins were detoxified separately by MCO. After detoxification, the *in vitro* cytotoxicity (TC_{50}) of deTcdA and deTcdB on Vero cells was reduced by more than $10^{4.9}$-fold and $10^{7.4}$-fold, respectively (Table 1). Samples of deTcdA and deTcdB were analyzed by SDS-PAGE to assess degradation and aggregation, and showed intense protein bands corresponding to the molecular weight of native TcdA of 308 kDa and native TcdB of 270 kDa, respectively, as well as some smaller fragmented protein bands (Fig. 1A and B). In addition, evaluation of antibody recognition of deTcdA and deTcdB using a panel of commercially available monoclonal and polyclonal antibodies specific to TcdA and TcdB, respectively, demonstrated overall intact binding efficacy (Supplementary Fig. S1A and B).

2.2. Immunogenicity

Immunogenicity and safety of different antigen-adjuvant formulations (Table 2) was investigated in rabbits. Groups of animals were given three intramuscular (i.m.) injections with 2-week intervals (Day 0, 14 and 28) and blood samples were collected on Day 0, 14, 28, 42, 63, and 84 for immunological and hematological analyses. Histopathological examination of the injection site was conducted on Day 84. The standard dose of antigens per injection was 5 μg deTcdA + 20 μg deTcdB. Group 5 received a 4-fold higher dose of both antigens to increase responses and to facilitate comparison with similar doses of toxoids investigated in clinical trials [59,66,67]. The dose of alum was 0.5 mg per injection and that of CMS was 2 or 8 mg per injection.

Serum antibody responses against TcdA (α-TcdA) and TcdB (α-TcdB) were measured by an indirect ELISA and by toxin neutralization assay

Table 1
Cytotoxicity of native and MCO-detoxified TcdA and TcdB on Vero cells.

Toxin	TC_{50} (μg/ml)	^{10}log reduction in cytotoxicity
Native TcdA	2.7×10^{-4}	–
Detoxified TcdA (deTcdA)	>20	>4.9
Native TcdB	1.2×10^{-6}	–
Detoxified TcdB (deTcdB)	>30	>7.4

Cytotoxicity of toxins and toxoids was measured on Vero cells, and cells were inspected visually after 48 h after exposure.

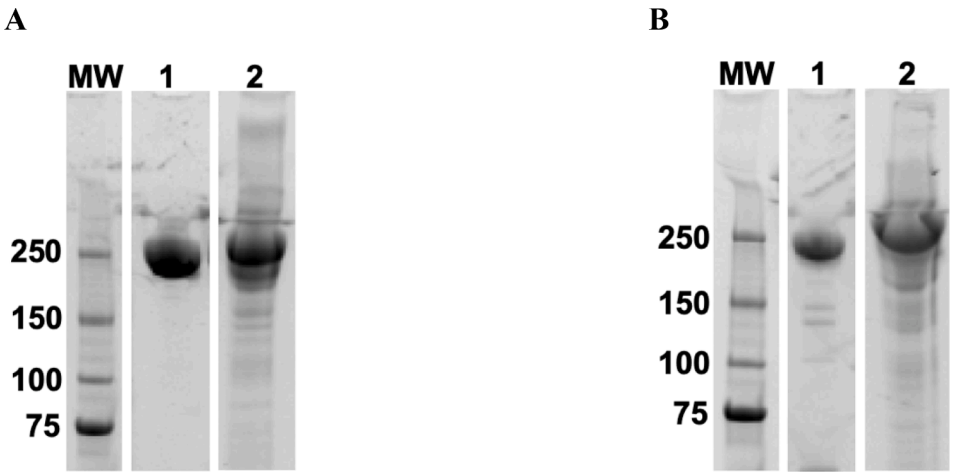

Fig. 1. SDS-PAGE analysis of native and MCO-detoxified TcdA and TcdB. SDS-PAGE gel showing **A)** TcdA and deTcdA or **B)** TcdB and deTcdB samples. Lane MW: molecular weight marker (kDa), Lane 1: native TcdA or TcdB, Lane 2: deTcdA or deTcdB.

Table 2
Composition of the vaccines tested in rabbits.

Group[a]	Colour code	ID Vaccine	Antigen[c]	Adjuvant[d]
1[b]	●	deTcdAB	5 μg deTcdA + 20 μg deTcdB	–
2	●	deTcdAB + alum	5 μg deTcdA + 20 μg deTcdB	0.5 mg alum
3	●	deTcdAB + CMS	5 μg deTcdA + 20 μg deTcdB	2 mg CMS
4	●	deTcdAB + 4xCMS	5 μg deTcdA + 20 μg deTcdB	8 mg CMS
5	●	4xdeTcdAB + CMS	20 μg deTcdA + 80 μg deTcdB	2 mg CMS

[a] Groups consist of 7 New Zealand White Rabbits.
[b] One rabbit in Group 1 was euthanized on Day 14 due to an untreatable neck wound exposing the jugular vein.
[c] Antigen doses per injection.
[d] Adjuvant dose per injection.

(TNA) at different time intervals after immunizations with deTcdAB with CMS or alum as adjuvant or without adjuvant (Table 3).

Significant ELISA IgG and nAb α-TcdA antibody titers appeared two weeks after the second immunization with alum (Group 2) or CMS (Group 3–5) (Table 3A). These titers further increased after the third immunization (at Day 42) and decreased in the subsequent 6-week period (Day 63 and 84). Without adjuvant (Group 1) low but significant α-TcdA titers were observed only after three immunizations. The 4-fold higher dose of antigen with CMS (Group 5) generated higher ELISA and nAb titers than the standard dose of antigen with CMS (Group 3) (Table 3A).

With CMS but not with alum or without adjuvant, high α-TcdB ELISA titers after the second immunization and higher nAb titers after the third immunization were noted (Table 3B). Titers reached a maximum at Day 42 and declined slowly in the following 6-week period (Day 63 and 84). In contrast to α-TcdA, α-TcdB nAb responses required three immunizations, a higher dose of CMS adjuvant resulted in higher α-TcdB responses (Group 4 versus Group 3), and no effect of a higher dose of deTcdB antigen was observed (Group 5 versus Group 3).

Overall, antibody titers reached a maximum two weeks after the third immunization (Day 42) with serum responses of each vaccine group ranked in the following increasing order; Group $1 < 2 < 3 = 4 < 5$ for IgG ELISA titers, and Group $1 < 3 < 4 < 2 < 5$ for nAb titers for α-TcdA (Fig. 2A and Table 3A), and Group $1 < 2 < 3 < 4 < 5$ for IgG ELISA titers, and Group $1 = 2 < 5 < 3 < 4$ for nAb titers for α-TcdB (Fig. 2B and Table 3B).

2.3. Safety: Effects on body weight and body temperature

Rabbits were weighed (Fig. 3A) and body temperatures monitored both rectally (Fig. 3B) and by dorsally implemented sensors (Fig. 3C) on each immunization day, the day after immunization and every seven days during the full experiment. Despite the immunizations, all groups exhibited a linear and stable increment in body weight during the study starting with a median weight of 1.45 kg (1.15–1.65 kg) and reaching a final median weight of 3.75 kg (3.05–4.45 kg) on Day 84.

Twenty-four hours post-immunizations, all the animals injected with deTcdAB + CMS vaccines demonstrated an overall increase in body temperature compared to the previous day, which returned to baseline seven days after. The body temperatures were highest one day after the first and second immunizations with no differences between the methods (Fig. 3B and C). After the first immunizations, median increases were 0.9 °C ($p < 0.01$) in Group 3, 0.5 °C ($p < 0.05$) in Group 4, and 0.3 °C ($p < 0.05$) in Group 5 when measured rectally (Fig. 3B), and 0.6 °C ($p < 0.01$) in Group 3 and 1.1 °C ($p < 0.01$) in Group 4 when measured by sensors (Fig. 3C). The same trend was observed after the second immunizations with slight but significant increases in body temperature in the animals injected with deTcdAB + CMS. After the third immunizations, body temperatures were less affected compared to the first and second immunizations. Compared to 2 mg of CMS, 8 mg with similar dose of antigens caused significantly higher body temperature increases (average of 0.7 °C, $p < 0.01$) (Fig. 3C). In contrast, a 4-fold higher dose of antigens with a fixed dose of 2 mg of CMS, did not result in increased body temperature.

2.4. Safety: Effects on hematology

To assess the effect of vaccination on blood parameters, hematology measurements were conducted during the study on the day of each immunization as well as one and seven days later and on Day 63 and 83. The number of red blood cells (Fig. 4A) and white blood cells (Fig. 4B) were measured as well as the % of lymphocytes (Fig. 4C), neutrophils (Fig. 4D) and monocytes (Fig. 4E) from the pool of white blood cells collected. During the first 24 h following the first but less after the second and third immunization with CMS, the white blood cell counts and % of neutrophils were increased, while % of lymphocytes were decreased ($p < 0.01$). The % of monocytes was also significantly decreased one day after the first immunization in the groups that received CMS compared to the other groups (Fig. 4E). However, after the second and third immunization, the number of monocytes was not significantly different between the groups. Other hematological parameters such as red blood cells (Fig. 4A), hematocrit, hemoglobin, and

Table 3
IgG and nAb titers at different time intervals after immunization with TcdAB with or without adjuvant in rabbits.

A

Group	Vaccine	Day 0	Day 14	Day 28	Day 42	Day 63	Day 84
		α-TcdA IgG titer[a]					
1	deTcdAB	177	139	346	8,776	5,192	4,657
2	deTcdAB + alum	389	1,705	17,676	44,060	29,664	17,061
3	deTcdAB + CMS	351	180	10,272	63,412	54,217	37,860
4	deTcdAB + 4xCMS	406	186	12,371	63,239	64,674	41,929
5	4xdeTcdAB + CMS	240	755	25,247	92,109	79,006	73,759
		α-TcdA nAb titer[b]					
1	deTcdAB	<100[c]	<100	<100	186 [27, 1292]	539 [302, 964]	635 [374, 1079]
2	deTcdAB + alum	<100	<100	635 [398, 1000]	6,509 [4170, 10,159]	5,563 [3500, 8842]	3,391 [2033, 5655]
3	deTcdAB + CMS	<100	<100	144 [25, 823]	3,254 [1787, 5925]	5,039 [2819, 9008]	3,533 [1771, 7049]
4	deTcdAB + 4xCMS	<100	<100	295 [106, 824]	4,133 [2416, 7071]	5,039 [2856, 8891]	3,901 [2394, 6357]
5	4xdeTcdAB + CMS	<100	<100	737 [404, 1345]	8,760 [4337, 17,695]	13,017 [8204, 20,655]	9,672 [5905, 15,841]

B

Group	Vaccine	Day 0	Day 14	Day 28	Day 42	Day 63	Day 84
		α-TcdB IgG titer					
1	deTcdAB	784	263	460	1,256	582	454
2	deTcdAB + alum	725	1,246	1,401	2,277	1,011	750
3	deTcdAB + CMS	649	343	6,120	15,980	13,265	10,360
4	deTcdAB + 4xCMS	878	685	5,537	32,566	35,255	29,445
5	4xdeTcdAB + CMS	419	671	3,870	39,837	24,527	16,229
		α-TcdB nAb titer					
1	deTcdAB	<100	<100	<100	<100	<100	<100
2	deTcdAB + alum	<100	<100	<100	<100	<100	<100
3	deTcdAB + CMS	<100	<100	<100	188 [28, 1243]	220 [33, 1482]	<100
4	deTcdAB + 4xCMS	<100	<100	<100	2,378 [1094, 5168]	2,282 [1009, 5162]	538 [203, 1430]
5	4xdeTcdAB + CMS	<100	<100	<100	107 [9, 1239]	326 [45, 2374]	107 [9, 1284]

Rabbits were immunized on Day 0, 14 and 28 and toxin-specific serum antibody titers were measured by ELISA (upper panel) or by TNA (lower panel). **A)** α-TcdA antibody titers **B)** α-TcdB antibody titers.

[a] ELISA IgG measurements were performed in technical duplicates of four-fold serial dilutions of pooled rabbit serum from each group.

[b] TNA measurements were performed in technical duplicates of two-fold serial dilutions of serum from each rabbit within the groups and values presented as geometric mean titers (GMTs) with [95% confidence intervals].

[c] Limit of detection of TNA was 100 (i.e., 1/100 fold pre-dilution of serum sample).

platelets (data not shown) remained unchanged during the study.

2.5. Safety: Effects at the site of injection

During the study, none of the animals showed detectable, adverse reactions at the injection site. Histologic specimens of tissue were collected from the injection sites (right thigh) to determine inflammation and alteration of skin and muscle tissue. Tissue from the left thigh of a rabbit (control tissue with no injections) is shown in Fig. 5A–E to visualize the different layers of skin and muscle tissue from a non-disturbed area. At the surface of the skin lies the epidermis (e), which consists for the most part of keratinocytes, but also melanocytes, Langerhans cells, Merkel cells and secondarily of lymphocytes and mast cells. Deeper in the skin lies the dermis (d) which is a dense network of collagen, elastin, reticular fibers arranged inside an intercellular matrix rich in water and proteoglycans (Fig. 5B). The smooth muscle layer, panniculus carnosus (pc), is found between the dermis and hypodermis on most histological sections (Fig. 5C). Next lies the hypodermis (h) which is a subcutaneous connective tissue containing fibrous structure and adipocytes (Fig. 5D). Finally, the hypodermis is connected to the muscle tissue (m), containing connective tissue between striated muscle cells (Fig. 5E). Histologic specimens of tissue from the injection site of all rabbits from vaccine Groups 1 to 5 were prepared on Day 84, i.e., 42 days after the last i.m. injection. A representative selection of specimens from each group is shown in Fig. 5F–J, while all the tissue specimens are shown in Supplementary Fig. S3A–E. In none of the rabbits, alterations of the skin or layers of tissue from epidermis to muscle were observed (Fig. 5F–J). Also, no inflammation, recruitment of immune cells or any indication for persistent alteration were visible in the histology sections of the muscle. Hence, none of the vaccines including those containing the novel and potent CMS adjuvant demonstrated local, adverse reactions 42 days after the last injections.

3. Discussion

Several nonclinical and clinical studies demonstrate that the level of neutralizing antibodies against TcdA and TcdB is crucial for immunological protection against CDI and recurrent infections [20,68–71]. Two toxoid-based vaccines both formulated with alum [59,69] have been evaluated in Phase III clinical trials but failed to prevent first episodes of CDI in the target population. The substantial burden of CDI and the lingering unmet need for an effective vaccine, prompted us to improve

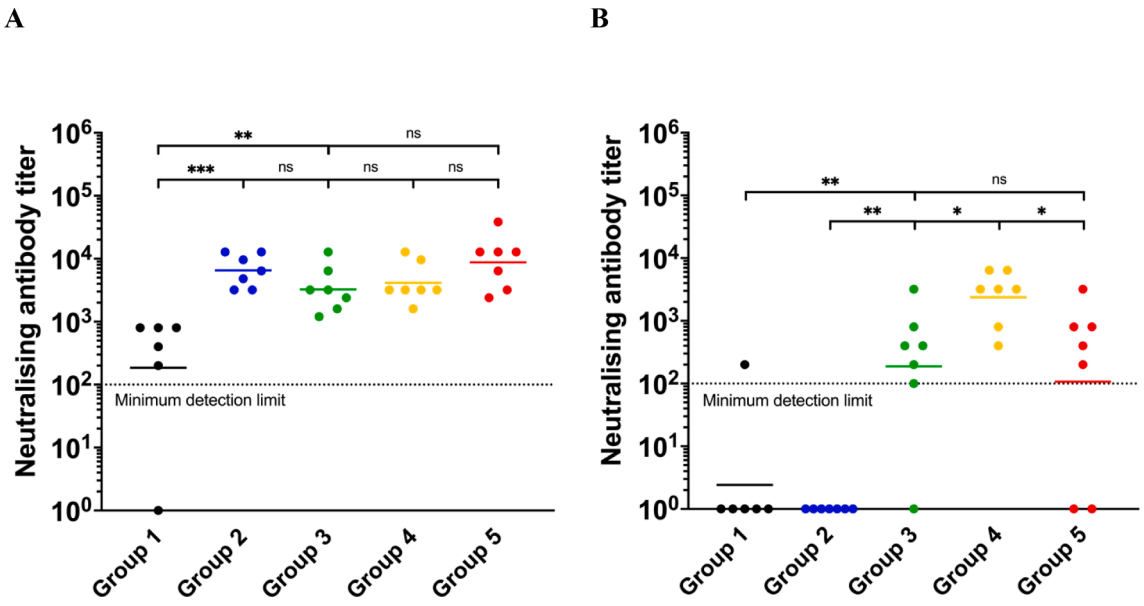

Fig. 2. Day 42 nAb responses in rabbits immunized with different test vaccines (see Table 2). Groups of 7 rabbits were immunised at Day 0, 14 and 28 and individual animal sera from Day 42 were analyzed by TNA for **A)** α-TcdA nAb and **B)** α-TcdB nAb. Unpaired Student's *t*-test (Mann-Whitney) was used to compare nAb titers. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = no significant difference.

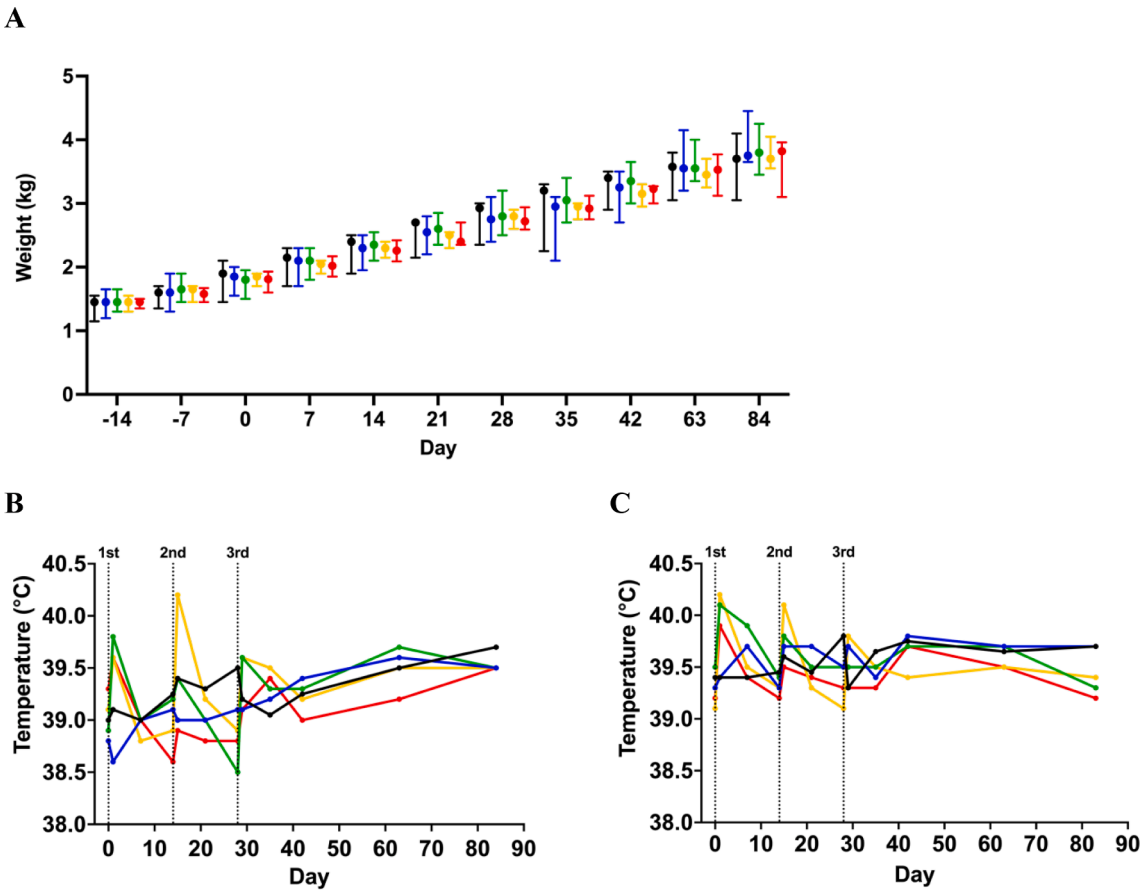

Fig. 3. Body weight and body temperature of rabbits during the immunization study. Groups of 7 rabbits were immunized as summarized in Table 2. Colored dots represent median values from each group of seven rabbits with error bars representing the range of values. **A)** Body weight increments **B)** Rectal temperature **C)** Temperature measured by dorsally implemented sensor.

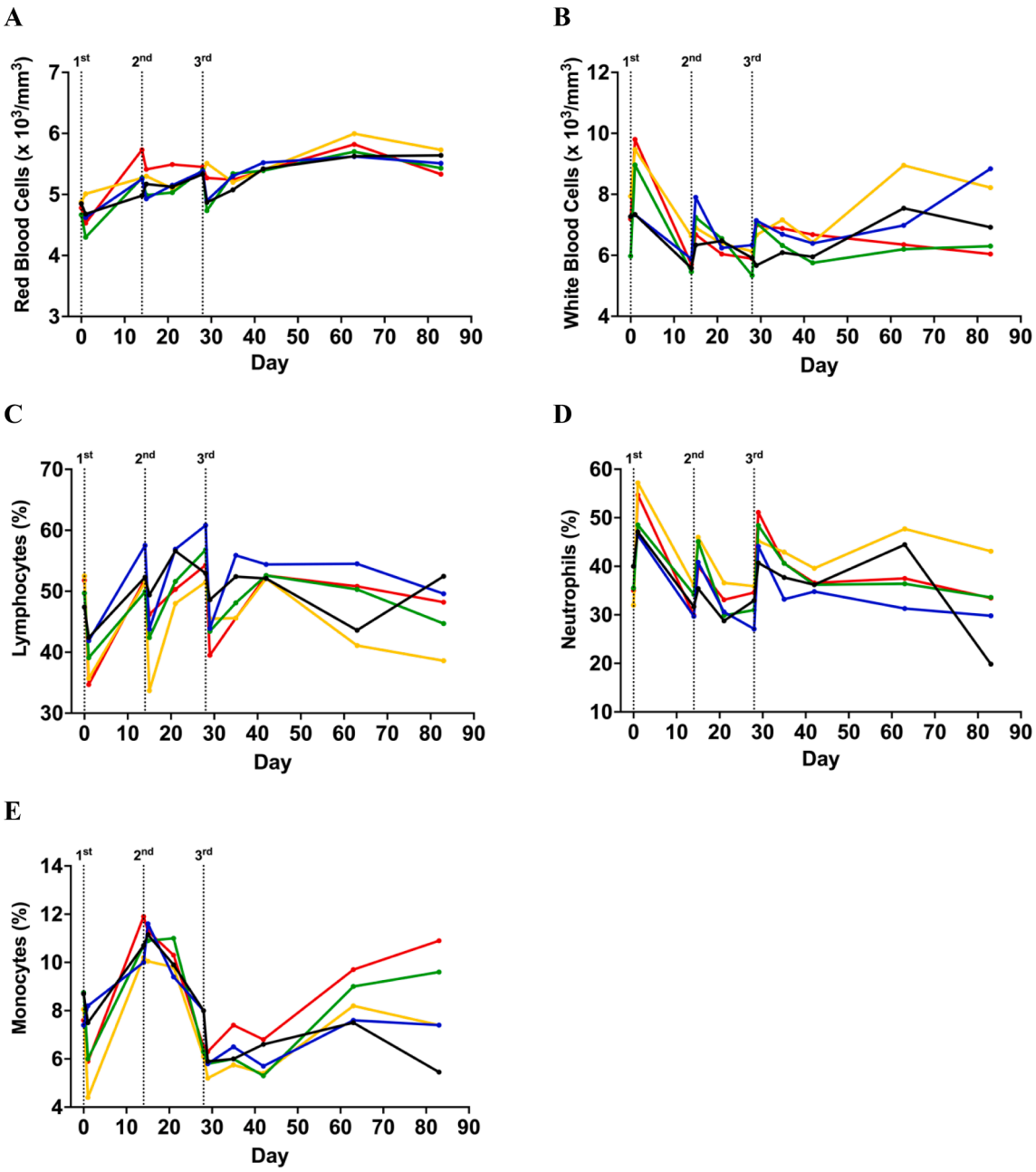

Fig. 4. Hematological measurements during the study. Rabbits were immunized as summarized in Table 2. Hematology measurements were performed on blood samples from Day 0, 1, 14, 15, 21, 28, 29, 35, 42, 63 and 83. Colored dots represent median values from each group. **A)** Red blood cells, **B)** White blood cells, **C)** Lymphocytes, **D)** Neutrophils, and **E)** Monocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunogenicity of these toxoids by using an alternative toxin inactivation method and a novel, potent vaccine adjuvant, CMS. This adjuvant has been shown to elicit 10 to 100-fold higher antibody titers than alum or an MF59-like adjuvant against malaria subunit and inactivated influenza virus in larger, non-rodent species [62,72].

In this study, we investigated immunogenicity and local and systemic safety of the test vaccines after one, two and three i.m. immunizations in rabbits, and compared different doses of antigens and the novel CMS adjuvant with the conventional alum.

Rabbits vaccinated with deTcdAB formulated with the CMS adjuvant induced high nAb titers against both TcdA and TcdB after three injections, while deTcdAB with alum gave a similar α-TcdA response but no measurable α-TcdB response (Table 3A and B). The deTcdAB injections without adjuvant only gave a weak nAb α-TcdA response and no measurable α-TcdB response. These results demonstrate the beneficial effects of the novel CMS adjuvant and especially for deTcdB. Interestingly, a 4-fold increase in the dose of CMS significantly enhanced the ELISA IgG and nAb α-TcdB responses but not the α-TcdA responses. Lower antibody responses to TcdB as compared to TcdA have been described in different animals and humans [39,59,66,67,73,74] and are thought to be at least partially due to lower immunogenicity of TcdB. To compensate for lower immunogenicity, we decided to use a higher dose of deTcdB (20 μg deTcdB versus 5 μg deTcdA), but still deTcdB required three immunizations while deTcdA only required two to generate nAb responses.

In addition to the standard doses of 5 and 20 μg, we also tested a 4-fold higher dose of both deTcdA and deTcdB, which lies within the range of doses used in clinical trials [59,66,67]. This 4-fold higher antigen

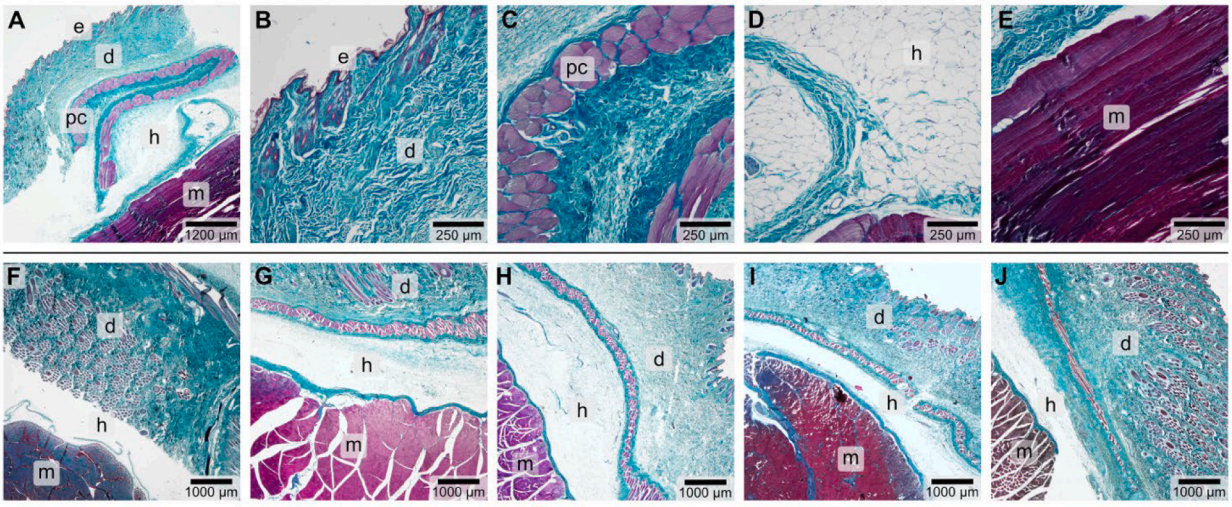

Fig. 5. Histology samples of vaccinated animals compared to tissue from a non-vaccinated area. The different layers of skin in a sample from a non-vaccinated rabbit (upper panels **A-E**). Masson-Goldner's trichrome staining of control tissue from a rabbit histologic specimen. **A)** The skin is made up of several layers: the epidermis (e), the dermis (d), panniculus carnosus (pc), and the hypodermis (h) which rests on muscle tissue (m). The thickness is not constant, and it appears thicker in the dorso-lumbar region, on the lateral faces of the limbs. Scale bar: 1200 µm. **B–E)** Close-up views of non-vaccinated epidermis, dermis, hypodermis, panniculus carnosus, and underlying muscle tissue. Scale bar: 250 µm. Lower panels (**F–J**). One representative histology sample from each of the five groups of vaccinated rabbits. Rabbits were immunized as summarized in Table 2. **F)** Group 1, **G)** Group 2, **H)** Group 3, **I)** Group 4, and **J)** Group 5. Scale bar: 1000 µm.

dose combined with 2 mg of CMS adjuvant (Group 5; 20 µg of deTcdA and 80 µg of deTcdB) generated higher nAb α-TcdA responses, but surprisingly no increase in nAb α-TcdB responses. This does not correlate with Group 5 obtaining the highest ELISA IgG responses toward both TcdA and TcdB compared to Group 3. This may suggest that crucial epitopes for developing neutralizing antibodies may be impaired by the MCO treatment. Compared to TcdA and depending on the type of mammalian cells used in the cytotoxicity assay, TcdB is 100–10.000 fold more effective in cell rounding [75], and it was necessary to use an increased concentration of H_2O_2 to obtain sufficient detoxification of TcdB for safe injection. Since deTcdAB with a 4-fold higher dose of CMS gave the best nAb response for α-TcdB, it is possible that combining 4-fold higher CMS with a 4-fold higher dose of deTcdAB could further improve the α-TcdB response.

Suboptimal responses in elderly or immunocompromised patients [76,77] complicate the development of effective CDI vaccines, which makes it crucial to make the proper choice of adjuvant to maximize immunity. Alum is still the gold standard and first candidate to be investigated. However, formulation including adsorption of antigens to alum requires detailed information on the surface charge of the antigens and precise control of the chemical and physical conditions of the process [78]. Alum gave high antibody responses to TcdA but not to TcdB, which could be the consequence of suboptimal adsorption of deTcdB to alum. The CMS adjuvanted vaccines resulted in significantly higher IgG responses to both toxins. In contrast to alum, adsorption of antigen to the CMS adjuvant is not a critical event, which facilitates the preparation of vaccines in a consistent manner.

Since CMS is a new adjuvant, we also examined the safety of the vaccines. Body temperature, body weight gain and hematological parameters such as red blood cell count (hematocrit, hemoglobin and platelets) were stable throughout the study indicating good general health condition and well-fare of the animals [79]. The CMS adjuvanted vaccines induced a significant but transient, dose-dependent increase in body temperature one day after the first i.m. injection (Fig. 3B and C). The temperature spikes occurred after the second but not the third injection. Similar temperature patterns have been observed previously in preclinical studies [62,72].

Total red and white blood cell populations including % lymphocytes, neutrophils and monocytes were monitored. After the first two immunizations, an increase in the total number of white blood cells was observed (Fig. 4B). This increase was stronger after the first immunization with the CMS adjuvanted vaccines. After each immunization with CMS but to a lesser extent with alum or without adjuvant, there was a decrease in the number of lymphocytes (Fig. 4C) and a strong increase in the neutrophils (Fig. 4D). This suggests that immunization with the CMS adjuvant induced a stronger immune stimulation than with alum or without adjuvant. These observations correlate with the immune response seen in other vaccination models with toxoids [80]. The use of an adjuvanted toxoid vaccine as an immunizing agent mimics the effect of an actual pathogen in stimulating the host immune response during an infection, which induces an early fall in the lymphocyte population and an increase in neutrophils. The decreased lymphocyte/neutrophil ratio reverses to baseline during the first days after each vaccination. The toxoid antigens are presented to T- and B lymphocytes which will be activated, and the latter will expand and produce antibodies. The monocyte population such as macrophages and dendritic cells, are part of the innate immunity which quickly responds to vaccine injections and is responsible for antigen uptake and presentation [81]. Therefore, it is likely that the sharp drop in monocytes immediately after the first immunization (Fig. 4E), is due to recruitment from the blood and accumulation in the muscle (injection site) and draining lymph nodes [82]. The decrease in peripheral blood monocytes is transient (one day post-injection) and highest for the 2 and 8 mg CMS vaccines, which suggests that it is an effect of the adjuvant rather than the antigen. However, several studies have suggested that monocytes are highly sensitive to and undergo apoptosis after phagocytosis of *C. difficile* toxins, possibly due to a residual activity of the glycosyltransferase domain of the toxins [83–85]. In case of residual glucosyltransferase activity in the MCO-treated deTcdA and deTcdB, this could potentially also play a role in the observed monocyte depletion in the blood.

In sum, a regime of three vaccinations with deTcdAB antigens combined with the CMS adjuvant was safe and effective in rabbits. Considerable nAb titers against the two toxins were noted and there were no local or systemic side effects. Remarkably, α-TcdB IgG and nAb responses were significantly improved by CMS while alum had limited or no effect. This suggests that the proper choice of adjuvant potentially could be the solution for obtaining a sufficient level of protective antibodies against the crucial and challengingly toxic TcdB. In addition,

since we are only using MCO detoxified antigens in this study, we cannot rule out that using a different detoxification method may result in antigens, in particular deTcdB, that provides an improved immune response. Further investigations are needed to confirm whether the presented immune response of this novel vaccine concept is sufficient for protection against CDI. Various nonclinical studies to demonstrate immunogenicity, safety, immunological protection, and stability in different animal species are ongoing.

4. Materials & methods

4.1. Toxin purification

Native TcdA and TcdB were obtained from the supernatant of *C. difficile* strain R20291 (NCTC 13366) using the dialysis bag method, as described previously [65]. Both toxins were purified from the culture supernatant grown in the dialysis bag, using two consecutive rounds of anion-exchange column chromatography with Q Sepharose and MonoQ resin, respectively, followed by gel-filtration chromatography into 50 mM Tris-HCl, pH 7.5. Immediately after gel-filtration, 20% (v/v) glycerol was added, and aliquots of purified TcdA and TcdB were stored in −80 °C until further use.

4.2. Preparation of MCO-toxoid antigens

The detoxification by MCO was essentially performed as previously described in Aminzadeh *et al.*, 2020 [42] but with some variations in the use of reagents. Briefly, deTcdA was prepared by mixing 1 mg/mL TcdA with a final concentration of 10 mM EDTA, 0.1 mM $FeSO_4$, 1 M urea, 5% (v/v) sucrose and 50 mM H_2O_2 followed by incubation for two hours at 37 °C. Similarly, deTcdB was prepared by mixing 1 mg/mL TcdB with a final concentration of 10 mM EDTA, 0.1 mM $FeSO_4$, 1 M urea, 5% (v/v) sucrose and 70 mM H_2O_2 followed by incubation for two hours at 37 °C. Both detoxification reactions were terminated by adding excess amounts of ice-cold 10 mM EDTA quickly followed by dialysis against 50 mM Tris-HCl, pH 7.5 using Amicon® 30 kDa cut-off centrifugal filters (Merck Millipore, Burlington, MA, USA) at 4 °C to remove the reaction components, and immediately transferring the detoxified toxins to −20 °C until further use.

4.3. Preparation of CMS adjuvant

CMS adjuvant was produced by LiteVax BV (Ophemert, the Netherlands) as described in Hilgers *et al.* [62]. Briefly, carbohydrate fatty acid monosulphate ester was synthesized, purified by liquid chromatography, and incorporated into a submicron emulsion of squalane-in-water. The adjuvant was sterilized by passage through a 0.22 μm filter and aliquoted in portions of 1 or 5 mL and stored at 4 ℃ until use.

4.4. Formulation of vaccines

On the day of immunization, toxoid antigens were thawed two hours before use and mixed in a deTcdA:deTcdB ratio of 1:4 before adding a final concentration of either 50 mM Tris-HCl, pH 7.5 buffer (Group 1), 1.0 mg/mL aluminum hydroxide (Alhydrogel®, Croda, Frederikssund, Denmark) (Group 2), 4 mg/mL CMS adjuvant (Groups 3 and 5) or 16 mg/mL CMS adjuvant (Group 4). The final antigen concentrations in the vaccines were 10 μg/mL deTcdA and 40 μg/mL deTcdB (Groups 1–4) or 40 μg/mL deTcdA and 160 μg/mL deTcdB (Group 5). Hence, each vaccine dose of 0.5 mL contained either 5 μg deTcdA and 20 μg deTcdB (Groups 1–4) or 20 μg deTcdA and 80 μg deTcdB (Group 5).

4.5. Ethical statement

All experiments were conducted in accordance with EU guidelines and French regulations (Directive 2010/63/EU, 2010; Rural Code, 2018; Decree No. 2013-118, 2013). All experimental procedures were evaluated and approved by the Ministry of Higher Education and Research (#27371-2020102812249764 v3 authorization). Procedures involving rabbits were evaluated by the ethics committee of the Val de Loire (CEEA VdL, committee number 19) and took place at the INRAE Experimental Infection Platform PFIE (UE-1277 PFIE, INRAE Centre de Recherche Val de Loire, Nouzilly, France (https://doi.org/10.15454/1.5535888072272498e12).

4.6. Immunization protocol

Female New Zealand White rabbits (HYPHARM, France), 7 to 8 weeks old and weighing approximately 1.45 kg were acclimatized for 14 days prior to the first immunization. Rabbits were divided into six groups of seven according to different vaccines and housed in wire mesh pens on the ground on sterilized crushed straw. Animals were housed under similar conditions with a light:dark cycle ratio of 12 h:12 h and the room temperature and humidity were between 20 and 22 °C and 60–70%, respectively. The rabbit pens on the ground were enriched before the installation of nest boxes (Techniplast, Louviers, France), on which a removable coplast roof was fitted (INRAE adaptation). Enrichments such as wooden gnawing objects (Zooplus®, Strasbourg, France) and hanging toys (Schippers®, Bédée, France) were used. All materials were autoclaved before the experiment. The rabbits were fed with sterile "Rabbit Confidence®" pellets, the composition of which is as follows: wheat bran, dehydrated alfalfa, wheat straw, barley, calcium carbonate, rapeseed meal, salt, vitamins A and D3, copper, iron, manganese, zinc, selenium, and cobalt (Axéréal, Neuillé-Pont-Pierre, France). Food and water were given *ad libitum*. Rabbits were immunized with a 0.5 mL vaccine dose on the same spot in the right thigh on days 0, 14, and 28 by intramuscular injection, and blood samples were collected on Day 0, 1, 14, 15, 21, 28, 29, 35, 42, 63, 83 and 84.

4.7. Telemetric measurements

Each rabbit was randomly identified using telemetric sensors (Biolog-Animal®, Paris, France) implanted subcutaneously in the dorsal region, under general anaesthesia with 4% isoflurane (Vetflurane®, Virbac, France). Before implanting the telemetric sensors, the rabbits were sheared (1 cm × 1 cm) in the dorsal implantation area, disinfected and a small amount of 1% Tronothane® gel (DELPHARM, L'Aigle, France) was applied to relieve the animal. After injection, a small massage at the injection site was performed to maintain the chip. In parallel, a rectal temperature measurement was also performed using a digital thermometer (Digital thermometer Digiflash).

4.8. Serum IgG titers measured by ELISA

Polystyrene MaxiSorp microtiter plates (Nunc, Denmark) were coated with 100 μL of a solution of either 1 μg/ml TcdA or TcdB in 0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6, and incubated overnight at 5 °C. The next day, wells were blocked with 300 μL of PBS, 1% (w/v) BSA, 0.05% (v/v) Tween 20, pH 7.4 (blocking buffer) and incubated for two hours at 37 °C. Technical duplicates of four-fold serial dilutions of pooled rabbit serum from each group were prepared in blocking buffer with 0.5% (w/v) BSA and added to the wells. HRP-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA) diluted 1:6000 in blocking buffer, was added to all wells and incubated for one hour at 37 °C. The quantity of conjugate bound was visualized by the addition of 100 μL TMB PLUS2 (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) and plates were incubated at room temperature for 10 min in the dark. The reaction was stopped by adding 100 μL of 0.2 M H_2SO_4, and absorbance was measured at 450 nm by using a SpectraMax® i3x microplate reader (Molecular Devices, San Jose, CA, USA). Between each step, plates were washed three times with 300 μL washing buffer (PBS, pH 7.4, containing 0.05% (v/v) Tween 20). A five-parameter

logistic curve was fitted to each pooled serum sample by plotting the average of the absorbance at 450 nm measured from the duplicated serial dilutions as a function of the serum dilution. IgG titers are expressed as EC_{50} values, which represent the dilution of the pooled group serum where the anti-toxin response is reduced by 50%.

4.9. Cell-based toxin neutralization assay (TNA)

One hundred μL of Vero cell culture in Dulbecco's Modified Eagle Medium (DMEM) was added to each well ($5x10^4$ cells/mL DMEM) in a 96-well Nunc™ MicroWell™ Nunclon Delta-Treated cell culture plate (Thermo Fisher Scientific, Waltham, MA, USA). Cell culture plates were incubated in a HeraCell 150i (Thermo Fisher Scientific) CO_2 incubator at 36.5 °C and 5% CO_2 for 24 hours to reach ca. 90% confluency prior to testing. Serial dilutions of TcdA and TcdB were tested to determine the toxin concentration causing 50% rounding of cells (TC_{50}) prior to each TNA study. In this TNA study, concentrations of 6 × TC_{50} for TcdA (1.6 ng/ml) or TcdB (7.4 pg/ml) were pre-incubated with duplicates of two-fold serial dilutions of individual sera from immunized rabbits for 90 min at 37 °C prior to addition to the Vero cell culture. Cell culture plates were emptied for media and immediately 100 μL of the pre-incubated toxin-sera mixture was added to the plates and incubated for 48 hours at 36.5 °C with 5% CO_2. Cell viability was assessed by visual inspection after cells were fixed with formaldehyde and stained with crystal violet. Briefly, plates were emptied for media and washed twice with 200 μL/well PBS, pH 7.5. After washing, 200 μL/well of formaldehyde (4%, v/v) in PBS were added and plates were incubated at room temperature for 10 min, followed by another washing step. Finally, the fixed cells in the wells were stained using 0.09% crystal violet (200 μL/well), incubated at room temperature for 10 min and washed gently with deionized water. Stained plates were photographed using a Bio-Rad Gel Doc Imager (Hercules, CA, USA) and qualitatively inspected. The nAb titer was defined as the highest dilution of sera where there was at least 50% cell survival 48 hours after adding the toxin-sera mixture. The detection threshold of the TNA was set to a minimum nAb titer of 100.

4.10. Hematology

Blood cells were counted with an MS9-5 Hematology Counter® (digital automatic hematological analyzer, Melet Schloesing Laboratories) [86]. Twenty-nine parameters were analyzed, which characterized three categories of blood cells: (1) total white blood cells (lymphocytes, monocytes; neutrophils; eosinophils; basophils and others white blood cells), (2) red blood cells and (3) platelets.

4.11. Histology

Rabbit skin samples were collected on Day 84 and fixed in (4%, v/v) formaldehyde (Carbo-Erba Reagents, Val de Breuil, France). Tissues were embedded in paraffin (Paraplast plus, Leica, France) using an automatic device (TP1020, Nanterre, Leica, France). Sections (5 μm) were cut with a rotary microtome (RM2235®, Leica, France) and mounted on Superfrost plus® glass slides (Thermo Fisher Scientific, Artenay, France). Specimens were deparaffinized in Histosol (Thermo Fisher Scientific, Artenay, France) followed by a descending alcohol series.

A topographic staining was chosen to demonstrate the tissue structure. For the Masson-Goldner's trichrome staining, the sections were incubated in Weigert's hematoxylin for nuclei (5 min), briefly washed with H_2O, incubated in fuchsin acid/ponceau xylidine for cytoplasm (5 min), briefly washed with water, incubated in phosphomolybdic acid (1%) for 10 min, in brilliant green for collagen (5 min) and washed in acetic acid 1% (1 min) and briefly descending alcohol series followed by histosol. Then, tissues were mounted in Eukitt® (Labelians, Nemours, France). Samples were visualized using a Nikon Eclipse 80i microscope and processed using the plugging ScientiFig in ImageJ-win64 [87].

4.12. Statistical analysis

Seven rabbits were included in vaccination of Group 1–5, however, in Group 1 one rabbit was euthanized on Day 14 because of an untreatable neck wound exposing the jugular vein. Statistical analyses were performed using GraphPad Prism software, versions 5.0, 6.0 and 9.3 (GraphPad, San Diego, CA, USA). Unpaired, nonparametric *t*-test (Mann-Whitney) was used to compare geometric mean antibody titers determined by TNA. A one-way ANOVA analysis was performed to show the effects of the vaccines on the measured biological parameters (temperature and hematology). In parallel, non-parametric statistical tests (Kruskal-Wallis tests) were carried out to evaluate differences at specific points of the vaccine kinetics.

Author contributions

AA performed toxin purification, preparation of antigens, ELISA, TNA, made and/or contributed to all figures and tables, and wrote the manuscript. **LH** and **PP** performed preparation of adjuvant, revised, and contributed intellectually to the manuscript. **MR** planned and administered the animal experiments, telemetric measurements, hematology and histology, revised and contributed intellectually to the manuscript. **NP, CR, AC, CB** performed the animal experiments, telemetric measurements, hematology and histology. **RJ** contributed to toxin purification and preparation of antigens, made and/or contributed to all figures and tables, revised, and contributed intellectually to the manuscript. **AA, RJ, LH and PP** all conceived the idea for the study and design of the animal experiments. All authors have read and approved the final version to be published.

Conflict of interest

AA and RJ are founders and shareholders of Proxi Biotech ApS. LH and PP are founders and shareholders of LiteVax BV holding the IP related to LiteVax Adjuvant.

CRediT authorship contribution statement

Aria Aminzadeh: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Luuk Hilgers:** Conceptualization, Project administration, Validation, Writing – review & editing. **Peter Paul Platenburg:** Investigation. **Mickaël Riou:** Investigation, Writing – review & editing. **Noémie Perrot:** Investigation. **Christelle Rossignol:** Investigation. **Axel Cauty:** Investigation. **Céline Barc:** Investigation. **René Jørgensen:** Conceptualization, Funding acquisition, Investigation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rene Jørgensen reports financial support was provided by European Vaccine Initiative. Rene Jørgensen reports a relationship with Eurostars that includes: funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.01.076.

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