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**Detoxification of Toxin A and Toxin B by copper ion-catalyzed oxidation in production of a toxoid-based vaccine against *Clostridioides difficile***

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20    **Abstract**

21    *Clostridioides difficile* infections (CDI) has emerged worldwide as a serious antimicrobial-resistant  
22    healthcare-associated disease resulting in diarrhea and pseudomembranous colitis. The two cytotoxic  
23    proteins, toxin A (TcdA) and toxin B (TcdB) are the major virulence factor responsible for the disease  
24    symptoms. We examined time-dependent oxidative detoxification of TcdA and TcdB using different  
25    molar ratios of protein / Cu<sup>2+</sup> / H<sub>2</sub>O<sub>2</sub>. The MCO reaction in molar ratios of 1:60:1000 for protein /  
26    Cu<sup>2+</sup> / H<sub>2</sub>O<sub>2</sub> at pH 4.5 resulted in a significant 6 log<sub>10</sub> fold reduction in cytotoxicity after 120-min  
27    incubation at 37 °C. Circular dichroism revealed that MCO- detoxified TcdA and TcdB had  
28    secondary and tertiary structural folds similar to the native proteins. The conservation of  
29    immunogenic epitopes of both proteins was tested using monoclonal antibodies in an ELISA,  
30    comparing our MCO-detoxification approach to a conventional formaldehyde-detoxification method.  
31    The oxidative detoxification of TcdA and TcdB led to an average 2-fold reduction in antibody binding  
32    relative to native proteins, whereas formaldehyde cross-linking resulted in 3-fold and 5-fold  
33    reductions, respectively. Finally, we show that mice immunized with a vaccine consisting of MCO-  
34    detoxified TcdA and TcdB were fully protected against disease symptoms and death following a *C.*  
35    *difficile* infection and elicited substantial serum IgG responses against both TcdA and TcdB. The  
36    results of this study present copper ion-catalyzed oxidative detoxification of toxic proteins as a  
37    method highly suitable for the rapid production of safe, immunogenic and irreversible toxoid antigens  
38    for future vaccine development and may have the potential for replacing cross-linking reagents like  
39    formaldehyde.

40

41    **Keywords:** *Clostridioides difficile*, CDI vaccine, Reactive oxygen species, Metal-catalyzed  
42    oxidation, Toxoid

## 43 1. Introduction

44 *C. difficile* infection is the leading cause of healthcare-associated diarrhea and is responsible  
45 for around 453,000 incidences and 29,000 deaths every year in the United States alone [1]. More than  
46 80% of CDI-related deaths occur in patients with age above 65 years leading to health care costs of  
47 approximately US\$ 6 billion per year in the United States [2]. This spore-forming, gram-positive  
48 anaerobic bacterium gives rise to a spectrum of disease symptoms, ranging from mild diarrhea to  
49 pseudomembranous colitis, toxic megacolon, and death [3,4]. The primary cause of pathogenicity by  
50 *C. difficile* is due to its clostridial toxins, TcdA and TcdB [5], which are large proteins with a  
51 molecular weight of 308 kDa and 270 kDa, respectively, sharing structurally similar functional  
52 domains [6]. Both toxins are transferred into the host cell cytoplasm by receptor-mediated  
53 endocytosis where low pH in the endosome triggers conformational changes of the toxins activating  
54 the translocation of a catalytic domain across the membrane. Once inside the toxins inactivate Rho  
55 GTPases by attaching a glucose moiety to a catalytically important residue of the GTPase. This causes  
56 a degradation of the actin cytoskeleton leading to cell death [7]. Although there have been some  
57 contradicting reports of the individual potency and cytotoxic effects of each toxin [8–11] most *in vivo*  
58 studies suggest that they both contribute to disease during a natural infection [8,12].

59 The primary treatment of CDI consists of narrow-spectrum antibiotics such as metronidazole,  
60 vancomycin, and fidaxomicin [13]. However, non-responders to metronidazole and vancomycin have  
61 been reported [14,15]. After treatment of the patient's first episode of CDI the risk of recurrence is  
62 20-30% and no approved antimicrobial treatment exists that provides a lower probability of secondary  
63 CDI recurrence, which occurs in 40-60% of patients overcoming the first recurrence [16]. Recurrent  
64 CDI is likely a consequence of resident and long-lasting spores, reinfection, or the disruption of  
65 healthy microbiota due to the antibiotic treatment(s) [16,17]. The importance of a commensal gut

microbiota against recurrent CDI is supported by successful reports of fecal transplantations, with disease resolution up to ca. 90% of patients [18,19].

Studies in both animals and humans have shown that vaccination with detoxified TcdA and TcdB protects against CDI symptoms [20–23]. Thus, neutralization of TcdA and TcdB by toxin-specific antibodies is potentially an efficient method for preventing disease symptoms [24,25], and several toxoid-based vaccine candidates have made it to clinical trials [26–28]. Conventional detoxification methods such as cross-linking by formaldehyde have previously been used to detoxify toxins for vaccine production [29–31]. For instance, formaldehyde is successfully used in licensed toxoid-based vaccines against tetanus and diphtheria [32]. Unfortunately, formaldehyde-based detoxification has several disadvantages including i) slow and time-consuming [29], ii) risk of toxic reversibility over time [33,34], iii) inherent carcinogenicity and toxicity associated with formaldehyde [35,36] and finally, iv) suboptimal immunogenicity in some vaccines due to intra- and intermolecular cross-linked toxoids [37–39]. Thus, there is a need for identifying alternative approaches for rapid formation of safe, stable and highly immunogenic toxoids for future vaccines.

Oxidizing agents including divalent metal ions [40] and  $\text{H}_2\text{O}_2$  [41] have long been used as antiseptics, disinfectants and for inactivation of virulence factors such as toxins [42]. Furthermore, it has been shown that the reactive oxygen species (ROS) produced via a  $\text{Fe}^{3+}/\text{H}_2\text{O}_2/\text{EDTA}$  system could effectively detoxify pertussis toxin. This method has produced a safe and irreversibly detoxified pertussis toxoid [43,44], with higher epitope conservation than the formaldehyde-detoxified vaccine [38]. Despite the widespread knowledge, ROSs are widely considered agents of irreversible damage to biomolecules and tissues and the full advantage of these active oxygen species for contributing to medical advances has not been realized fully. In this study, using pH-dependent conformational modulation of TcdA and TcdB combined with a controlled copper ion-catalyzed protein oxidation

method developed by us previously [45,46], we describe an efficient, permanent and safe method for producing highly immunogenic toxoids of TcdA and TcdB.

91

## 2. Materials and Methods

### 2.1 Chemicals and reagents

Stabilizer-free 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$  30%) and copper(II)chloride dihydrate ( $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$ ), was obtained from Merck Chemicals GmbH (Darmstadt, Germany). Whereas, iron(III) sulfate ( $\text{Fe}_2(\text{SO}_4)_3 \cdot 7 \text{H}_2\text{O}$ ), Trizma base, crystal violet solution and SYPRO orange dye was purchased from Sigma-Aldrich (St. Louis, MO, USA). Formaldehyde (4%, v/v) solution was obtained from VWR (Gliwice, Poland) and ethylenediaminetetraacetic acid disodium salt (2Na-EDTA) was obtained from BDH Ltd. (Poole, England). Monoclonal mouse anti-TcdA and anti-TcdB antibodies were purchased from tgcBIOMICS (Bingen, Germany). AP-conjugated goat anti-mouse IgG (H+L) was purchased from Dako A/S (Glostrup, Denmark). HRP-conjugated rabbit anti-mouse (H+L) was purchased from Southern Biotech (Birmingham, AL, USA). TMB PLUS2 was obtained from Kem-En-Tec Diagnostics A/S (Taastrup, Denmark). Dulbecco's Modified Eagle Medium (DMEM) was obtained from ThermoFisher (Waltham, MA, USA). Tryptone and Yeast Extract were obtained from Formedium (Norfolk, UK). Tryptone, yeast extract, mannitol (TYM) consists of 24 g/L tryptone, 12 g/L yeast extract, 10 g/L mannitol, 1 g/L glycerol where tryptone, yeast extract, sodium thioglycolate (TYS) consists of 30 g/L tryptone, 20 g/L yeast extract, 1 g/L sodium thioglycolate. HiTrap Q FF column (4 x 5 mL serially connected), MonoQ 10/100 GL column and HiPrep 16/60 Sephacryl S-300 column were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA).

110

## 111    **2.2 Purification of *C. difficile* TcdA and TcdB**

112            TcdA and TcdB toxins from *C. difficile* Ribotype 027 (NCTC 13366) were purified using the  
113    dialysis bag method as described previously [47]. Briefly, an overnight anaerobic culture of *C.*  
114    *difficile* in TYM medium was inoculated (1%, v/v) into 2 L of sterile 0.9% saline in a dialysis bag  
115    suspended in 15 L of TYS. The media were pre-reduced with nitrogen and autoclaved before  
116    inoculation. Cultures were grown for 72 hours at 37 °C, centrifuged at 18.500 x g for 20 min at 4 °C  
117    and dialyzed using a Quattro 1000 Ultrafiltration/Diafiltration with a 50 kDa cut-off membrane in 50  
118    mM Tris-HCl (pH 7.5). Separation of TcdA and TcdB from the dialyzed supernatants was achieved  
119    using a HiTrap Q FF anion-exchange column, integrated on a fast protein liquid chromatography  
120    (FPLC). The toxins were eluted with a linear 0 to 1 M NaCl gradient, with TcdA eluting at 150 - 200  
121    mM NaCl and TcdB at 400 - 450 mM NaCl. Fractions were visualized on SDS-PAGE and protein  
122    sizes corresponding to either TcdA or TcdB were pooled and further purified using a HiPrep 16/60  
123    Sephacryl S-300 size-exclusion column. In the final step, a high-resolution anion-exchange MonoQ  
124    10/100 GL column was used.

125

## 126    **2.3 Differential scanning fluorimetry (DSF)**

127            Using a 96-well plate (MicroAmp, applied biosystems, USA), 2 µL of SYPRO Orange dye (62x  
128    concentrated stock) was mixed with 1.25 µM TcdA or 0.8 µM TcdB in individual pH-adjusted buffers  
129    to a final volume of 25 µL. The plate was centrifuged for 1 min at 2300 x g before placing it into the  
130    ABI 7500 Real-Time Polymerase Chain Reaction machine. The temperature gradient was set to run  
131    from 20 to 95 °C with an increase of 1 °C/min, as described previously [48]. The fluorescence signal  
132    was recorded and the obtained data were analyzed and processed on Graphpad Prism software version  
133    8 (San Diego, CA, USA).

134

## 135    **2.4 Circular dichroism (CD) spectroscopy**

136    Secondary and tertiary structural changes in TcdA and TcdB were probed using far-UV (200–260  
137    nm) and near-UV (250–320 nm) CD spectroscopy. A Jasco J-815 spectropolarimeter equipped with  
138    a Peltier-element-controlled thermostat was used for all studies. All CD measurements were  
139    performed with a spectral bandwidth of 2 nm and a scanning speed of 50 nm min<sup>-1</sup>. Far-UV  
140    measurements were performed using a cell of 0.1-cm path length and near-UV measurements were  
141    performed using a cell of 1.0-cm path length. The temperature stability studies were performed by  
142    heating TcdA or TcdB to the desired temperature, incubating for 5 min before measuring the CD  
143    spectrum, followed by heating to the next temperature and measuring a new CD spectrum. The  
144    studies monitoring the secondary/tertiary structure of the toxins/toxoids after metal-catalyzed  
145    oxidation were measured at 25 °C. The final CD spectra were obtained by subtracting the spectrum  
146    of the sample buffer from the mean sample spectrum of two individual scans using Jasco Spectra  
147    Analysis software, with a Savitzky-Golay algorithm of convolution width 11 applied as described  
148    previously [45,46]. Molar ellipticity ([ $\theta$ ]) in units of mdeg cm<sup>2</sup> dmol<sup>-1</sup> was calculated as

$$149 \quad [\theta] = \frac{\text{mdeg} \cdot M_w}{10 \cdot L \cdot c}$$

150    where [ $\theta$ ] is calculated molar ellipticity, *mdeg* is experimentally measured ellipticity in mdeg,  $M_w$  is  
151    protein molecular weight (g/mol),  $L$  is the optical path length (cm),  $c$  is the protein concentration  
152    (mg/ml).

153



## 154 2.5 Metal-catalyzed oxidation of TcdA and TcdB

155 Inactivation of TcdA and TcdB was achieved by  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  mediated metal-catalyzed oxidation  
156 (MCO) as previously described [45,46]. Briefly, in a pilot experiment MCO reactions with varying  
157  $\text{Cu}^{2+}$  (15, 30 and 37.5  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (50, 250, 500 and 1000  $\mu\text{M}$ ) concentrations were set to  
158 oxidatively modify TcdA (0.5  $\mu\text{M}$ ) at four pH values (4, 4.5, 5, 7.5) for 2 h at 37 °C and the MCO  
159 reaction was terminated by adding an optimized 2 mM EDTA [45,46] and incubation on ice  
160 (Supplementary Table S1). Protein concentrations were measured by direct absorbance at 280 nm  
161 using a NanoDrop ND-1000 spectrophotometer. All buffers consisted of 50 mM Tris with pH  
162 adjusted using acetic acid. From the pilot experiment, the best condition showing the highest levels  
163 of TcdA inactivation was further subjected to time-dependence trials at four-time points (30, 60, 90,  
164 120 min) as shown in Supplementary Fig. S1. Around 3 - 4  $\mu\text{M}$  of the toxin was mixed with pH-  
165 adjusted MCO components (toxin:  $\text{Cu}^{2+}$ :  $\text{H}_2\text{O}_2$ ) in a molar ratio of 1:60:1000, which were the optimal  
166 conditions for the final preparation of the toxoids. The reaction mixtures were mixed gently and  
167 transferred to a 37 °C heating block. Control samples were also prepared at each pH value without  
168  $\text{CuCl}_2$  and  $\text{H}_2\text{O}_2$ . Further control samples were prepared at pH 4.5 and 7.5 each containing one  
169 component of the reaction system (Supplementary Fig. S2 and S3). All samples were either analyzed  
170 immediately or after being stored a maximum of 2 h on ice.

171

## 172 2.6 *In vitro* cytotoxicity

173 Cell toxicity of native and MCO-detoxified TcdA and TcdB was tested using Vero cell culture  
174 ( $5 \times 10^4$  cells/ mL DMEM) [49]. After adding 150  $\mu\text{L}$  Vero cell culture to each well in a 96-well  
175 microtiter plate the plates were incubated in a HeraCell 150i  $\text{CO}_2$  incubator at 36.5 °C and 5%  $\text{CO}_2$   
176 for 24 h prior to cytotoxicity testing. Native toxin and/or MCO-detoxified samples (10  $\mu\text{L}$ ) were

177 added to the first well in each row, followed by serial dilution. After 48 h of incubation at 36.5 °C the  
178 level of cell rounding was assessed by visual inspection using a microscope. To further verify the  
179 visual assessment the plates were emptied for media and washed twice with 200 µL/well PBS buffer.  
180 After washing, 200 µL/well (4%, v/v) formaldehyde was added and incubated at room temperature  
181 for 10 min, followed by another washing step. Finally, the fixed cells inside the wells were stained  
182 using 0.1% crystal violet (200 µL/well), placed at room temperature for 10 min and washed gently  
183 with deionized water. Stained plates were photographed using a Bio-Rad Gel Doc Imager and  
184 qualitatively inspected.

185

## 186 **2.7 SDS-PAGE and Western Blot analysis**

187 TcdA and TcdB samples were visualized by reducing SDS-PAGE using TGX Stain-free™  
188 mini-protein gels (Bio-Rad, Hercules, CA, USA). Fifteen µL sample (0.5-1 µM/well) was mixed with  
189 5 µL of 2 x Laemmli Sample Buffer (Bio-Rad, USA), and incubated for 20 min at room temperature.  
190 Electrophoresis was carried out using TGS SDS Buffer (Bio-Rad, USA) for 30 min at 200 V, 500  
191 mA. Bio-Rad Precision Plus Protein Standard (4 µL/well) was used as a molecular weight marker.

192 For Western Blot analysis, SDS-PAGE gel bands were transferred to a Trans-Blot Turbo 0.2  
193 µm nitrocellulose membrane (Bio-Rad, USA) using electroblotting on a Bio-Rad Trans-Blot Turbo  
194 Transfer System for 7 min at 25 V, 2.5 A. Subsequently, the nitrocellulose membrane was blocked  
195 with 5% w/v skim milk/TBS buffer for 30 min at 37 °C with shaking, and thereafter washed 3x5 min.  
196 in TBS at 37 °C with shaking. After washing, the membrane was incubated for 1 h at 37 °C with  
197 mouse anti-TcdA or anti-TcdB antibodies diluted 1:100,000 in skim milk/TBS. Another washing step  
198 was performed, where after the blots were incubated for 1 h at 37 °C with goat anti-mouse AP-  
199 conjugated antibody diluted 1:1000 in skim milk/TBS, followed by a final washing step. For

200 visualization of antibody binding, SigmaFast BCIP/NBT tablets (Sigma-Aldrich, St. Louis, MO,  
201 USA) were used.

202

## 203 **2.8 Stability study**

204 Native and MCO-detoxified TcdA and TcdB samples were incubated at -20 °C, 4 °C and 25 °C  
205 for 26 or 28 days. Detoxified TcdA was kept at pH 4.5 during storage, whereas detoxified TcdB was  
206 adjusted to pH 7.5 for storage. Cytotoxicity was measured for all samples using Vero cells.  
207 Furthermore, secondary structure was analyzed for all samples before and after incubation at the  
208 various temperatures using far-UV CD at 200 – 260 nm.

209

## 210 **2.9 Epitope recognition study**

211 Polystyrene MaxiSorp microtiter plates (Nunc) were coated with 100 µl of either 1 µg/ml native  
212 or detoxified TcdA and TcdB, respectively, in 0.05 M Na<sub>2</sub>CO<sub>3</sub>, 0.05 M NaHCO<sub>3</sub> (pH 9.6) and  
213 incubated overnight at 5 °C. The next day, wells were blocked with 300 µl of 1% (w/v) BSA in PBS-  
214 0.05% (v/v) Tween (pH 7.4) and incubated for 2 h at 37 °C. One hundred µl of serially diluted  
215 monoclonal antibody (1:4 in 1% BSA in PBS-0.05% Tween) was added to each well in triplicates  
216 and incubated for 1 h at 37 °C. HRP-conjugated rabbit anti-mouse IgG diluted 1:5000 in 100 µL of  
217 1% BSA in PBS-0.05% Tween was added to each well, followed by incubation for 1 h at 37 °C.  
218 Antibody binding was visualized by the addition of 100 µL TMB PLUS2 substrate and incubation at  
219 room temperature for 15 min, and the reaction was stopped by adding 50 µL of 0.2 M H<sub>2</sub>SO<sub>4</sub>.  
220 Absorbance was measured at 450 nm using a POLARstar OPTIMA microplate reader (BMG

laboratories). Plates were washed 5 times with 250  $\mu$ L washing buffer (PBS-0.05% Tween 20) between each step.

## **2.10 Toxoid preparation for mouse challenge study**

The MCO-detoxified vaccine was prepared by individually mixing 11.2  $\mu$ M and 11.9  $\mu$ M TcdA and TcdB, respectively, with pH 4.5-adjusted MCO components in a molar ratio of 1:60:1000 for toxin: $\text{Cu}^{2+}$ : $\text{H}_2\text{O}_2$  and incubated at 37  $^\circ\text{C}$  for 2 h. MCO reactions were terminated by adding EDTA to a final concentration of 2 mM, adjusting pH to 7.5 and transferring the tubes to 4  $^\circ\text{C}$ . The formaldehyde-detoxified vaccine was prepared by individually dialyzing 5.5  $\mu$ M TcdA and TcdB into 0.1 M phosphate buffer, pH  $7\pm 0.2$  using 30 kDa cut-off centrifugal filters (Amicon). Then formaldehyde was added to a final concentration of 0.45% (v/v) and the samples incubated at room temperature (25  $^\circ\text{C}$ ) for 7 days. Samples were then dialyzed against 0.1 M phosphate, 0.1 M NaCl, pH  $7\pm 0.2$  using 30 kDa cut-off centrifugal filters at 4  $^\circ\text{C}$  to remove the formaldehyde. Formaldehyde was added to a final concentration of 0.016% (v/v) after dialysis to prevent the reversion of toxicity, and samples were stored at 4  $^\circ\text{C}$ . Each individual toxin from the MCO- and formaldehyde-detoxified samples were diluted to 0.2 mg/ml and mixed with aluminium hydroxide (Alhydrogel®) to a final concentration of 2 mg/ml and incubated for 24 h at 4  $^\circ\text{C}$  shaking at 250 rpm. The next day, aluminium hydroxide-adsorbed TcdA and TcdB samples were mixed in equal ratios of TcdA and TcdB, in a final vaccine formulation consisting of 0.1 mg/ml TcdA, 0.1 mg/ml TcdB and 2 mg/ml aluminium hydroxide. Each vaccine dose of 50  $\mu$ L contained 5  $\mu$ g TcdA and 5  $\mu$ g TcdB, detoxified with either MCO or formaldehyde.

## 2.11 Mouse challenge model of CDI

The experimental protocol for this animal study was approved by The Danish Experimental Animal Inspectorate (No. 2018-15-0201-01387), and all applicable national guidelines for the care and use of animals were followed. Female C57BL/6J-OlaHsd mice, 8- to -10 weeks old and weighing approximately 18 g (Envigo, UK) were housed in three groups of 8 per cage under similar conditions. Food (Teklad 2916 Global 16% protein rodent diet, Envigo), bedding (Tapvei aspen), nesting material (Enviro-dri), cage enrichments (cardboard house, dried corn, peanuts, sunseed – given twice a week) were all irradiated before use. Food and water were given ad libitum. Housing was a Type III cage and washed prior to use. The experimental model used in this study was based on the model developed by Chen *et al.* [50] and Erikstrup *et al.* [51]. Mice were immunized with a 50  $\mu$ L vaccine dose two times, on days 0 and 21 by intramuscular injection. The animals received either a formulation with MCO-detoxified vaccine (n = 8), formaldehyde-detoxified vaccine (n = 8) or an aluminium hydroxide (mock) control (n = 8). Blood samples were collected on days 0, 21, 49 and 60. In order to establish CDI, the normal enteric microbiota was disrupted by pretreating the mice with an antimicrobial mixture in the drinking water, for 3 days starting on day 50, containing kanamycin (40 mg/kg), gentamycin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg) and vancomycin (4.5 mg/kg). The concentration of the antimicrobial mixture was calculated based on the average weight of the mice and their expected water consumption. On day 53, the mice were switched back to regular drinking water and on day 55 all mice were intraperitoneally injected with a 200  $\mu$ L single dose of clindamycin (25 mg/kg). On day 56, all mice were challenged with 250  $\mu$ L of  $0.3 \times 10^7$  colony-forming units (CFU) of vegetative *C. difficile* Ribotype 027 (NCTC 13366) by oral gavage. The mice were monitored for signs of disease (diarrhea, wet tail, weight loss) and death. A clinical scoring system based on activity level, weight loss, changed breathing, appearance of eyes and fur was used at least 5 times per day with strict criteria to euthanize moribund animals.

267

## 268 **2.12 Serum IgG measurements by ELISA**

269 Polystyrene MaxiSorp microtiter plates (Nunc, Denmark) were coated with 100 µl of either 1  
270 µg/ml TcdA or TcdB in 0.05 M Na<sub>2</sub>CO<sub>3</sub>, 0.05 M NaHCO<sub>3</sub> pH 9.6 and incubated overnight at 5 °C.  
271 The next day, wells were blocked with 300 µl of 1% BSA in PBS pH 7.4 and incubated for 2 h at 37  
272 °C. Each mouse serum was 3-fold serially diluted in 0.5% BSA-PBS and 100 µl was added to each  
273 well in triplicates and incubated for 1 h at 37 °C. HRP-conjugated rabbit anti-mouse IgG diluted  
274 1:5000 in 100 µL 0.5% BSA-PBS was added to each well, followed by incubation for 1 h at 37 °C.  
275 The antibody binding was visualized by the addition of 100 µL TMB PLUS2 substrate and incubation  
276 at room temperature up to 10 min, and the reaction was stopped by adding 100 µL of 0.2 M H<sub>2</sub>SO<sub>4</sub>.  
277 Absorbance was measured at 450 nm using a POLARstar OPTIMA microplate reader (BMG  
278 laboratories). Plates were washed 5 times with 250 µL washing buffer (PBS, pH 7.4, containing  
279 0.05% (v/v) Tween 20) between each step.

280

## 281 **2.13 Toxin Neutralization Assay (TNA)**

282 One hundred µL cell culture in DMEM was added to each well in a 96-well microtiter plate and  
283 incubated in a HeraCell 150i CO<sub>2</sub> incubator at 36.5 °C and 5% CO<sub>2</sub> for 24 h prior to testing. Titrations  
284 of TcdA and TcdB were tested for the toxin concentration causing 50% rounding of cells (TC<sub>50</sub>) prior  
285 to TNA studies. A concentration of 4 x TC<sub>50</sub> for TcdA (4 ng/ml) or TcdB (7 µg/ml) was pre-incubated  
286 with a 2-fold serial dilution of sera from immunized animals for 90 min at 36.5 °C with 5% CO<sub>2</sub> prior  
287 to their addition to the cell culture. One hundred µL of toxin-sera mixture was added to each well  
288 containing 100 µL cell culture and the plates were incubated for 48 h at 36.5 °C with 5% CO<sub>2</sub>. Cell  
289 rounding was inspected as described in the “*In vitro* cytotoxicity” paragraph.

290

## 291 **2.14 Statistical analysis**

292 DSF curves and all ELISA titers are presented as the mean of three individual replicates. All  
293 statistical analysis of the data was performed using GraphPad Prism 8 software. Unpaired Student's  
294 t-test was used to calculate p-values for IgG titers and mean relative weights, whereas Mantel-Cox  
295 log-rank test was conducted on the Kaplan-Meier survival curves. P-values < 0.05 was taken as  
296 significant.

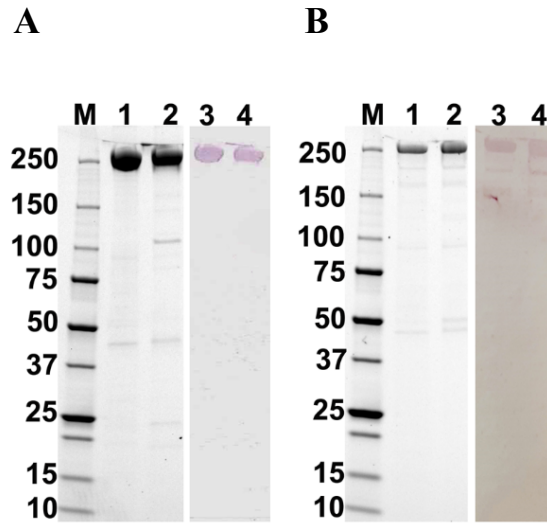
297

## 298 **3. Results**

### 299 **3.1 Native expression of TcdA and TcdB**

300 Native forms of TcdA and TcdB were expressed using the *C. difficile* Ribotype 027 strain  
301 (NCTC 13366). Brain Heart Infusion (BHI) broth is generally used as a growth medium for *C. difficile*  
302 for native toxin expression [52,53], however, we found that using a growth medium containing  
303 tryptone, yeast extract and sodium thioglycolate (TYS) produced a higher yield of toxins compared  
304 to BHI (Supplementary Fig. S4). After 72 h of incubation at 37 °C under anaerobic conditions, the  
305 toxins were purified from the culture supernatant using FPLC chromatography. Purified TcdA and  
306 TcdB were evaluated by SDS-PAGE and western blot (WB) analysis in a neutral and acidic buffer  
307 respectively, to evaluate if the toxins were degraded under the harsher acidic conditions (Fig. 1A and  
308 1B). SDS-PAGE and antibody recognition of the neutral and acidic stored toxins confirmed the  
309 presence of intact protein bands for both TcdA and TcdB.

310



**Figure 1. SDS-PAGE and WB analysis of native TcdA and TcdB.** TcdA (0.9  $\mu$ M) and TcdB (0.6  $\mu$ M) were stored for 2 hours at 37  $^{\circ}$ C in either neutral or acidic conditions prior to analysis. **A:** TcdA samples; lane M: molecular weight markers (kDa), lane 1: TcdA in pH 7.5 (protein stain), lane 2: TcdA in pH 4.5 (protein stain), lane 3: TcdA in pH 7.5 (western), lane 4: TcdA in pH 4.5 (western). **B:** TcdB samples; lane M: molecular weight markers (kDa), lane 1: TcdB in pH 7.5 (protein stain), lane 2: TcdB in pH 4.5 (protein stain), lane 3: TcdB in pH 7.5 (western), lane 4: TcdB in pH 4.5 (western).

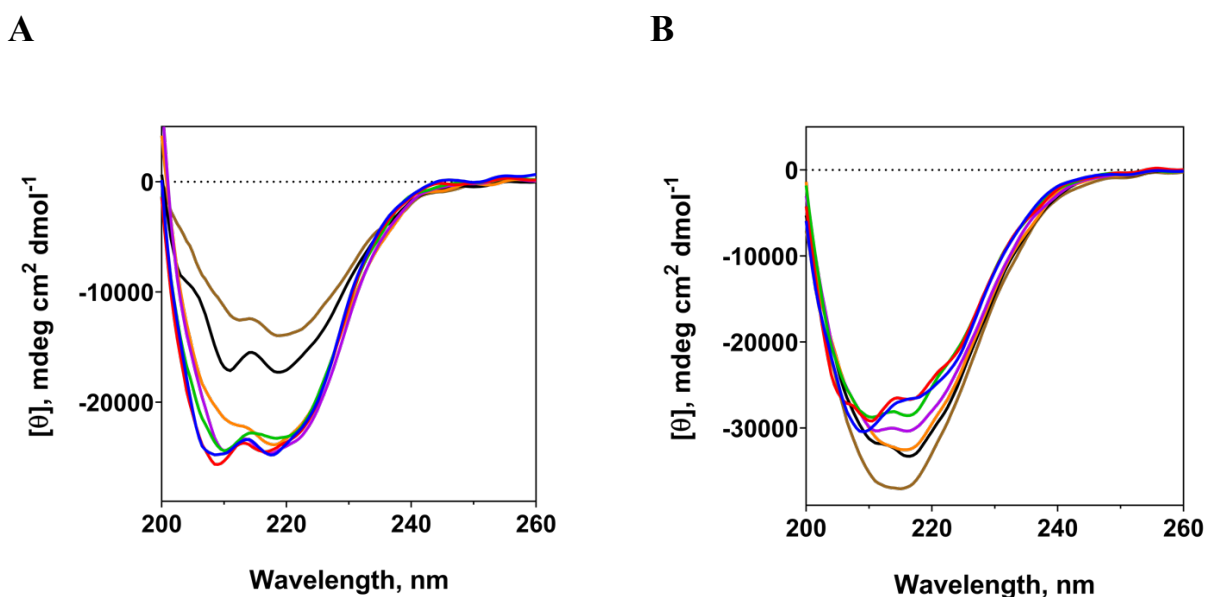
311

### 312 3.2 Temperature stability of native purified TcdA and TcdB

313 The changes in the secondary structure of TcdA (Fig. 2A) and TcdB (Fig. 2B) with increasing  
314 temperature (25 to 80  $^{\circ}$ C) were monitored using circular dichroism (CD) in the 200 - 260 nm region.  
315 TcdA shows well-defined far-UV CD spectra from 25  $^{\circ}$ C to 37  $^{\circ}$ C with similar spectral shapes and  
316 two negative peaks at 208 and 218 nm (Fig. 2A). This indicates that the secondary structure of TcdA  
317 is stable and largely intact during heating to 37  $^{\circ}$ C. However, at 45  $^{\circ}$ C and 50  $^{\circ}$ C the spectra show a  
318 slight change in the 208 nm region with a beginning loss of the negative peak at 208 nm. By further  
319 heating to 60  $^{\circ}$ C, the loss of this characteristic peak is more severe, which is seen by the complete  
320 loss of the negative peak at 208 nm, indicating the unfolding of secondary protein structure. Heating  
321 TcdA to 70  $^{\circ}$ C and then to 80  $^{\circ}$ C, the far-UV CD spectra have now completely lost any well-defined  
322 shape and the intensity of the CD spectra are significantly reduced overall, indicating denaturation of



323 TcdA. TcdB also shows a well-defined far-UV CD spectrum during heating from 25 °C to 37 °C with  
 324 no significant changes in the spectrum, and the presence of two negative peaks at 208 nm and 218  
 325 nm (Fig. 2B). At 45 °C there is a slight increase in the intensity of the negative peak at 218 nm,  
 326 showing the beginning of minor structural changes at this temperature. However, TcdB does not seem  
 327 to show significant spectral changes in the 208 nm region at 45 °C, unlike what is seen for TcdA (Fig.  
 328 2A), instead there are slight changes in the 218 nm region. Further heating to 50 °C induces significant  
 329 changes in the secondary structure, which is seen by a more profound increase in the negative peak  
 330 at 218 nm. This trend continues and increases during further heating to 60 °C and 70 °C, but at 80 °C  
 331 the far-UV CD spectrum has changed drastically and completely lost the two negative peaks. At 80  
 332 °C, the CD spectrum shows a sharp rounded shape with a minimum of around 216 nm, and a  
 333 significant increase is seen in the spectral intensity compared to the initial spectrum before heating.



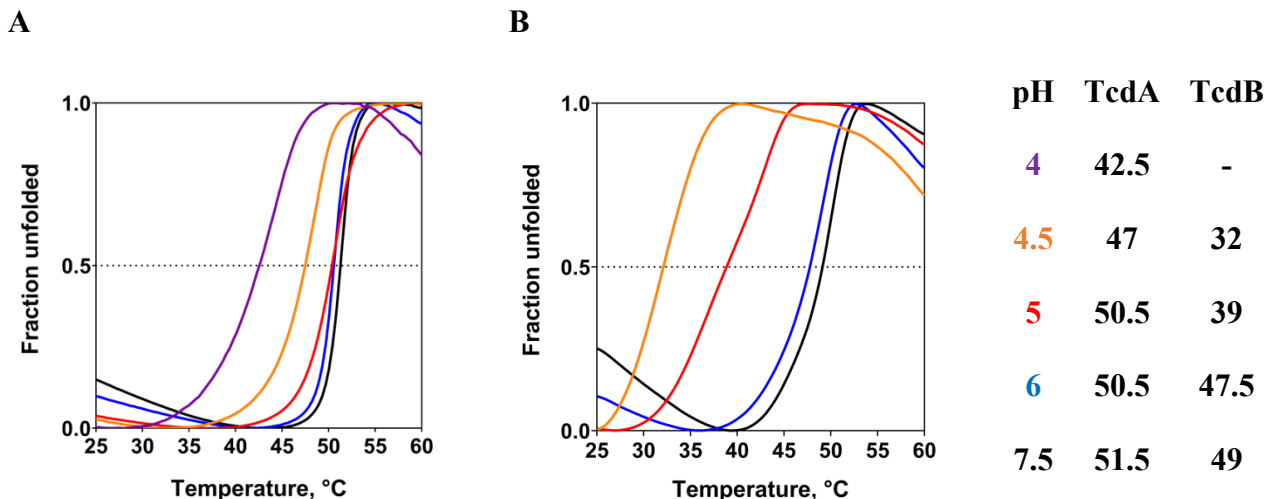
**Figure 2. Effect of temperature on the secondary structure of TcdA and TcdB.** Circular dichroism analysis of TcdA and TcdB, showing the change in the secondary structure during heating. The samples are kept for 5 min at each temperature before measurement, and the measurements are cumulative. **A:** 0.65  $\mu$ M TcdA, **B:** 0.75  $\mu$ M TcdB. Blue: 25°C, red: 37°C, green: 45°C, purple: 50°C, orange: 60°C, black: 70°C, brown: 80°C.

334

### 335 3.3 pH-induced thermostability changes

336 Differential Scanning Fluorimetry (DSF) analysis was performed on TcdA (Fig. 3A) and TcdB  
337 (Fig. 3B), in temperature ranges from 25 to 95 °C with an increase of 1°C/min. Both toxins were  
338 tested at five different pH conditions, ranging from pH 4 to 7.5, and the resulting melting temperatures  
339 ( $T_m$ ) are listed in (Fig. 3C). The melting temperature ( $T_m$ ) for TcdA at pH 7.5 is 51.5 °C, which is in  
340 alignment with the result obtained from the far-UV CD spectrum (Fig. 2A) showing initiation of  
341 unfolding at 45 - 50 °C. When lowering the pH to 6 and 5 respectively, no significant changes in the  
342  $T_m$  values are observed. We only see a slight decrease of 1 °C which lowers the  $T_m$  of TcdA in pH 6  
343 and 5 to 50.5 °C, indicating that the thermal stability of TcdA is not significantly affected in the pH  
344 range of 7.5 to 5. However, by lowering the pH further to acidic levels of 4.5 and 4, a significant  
345 decrease can be seen in the  $T_m$  for TcdA. At pH 4.5 there is a 4.5 °C decrease in the  $T_m$  reaching 47  
346 °C, and at pH 4 the decrease in  $T_m$  is as high as 9 °C reaching a melting temperature of only 42.5 °C.  
347 TcdB has a slightly lower melting temperature at neutral pH than TcdA, with a  $T_m$  of 49 °C. This also  
348 correlates with the data from the far-UV CD spectra (Fig. 2B), where the structural changes of TcdB  
349 at 50 °C are more significant than for TcdA (Fig. 2A) confirming that TcdB has a lower melting  
350 temperature. At pH 6 the  $T_m$  is 47.5 °C, showing that TcdB is relatively stable when lowering the pH  
351 from 7.5 to 6, but not as stable as TcdA. However, when lowering the pH further to 5, there is a  
352 significant decrease in the  $T_m$  to 39 °C, which is much lower than the  $T_m$  of TcdA at pH 5. When the  
353 pH is lowered to 4.5 the  $T_m$  of TcdB is further decreased by 7 °C reaching 32 °C. At pH 4, the  $T_m$   
354 cannot be calculated as the melting curve had no visible transition phase during heating. From the  
355 DSF analysis (Fig. 3A and 3B), it is clear that at acidic pH around 4.5, TcdA and TcdB have lower  
356 melting temperatures than at neutral pH.

357



**Figure 3. The effect of pH on the thermal stability of TcdA and TcdB.** DSF was conducted using real-time PCR with a temperature gradient from 20 to 95 °C with an increase of 1°C/min (only 25 to 60 °C is shown). **A:** Each well contained 1.25 μM of TcdA, pH-adjusted buffer, and 2 μl of SYPRO orange dye (from 62x concentrated stock) in a final volume of 25 μl. **B:** Each well contained 0.8 μM of TcdB, pH-adjusted buffer, and 2 μl of SYPRO orange dye (from 62x concentrated stock) in a final volume of 25 μl. Black: 50 mM Tris-HCl pH 7.5, blue: 50 mM Na-citrate pH 6, red: 50 mM Na-acetate pH 5, orange: 50 mM Na-acetate pH 4.5, purple: 50 mM Na-acetate pH 4. The melting temperatures ( $T_m$ ) are given in °C on the right.

### 3.4 Metal-catalyzed oxidation of TcdA and TcdB

Different pH ranges and MCO components were tested in order to identify and optimize mild oxidative conditions for detoxification of TcdA and TcdB. A concentration of TcdA (0.5 μM) at 37 °C was kept constant in the reaction mixture, and MCO treatment was tested at different pH values (4, 4.5, 5 and 7.5). The MCO components were also varied; CuCl<sub>2</sub> (15 to 37.5 μM) and H<sub>2</sub>O<sub>2</sub> (50 to 1000 μM) as shown in Supplementary Table S1. All conditions were evaluated by SDS-PAGE, western blot analysis and Vero cell cytotoxicity assay (data not shown). These studies led to the optimal values of the MCO reaction components, which were determined to be molar ratios of 1:60:1000 for TcdA:Cu<sup>2+</sup>:H<sub>2</sub>O<sub>2</sub>, respectively, with a reaction pH of 4.5 and a concentration of TcdA of 0.5 μM. Then the optimal conditions such as molar ratios of oxidants, buffer and pH obtained on TcdA were transferred to TcdB in further studies. With this knowledge, we proceeded to test MCO

369 on TcdA at pH 4.5 and at 37 °C with a range of different metal ions. All MCO samples were tested  
370 for cytotoxicity (Supplementary Fig. S5) and the results are summarized in Table 1. Of all the tested  
371 metal ions only  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  system was able to induce a significant inactivation of TcdA with our  
372 MCO method, resulting in 6  $\log_{10}$  reductions of cytotoxicity relative to native TcdA. A similar level  
373 of TcdB inactivation was also achieved using  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  system at pH 4.5 and 37 °C. The  $\text{Fe}^{2+}/\text{H}_2\text{O}_2$   
374 system and  $\text{Fe}^{3+}/\text{H}_2\text{O}_2$  systems were only capable of reducing the cytotoxicity by a negligible 50-fold  
375 and 7-fold, respectively. None of the other metal ions had any significant effect on the cytotoxicity  
376 of TcdA as seen in Table 1. All conditions were evaluated by SDS-PAGE, western blot analysis and  
377 Vero cell cytotoxicity assay. TcdA and TcdB after MCO treatment at the optimal conditions were  
378 tested by cytotoxicity assay to determine the extent of reduction of cytotoxicity, in comparison to  
379 corresponding control samples (Supplementary Fig. S6 and S7) and summarized in Table 2.

380 The MCO detoxification of TcdA and TcdB at neutral pH using  $\text{Cu}^{2+}$  did not affect the  
381 cytotoxicity, whereas the same treatment at pH 4.5 showed more than 6  $\log_{10}$  fold reduction of the  
382 cytotoxicity for both toxins. It was also tested whether the individual components of the MCO  
383 reaction, such as  $\text{Cu}^{2+}$ ,  $\text{H}_2\text{O}_2$  or the acidification in itself had any significant detoxifying effect on the  
384 toxins (Supplementary Fig. S3), but the results showed that only the specific combination of  $\text{Cu}^{2+}$ ,  
385  $\text{H}_2\text{O}_2$ , and acidic pH range (4.0 to 4.5) is able to produce highly detoxified TcdA and TcdB toxoids.  
386 Finally, we studied the effect of different temperatures (25 to 37 °C) and incubation times (30 to 120  
387 min) on the efficacy of MCO inactivation (Supplementary Figs. S1 and S8). We found that the optimal  
388 temperature and incubation time for effective inactivation of TcdA and TcdB is 37 °C and 120 min  
389 respectively.

390

**Table 1.** Effect of various metal ions on the MCO detoxification of TcdA

Metal salt	Metal-ion	Fold reduction in cytotoxicity
CuCl <sub>2</sub>	Cu <sup>2+</sup>	> 1,000,000
MgCl <sub>2</sub>	Mg <sup>2+</sup>	7
CoCl <sub>2</sub>	Co <sup>2+</sup>	7
MnCl <sub>2</sub>	Mn <sup>2+</sup>	7
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	Fe <sup>3+</sup>	7
FeSO <sub>4</sub>	Fe <sup>2+</sup>	50
CaCl <sub>2</sub>	Ca <sup>2+</sup>	7
LiCl	Li <sup>+</sup>	7
NiCl <sub>2</sub>	Ni <sup>2+</sup>	7
AgNO <sub>3</sub>	Ag <sup>+</sup>	7

All MCO reactions were conducted at pH 4.5 and each sample consisted of TcdA (1.2 μM), a metal salt (72 μM) and H<sub>2</sub>O<sub>2</sub> (1200 μM) which were incubated for 2 hours at 37 °C and measured for cytotoxicity on Vero cells (Fig. S5).

**Table 2.** Effect of Cu<sup>2+</sup>-catalyzed oxidation on the cytotoxicity of TcdA and TcdB

Toxin	Final toxin concentration (μM)	pH	Molar ratios	Fold reduction in cytotoxicity
			(Toxin:Cu <sup>2+</sup> :H <sub>2</sub> O <sub>2</sub> )	
TcdA native	1.3	7.5	1:0:0	1
TcdA native	1.3	4.5	1:0:0	50
TcdA MCO-detoxified	1.3	4.5	1:60:1000	> 1,000,000
TcdA MCO-detoxified	1.3	7.5	1:60:1000	5
TcdB native	1.5	7.5	1:0:0	1
TcdB native	1.5	4.5	1:0:0	350
TcdB MCO-detoxified	1.5	4.5	1:60:1000	> 1,000,000
TcdB MCO-detoxified	1.5	7.5	1:60:1000	5

All MCO reactions were incubated for 2 hours at 37 °C and measured for cytotoxicity on Vero cells (Figs. S6 and S7).

### 3.5 MCO-induced structural changes

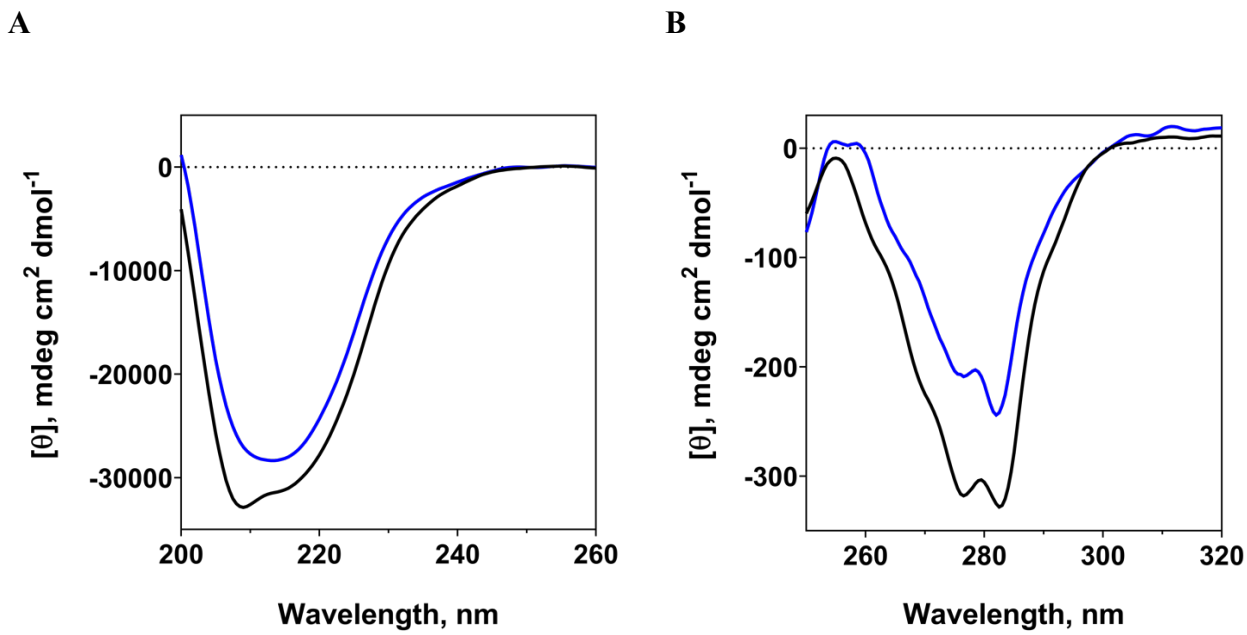
The secondary structure of native and MCO detoxified TcdA and TcdB was monitored by far-UV CD in the 200 - 260 nm region. Differences are observed in the 200 - 220 nm region between MCO-detoxified and active TcdA (Fig. 4A). There is a slight loss of overall CD spectrum intensity after MCO treatment, and the characteristic negative peak at 208 nm is lost, indicating some changes in the α-helical structure of TcdA after oxidation. The same trend is seen for TcdB with a loss of the characteristic negative peak at 208 nm for the oxidized TcdB at pH 4.5 (Fig. 5A). The CD results

align with the DSF results, showing that the toxins are more prone to structural changes at pH 4.5 (Fig. 3A and 3B). However, as seen in Fig. 5A (red line), the CD spectrum of MCO-detoxified TcdB could be reversed to the native-like state by raising the pH to 7.5 after oxidation. Surprisingly, when adjusting the pH of MCO-detoxified TcdA to 7.5, as we did for TcdB, we saw a significant reduction of CD spectrum intensity, indicating a degree of precipitation caused by the pH change.

The tertiary structure of native and MCO-detoxified TcdA and TcdB were monitored by near-UV CD. The spectra for native and MCO-detoxified TcdA both show an overall similar shape with two negative peaks at 275 and 282 nm (Fig. 4B). The CD spectrum of MCO-detoxified TcdA, however, has lower CD signal intensity compared to native TcdA, which could indicate that changes of the aromatic residues have occurred or that the protein has slightly precipitated. The near-UV CD spectrum of MCO-detoxified TcdB was monitored after readjusting pH to 7.5. The CD spectrum shows a similar shape and finer features compared to native TcdB with two negative minima at 275 and 282 nm (Fig. 5B). Like TcdA, the MCO-detoxified TcdB CD spectrum has lower signal intensity compared to the native TcdB spectrum.

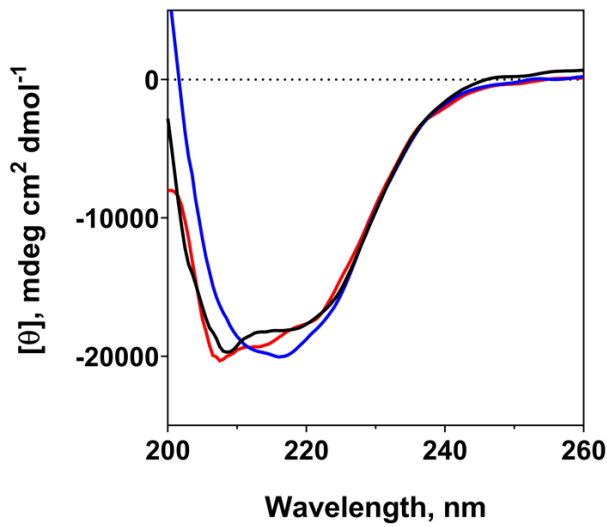
Furthermore, to separate low pH and oxidative modification in the spectral changes of the CD, we monitored far-UV CD (Supplementary Fig. S9) and near-UV (Supplementary Fig. S10) of TcdA continuously during the MCO reaction at pH 4.5. CD measurements were conducted every 3 min to follow the progression of spectral changes. Interestingly, in the far-UV CD we see that already in the first spectrum after initiating oxidation there is a change between native TcdA at pH 4.5 to the MCO-detoxified TcdA at pH 4.5, meaning that MCO immediately causes more changes to the secondary structure than pH 4.5 alone. TcdA at pH 4.5 and the MCO-detoxified TcdA at pH 4.5, both show a progression of spectral changes over time, however, the spectral changes happen faster and are more extensive for the MCO-detoxified TcdA compared to TcdA at pH 4.5. The spectral changes progress until reaching a plateau around the 15 min time point. Near-UV CD spectra were also monitored for

426 the MCO reaction, and here we see an immediate change in the spectrum during the very first minutes  
427 of the MCO reaction (Supplementary Fig. S10).

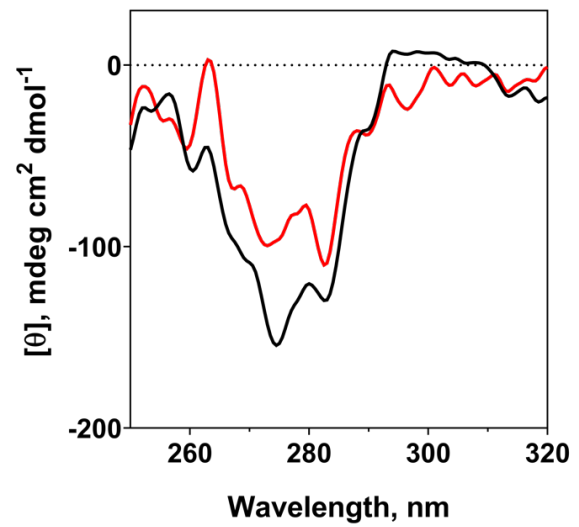


**Figure 4. Circular dichroism analysis of native and MCO-detoxified TcdA.** All samples are shown as an average of duplicate measurements with the buffer spectrum (blank) subtracted. **A:** Far-UV CD spectra ranging from 200 – 260 nm. The sample consisted of 1  $\mu\text{M}$  TcdA. **B:** Near-UV CD spectra ranging from 250 – 320 nm. The sample consisted of 3.15  $\mu\text{M}$  TcdA. Black: native TcdA pH 7.5, blue: MCO-detoxified TcdA pH 4.5.

A



B



**Figure 5. Circular dichroism analysis of native and MCO-detoxified TcdB.** Far-UV CD samples are shown as an average of duplicate measurements with the buffer spectrum (blank) subtracted. Near-UV CD samples are shown as an average of 20 spectra with the buffer spectrum (blank) subtracted. **A:** Far-UV CD spectra ranging from 200 – 260 nm. The sample consisted of 0.75  $\mu$ M TcdB. **B:** Near-UV CD spectra ranging from 250 – 320 nm. The sample consisted of 0.75  $\mu$ M TcdB. Black: native TcdB pH 7.5, blue: MCO-detoxified TcdB pH 4.5, red: MCO-detoxified TcdB readjusted to pH 7.5.

429

### 430 3.6 Epitope recognition after MCO detoxification

431 The comparison between the binding of the mAbs to native and detoxified TcdA and TcdB are  
 432 shown in Table 3 and 4, respectively. The oxidation of TcdA slightly affected the epitope binding to  
 433 the six different mAbs. The ranges of mAb binding are between 12% and 79% with an average of  
 434 52%, relative to the binding of the mAbs to native TcdA. Formaldehyde detoxification has a  
 435 significantly more deleterious effect on the TcdA epitopes, as the binding capacity of the mAbs is  
 436 between 8% and 38% with an average of 21%. Detoxification of TcdB with either MCO or  
 437 formaldehyde follows a similar trend as TcdA, where MCO detoxification of TcdB leads to higher  
 438 epitope recognition by the mAbs compared to formaldehyde. The binding efficacy of the five mAbs



to MCO-detoxified TcdB is between 51% and 65% with an average of 57%, compared to binding to formaldehyde detoxified TcdB which is between 0% and 69% with an average of only 31%.

**Table 3.** Recognition of native and detoxified TcdA (TxdA) by monoclonal anti-TcdA antibodies

mAb (target)	Toxin		
	TcdA-native	TcdA-MCO	TcdA-formaldehyde
A-21 (C-terminal)	1.00	0.43	0.38
A-22 (C-terminal)	1.00	0.47	0.09
A-23 (C-terminal)	1.00	0.79	0.37
A-26 (C-terminal)	1.00	0.5	0.08
A-24 (N-terminal)	1.00	0.12	0.24
A-25 (N-terminal)	1.00	0.79	0.09

The recognition of MCO- or formaldehyde-detoxified TcdA by the mAbs is expressed as the ratio between the ELISA titer of the detoxified TcdA relative to the ELISA titer of native TcdA. The ELISA titer is defined as the endpoint titer, which is the highest dilution of mAb showing at least twice the A<sub>450</sub> value of the blank wells. The ELISA titer of native TcdA is defined as 1.00. Formaldehyde-detoxification was conducted with 0.45% (v/v) formaldehyde, 30 mM lysine in 50 mM Tris pH 7.5 for 7 days at room temperature (25 °C).

**Table 4.** Recognition of native and detoxified TcdB by monoclonal anti-TcdB antibodies

mAb (target)	Toxin		
	TcdB-native	TcdB-MCO	TcdB-formaldehyde
B-72 (C-terminal)	1.00	0.65	0.37
B-75 (C-terminal)	1.00	0.57	0.1
B-76 (C-terminal)	1.00	0.57	0.38
B-71 (N-terminal)	1.00	0.51	0.69
B-74 (N-terminal)	1.00	0.55	0

The recognition of MCO or formaldehyde-detoxified TcdB by the mAbs is expressed as the ratio between the ELISA titer of the detoxified TcdB relative to the ELISA titer of native TcdB. The ELISA titer is defined as the endpoint titer, which is the highest dilution of mAb showing at least twice the A<sub>450</sub> value of the blank wells. The ELISA titer of native TcdB is defined as 1.00. Formaldehyde-detoxification was conducted with 0.45% (v/v) formaldehyde, 30 mM lysine in 50 mM Tris pH 7.5 for 7 days at room temperature (25 °C).

### 3.7 Stability

Native and MCO-detoxified TcdA and TcdB were analyzed by far-UV CD and cytotoxicity testing after being stored for 26-28 days at either -20 °C, 4 °C, and 25 °C. Neither native nor MCO-detoxified TcdA shows significant changes in the shapes of the CD spectra at day 28 compared to day 0 when stored at any of the different temperatures (Fig. 6A-C). However, at all storage conditions, there is a significant reduction of the overall spectral intensity after 28 days for both native and MCO-

451 detoxified TcdA. TcdA samples (native and MCO-detoxified) show roughly the same level of spectral  
452 intensity loss when stored at -20 °C (Fig. 6A) and 4 °C (Fig. 6B) over 28 days, indicating that either  
453 precipitation and/or degradation is occurring equally for both. Surprisingly, native TcdA stored at 25  
454 °C shows a more severe loss of CD spectrum intensity after 28 days, compared to the MCO-detoxified  
455 TcdA sample at 25 °C.

456 MCO-detoxified TcdB also shows well-preserved CD spectral features after either 26 or 28  
457 days of storage (Fig. 6D-F). Similar to the TcdA samples, the CD spectra for both native and MCO-  
458 detoxified TcdB lose intensity overall during the storage period, likely caused by precipitation.  
459 Interestingly, after storage at 25 °C for 26 days the CD spectrum for native TcdB has significantly  
460 lower signal intensity compared to the MCO-detoxified TcdB CD spectrum, which is also seen for  
461 TcdA at 25 °C. None of the MCO-detoxified TcdA or TcdB samples show a reversal of cytotoxicity  
462 during the storage period at any of the storage conditions. A small decrease in cytotoxicity is observed  
463 during the storage period for both native and MCO- detoxified TcdA and TcdB, likely due to protein  
464 precipitation as mentioned above.

465

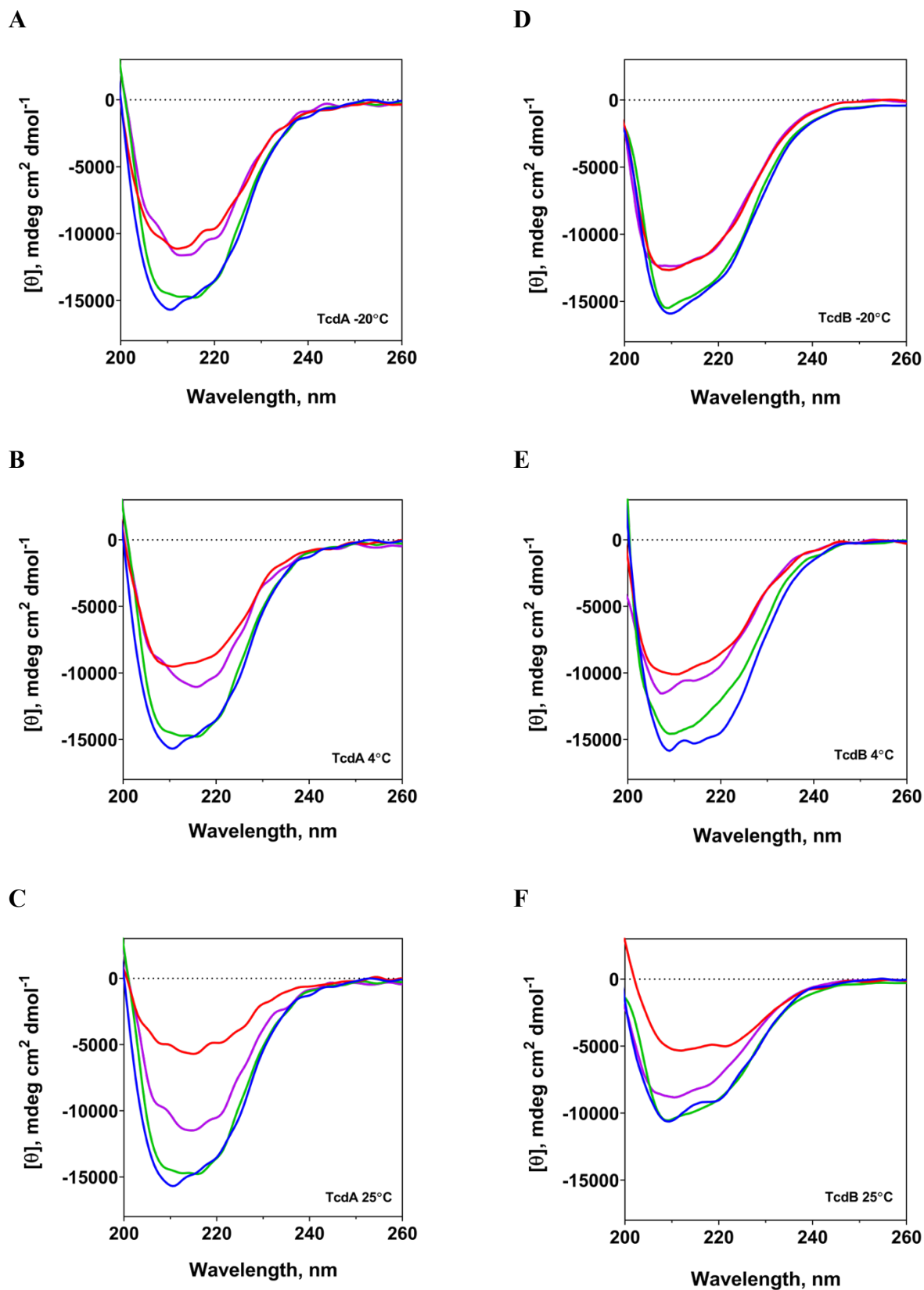


Figure 6. Long-term stability of native and MCO-detoxified TcdA and TcdB

Samples were analyzed using far-UV CD (200 – 260 nm) on day 0 and again on either day 26 or 28 after storage at different temperatures. **A:** 0.6  $\mu$ M TcdA stored at -20 °C. **B:** 0.6  $\mu$ M TcdA stored at 4 °C. **C:** 0.6  $\mu$ M TcdA stored at 25 °C. Blue: native TcdA day 0, red: native TcdA day 28, green: MCO-detoxified TcdA day 0, purple: MCO-detoxified TcdA day 28. **D:** 0.7  $\mu$ M TcdB stored at -20 °C. **E:** 0.7  $\mu$ M TcdB stored at 4 °C. **F:** 0.7  $\mu$ M TcdB stored at 25 °C. Blue: native TcdB day 0, red: native TcdB day 26/28, green: MCO-detoxified TcdB day 0, purple: MCO-detoxified TcdB day 26/28.

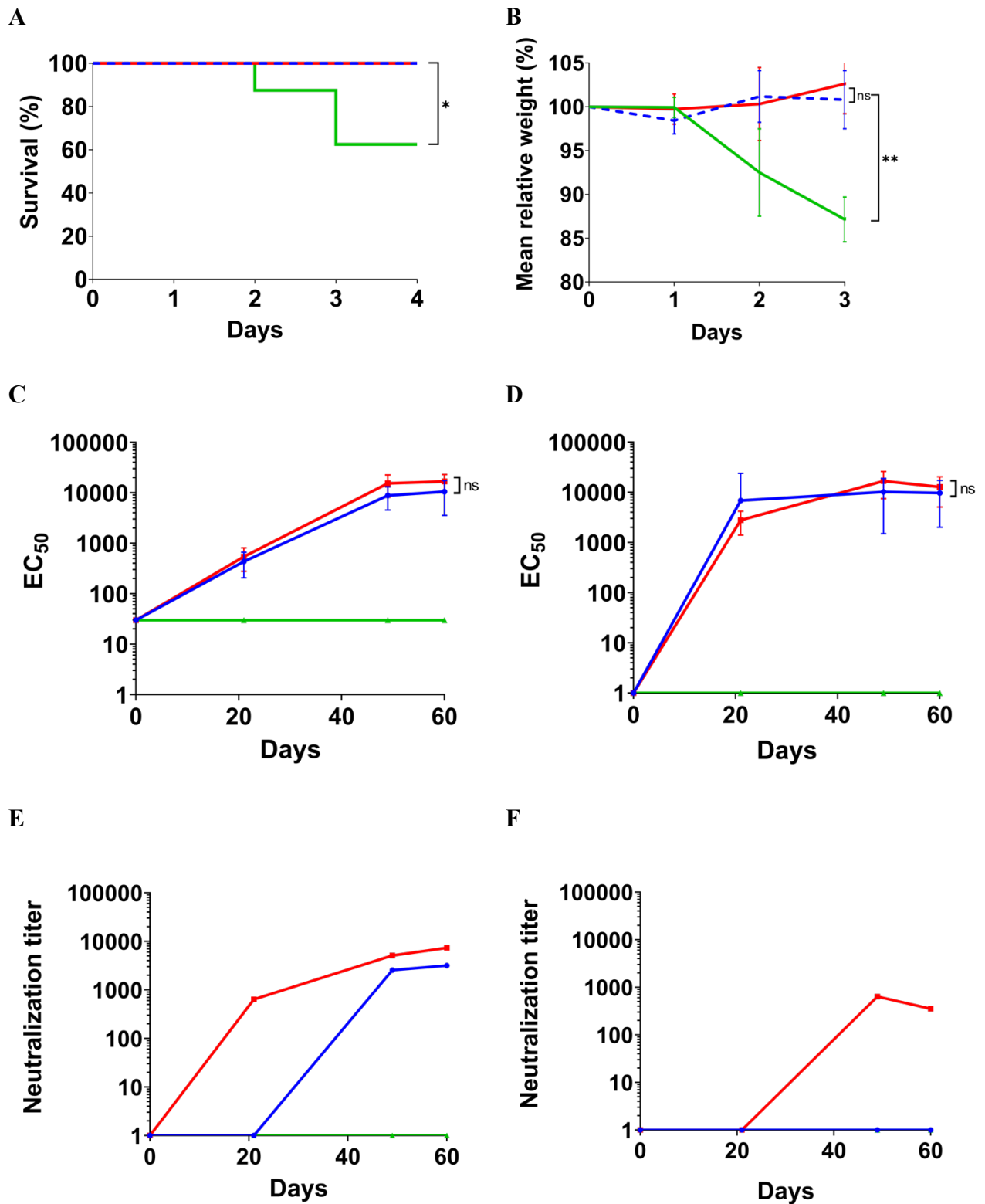
### 3.8 Immunogenicity and protective efficacy

Both vaccinated groups showed no visible side effects from the vaccine injections. No swelling around the injection site and no change in either weight or temperature. The MCO-detoxified vaccine and the formaldehyde-detoxified vaccine both fully protected against the oral challenge given on day 56, and all mice survived (Fig. 7A) while showing no signs of CDI disease symptoms such as diarrhea or weight loss (Fig. 7B). On the other hand, the unvaccinated control mice all exhibited CDI symptoms and three out of the eight mice were moribund and had to be euthanized within 3 days post-challenge.

To assess the development of toxin-specific IgG and neutralizing antibody responses in mice, sera samples were collected on days 0, 21, 49 and 60 after the primary immunization, and analyzed for levels of antibodies against native TcdA and TcdB, respectively, by ELISA and TNA. Immunization with both MCO- and formaldehyde-detoxified vaccine formulation elicited substantial anti-TcdA and anti-TcdB IgG responses, whereas control mice with mock injections had no detectable levels of antibodies in their sera (Fig. 7C and 7D). At day 60, mice immunized with the MCO-detoxified vaccine had mean anti-TcdA and anti-TcdB EC<sub>50</sub> titers of around 4 log<sub>10</sub>, whereas the formaldehyde-detoxified vaccine-elicited mean anti-TcdA and anti-TcdB EC<sub>50</sub> titers of around 4.2 log<sub>10</sub> and 4.17 log<sub>10</sub> respectively. There is no significant statistical difference between the mean anti-TcdA and TcdB EC<sub>50</sub> titers induced by the MCO- and formaldehyde-detoxified vaccines, respectively. The same serum samples were tested for toxin neutralizing activity on Vero cells, where

486 native TcdA or TcdB were pre-incubated with serial dilutions of pooled sera for 90 min and added to  
487 cells. The MCO- detoxified vaccine was less efficient at eliciting neutralizing antibodies against  
488 TcdA compared to the formaldehyde-detoxified vaccine, with mean anti-TcdA neutralization titers at  
489 day 60 of around 3100 and 7300 respectively (Fig. 7E). We could not detect any anti-TcdB  
490 neutralizing antibodies in the sera of mice immunized with the MCO-detoxified vaccine, whereas the  
491 formaldehyde-detoxified vaccine was able to elicit a low mean anti-TcdB neutralization titer of  
492 around 600 at day 49 (Fig. 7F).

493



**Figure 7. Survival and antibody response of vaccinated mice**

Mice were immunized with either MCO-detoxified vaccine (blue), formaldehyde-treated vaccine (red) or an adjuvant control (green) before being challenged with *C. difficile* (n = 8 for all groups). Sera from days 0, 21, 49 and 60 were tested for anti-TcdA and anti-TcdB IgG titers by ELISA and neutralizing antibodies by TNA. For ELISA, a four-

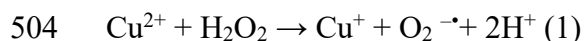
parameter logistic curve was fitted to each serum sample by plotting the absorbance at 450 nm as a function of the serum dilution. **A:** Kaplan-Meier survival curve, with day 0 representing the day of *C. difficile* challenge. Statistical analysis of survival curves was performed using Mantel-Cox log-rank test ( $p = 0.034$ ). **B:** Mean relative weight graph, where the relative weight of each mouse is based on its weight on the day of the challenge. Unpaired Student's t-test was used to compared weight curves. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , ns = no significant difference. **C:** Anti-TcdA IgG titers are shown as  $EC_{50}$  values, representing the serum dilution where the anti-TcdA response is reduced by 50%. Unpaired Student's t-test was used to compare  $EC_{50}$  values at day 60 ( $p = 0.079$ ). **D:** Anti-TcdB IgG titers are shown as  $EC_{50}$  values, representing the serum dilution where the anti-TcdB response is reduced by 50%. Unpaired Student's t-test was used to compare  $EC_{50}$  values at day 60 ( $p = 0.44$ ). **E:** Pooled sera were tested for anti-TcdA neutralization titers, which represents the highest dilution of sera where there is at least 50% cell survival after 48 h of adding the toxin-sera mixture. **F:** Pooled samples were tested for anti-TcdB neutralization titers, which represents the highest dilution of sera where there is at least 50% cell survival after 48 h of adding the toxin-sera mixture.

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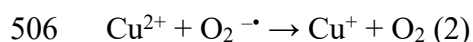
## 495 4. Discussion

496 In this study, a mild MCO condition is used as an efficient method to detoxify TcdA and TcdB without  
 497 altering structural epitopes. MCO detoxification of TcdA and TcdB most likely occurs when metal  
 498 ions, typically  $Cu^{2+}$ ,  $Fe^{2+}$  or  $Fe^{3+}$ , interact with exposed functional sites on native TcdA and TcdB.  
 499 The oxidizing species produced by reaction of copper with  $H_2O_2$  remains contentious. However, in  
 500 our previous studies [45,46] we have shown that  $Cu^{2+}$  and  $H_2O_2$  mediate radical production and could  
 501 lead to alterations in structure and function of the target proteins. The mechanism for the radical  
 502 production in the presence of  $Cu^{2+}$  and in the absence of any reductant is suggested to be as following:

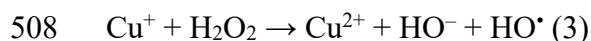
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507



509

510 Hence the reduction of  $Cu^{2+}$  to  $Cu^+$  can take place by either  $H_2O_2$  (reaction 1) or by superoxide  
 511 radical anions (reaction 2). Furthermore, the reduced  $Cu^+$  (cuprous ions) could initiate a Fenton-like  
 512 reaction with surplus  $H_2O_2$  (reaction 3). This reaction could generate  $Cu^{2+}$ - $HO^{\cdot}$  or its ionized

equivalent,  $\text{Cu}^{2+}\text{-O}^\bullet$ , as suggested by [54]. Since we have not examined the type of radical produced by the  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  reaction in this study, it is possible that other active species might be produced and involved in the radical mediated reactions [55]. Hydroxyl radicals and other active species will react almost instantaneously with amino acid side-chains near the interaction site. For this reason, the MCO can be very protein specific depending on specific metal ion interactions sites in the protein as we have seen for TcdA and TcdB in this study.

#### 4.1 Temperature-induced unfolding of TcdA and TcdB

The temperature study was performed to determine the structural changes in the toxins during increasing temperatures, and thereby determine the highest temperature we could use during the inactivation reaction without altering their structure. The far-UV CD spectroscopy during increasing temperatures show the presence of the characteristic double minima at 208 and 218 nm for TcdA (Fig. 2A) and TcdB (Fig. 2B) at 25 °C and 37°C, indicating that the toxins possess a well-defined secondary structure. The observed far-UV CD spectra can be explained by the diverse multi-domain structure of the toxins, where each domain has a unique contribution [56,57]. The diverse secondary structure composition is also confirmed from the crystal structures of TcdA [58] and TcdB [59]. The effect of heating on the secondary structure of TcdA and TcdB is very different. Even though both toxins are losing their characteristic and well-defined far-UV CD spectra at temperatures higher than 45 °C, the progression of this change is different. The spectral curve for TcdA displays a progressing decrease in molar ellipticity during heating (Fig. 2A), indicating a shift towards more disordered structure and unfolding of the  $\alpha$ -helical and  $\beta$ -sheet structures [57,60]. In contrast, the spectral curve for TcdB increases in molar ellipticity and instead progresses into a sharper negative minimum at 216 nm during heating (Fig. 2B), which could indicate an increase in  $\beta$ -sheet content [56]. These results



suggest that thermal denaturation of TcdA and TcdB have different unfolding patterns, in which TcdA unfolds and loses both its  $\alpha$ -helical and  $\beta$ -sheet structure, whereas TcdB loses  $\alpha$ -helical structure and gains a significant amount of new  $\beta$ -sheet structure. This indicates that TcdB likely aggregates at higher temperatures leading to intermolecular  $\beta$ -sheet interactions as shown for TcdB in a previous study [57] and also for other proteins [61–63]. Furthermore, the effects of thermal denaturation on TcdA and TcdB is an irreversible process, as cooling the toxins from 50 °C back to 25 °C had no effect on restoring their native-like CD spectra, which has also been reported previously by another group [57]. Temperatures between 25 °C and 37 °C were concluded to be favorable for detoxification studies, as the toxins were both structurally unmodified until at least 37 °C, while at 45 °C changes in their secondary structures started to occur.

546

#### 4.2 TcdB is more sensitive to acidic pH changes than TcdA

The effect of pH on the thermostability of both toxins were tested by DSF analysis and suggests that the two toxins have quite different pH sensitivity. TcdA is slightly more heat resistant than TcdB at neutral pH with a  $T_m$  of 51.5 °C compared to 49 °C for TcdB. Furthermore,  $T_m$  for TcdA in the native folded state is up to 50 °C in the pH range between 5 and 7.5 (Fig. 3A), whereas TcdB in the native folded state is up to 47 °C only between pH 6 and 7.5 (Fig. 3B). These values are consistent with previous studies where Tam *et al.* [64] found a  $T_m$  of 49 °C for TcdB and Salnikova *et al.* [57] found  $T_m$  values of 52 °C and 47 °C for TcdA and TcdB respectively. However, in acidic conditions at pH 4.5, there is a dramatic difference between the folded state of the toxins. TcdA is partially unfolded around 47 °C, and TcdB is partially unfolded at only 32 °C. This is physiologically relevant, as pH 4.5 mimics the environment in the endosomes, and it is known that structural changes occur for both TcdA [65] and TcdB [59,66] when the pH environment in the endosome is lowered. The  $T_m$

changes we see at pH 4.5 in the DSF analysis is, therefore, a result of the toxins' natural response to acidification, where a structural change occurs to activate translocation across the endosomal membrane [66–68]. TcdB seems to be much more affected by the acidic conditions, as it partially unfolds at a much lower temperature than TcdA. At pH 4, the high initial fluorescence signal and lack of transition phase in the DSF analysis of TcdB, suggests that the toxin is likely unfolded and/or aggregated at lower temperatures [48]. This confirms that acidic conditions (pH 4 and 4.5) have a significant effect on the unfolding behavior of both toxins, with TcdB being more sensitive to low pH than TcdA.

567

#### 568 **4.3 Acidification induced open conformation and Cu<sup>2+</sup> catalyzed oxidation of TcdA and TcdB**

It is our hypothesis that partially exposed toxic regions of the TcdA and TcdB would allow maximum accessibility to the produced ROS and thereby inactivation of TcdA and TcdB. Several strategies (see Materials and Methods) to unfold and inactivate were investigated. However, at neutral pH we did not observe any significant reduction in cytotoxicity albeit loss of protein band intensity on SDS-PAGE was often observed. Thus, we turned our focus on the inherent structural plasticity of the toxins at acidic pH. Our results from the CD (Fig. 2A and 2B) and DSF (Fig. 3A and 3B) in combination with known literature [59,65–68], led us to hypothesize that mimicking the natural environment of the toxins during their cytotoxic mode of action, might facilitate exposure of critical residues necessary for effective oxidative detoxification. Indeed, by lowering the pH to 4.5, we could successfully detoxify both TcdA and TcdB using MCO with Cu<sup>2+</sup> (Supplementary Fig. S6 and S7). Subsequent control experiments where each component of the MCO reaction, such as H<sub>2</sub>O<sub>2</sub>, Cu<sup>2+</sup> and pH 4.5 were tested separately, confirmed that detoxification only occurred when a combination of the three components was used. Low pH alone only had a small effect on the

582 cytotoxicity of TcdA and TcdB with reductions of around 50-fold and 350-fold respectively  
 583 (Supplementary Fig. S2 and S3). This is in alignment with our DSF analysis (Fig. 3A and 3B)  
 584 showing a partial unfolding of the toxins at pH 4.5. However, 50-fold and 350-fold reductions in  
 585 cytotoxicity are insufficient for using the toxoids as safe vaccine antigens, as they are still extremely  
 586 cytotoxic. The efficacy of our novel approach was only seen when  $\text{Cu}^{2+}$  was used in the MCO  
 587 system, as the usual Fenton catalysts,  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  in the same system could only reduce the  
 588 cytotoxicity by 50-fold and 7-fold respectively (Table 2, Supplementary Fig. S5). These differences  
 589 are mainly attributed to  $\text{Cu}^{2+}$  being a more efficient ROS-generating metal ion compared to  $\text{Fe}^{3+}$  and  
 590 less to the ability of  $\text{Cu}^{2+}$  to bind non-specifically to proteins [69–71]. TcdA and TcdB are known to  
 591 bind metal ions, both having a specific  $\text{Zn}^{2+}$  binding site in the autoprotease domain (APD), a  
 592 requirement for autoprocessing [65].  $\text{Mn}^{2+}$  has also been demonstrated to bind TcdA in the catalytic  
 593 core of the glucosyltransferase domain (GTD), which consist of a classical Rossman fold [72,73].  
 594 Similarly, TcdB and the closely related *C. sordellii* Lethal Toxin (TcsL) have both been shown to  
 595 have specific binding requirements for  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Mg}^{2+}$ , necessary for UDP-glucose hydrolysis  
 596 and activating their cytotoxic effects [74]. However, in the same study,  $\text{Cu}^{2+}$  was shown to be the  
 597 least effective metal ion for cytotoxic activation of both TcdB and TcsL among a range of tested  
 598 divalent metal ions. In summary, there are no reports of TcdA and TcdB having specific  $\text{Cu}^{2+}$ -  
 599 binding sites, and the likely reason for the  $\text{Cu}^{2+}$ -specific effects in our MCO system is due to the  
 600 much higher capacity of  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  systems to generate ROS and promote oxidative damage to the  
 601 toxins.

602 Finally, we also determined that the molar ratios of 1:60:1000 for TcdA/B: $\text{Cu}^{2+}$ : $\text{H}_2\text{O}_2$   
 603 respectively, were optimal for the MCO components to achieve efficient detoxification of TcdA and  
 604 TcdB. The concentration of  $\text{H}_2\text{O}_2$  in our MCO system is at least 100-fold lower than previously  
 605 reported for detoxification of Pertussis toxin [75] and whole-cell bacteria [76], and around 1000-fold

606 lower than a hydrogen peroxide-detoxified viral vaccine [77]. Thus, the method described here for  
607 detoxification of TcdA and TcdB is orders of magnitude more efficient compared to previously used  
608 oxidation methods.

#### 609 610 **4.4 Oxidatively induced structural modifications in TcdA and TcdB**

611 To assess the structural changes induced by MCO in TcdA and TcdB we conducted far-UV CD  
612 to study the secondary structure and near-UV CD to study the tertiary structure. These structural  
613 studies were complemented with ELISA studies of the epitope integrity with multiple monoclonal  
614 antibodies, to evaluate how these structural changes would affect the antibody recognition of various  
615 TcdA and TcdB epitopes. According to the far-UV CD spectra, MCO-detoxified TcdA (Fig. 4A) and  
616 MCO-detoxified TcdB (Fig. 5A) both have lost the characteristic minimum at 208 nm. This indicates  
617 that the MCO detoxification modifies the  $\alpha$ -helices of TcdA and TcdB. We also see a slight loss in  
618 the magnitude of the molar ellipticity over the whole CD spectrum of TcdA, likely caused by  
619 increased disordered structure or some degree of precipitation [57,60]. TcdB does not show the same  
620 trend, instead, we see a more intense negative peak formed around 216 nm, which we also see in the  
621 thermal denaturation experiment (Fig. 2B). It should be kept in mind that the MCO-detoxified TcdA  
622 and TcdB are still at pH 4.5, and therefore the CD spectral changes might also be influenced by  
623 structural modifications due to pH alone. Interestingly, raising the pH of MCO-detoxified TcdB back  
624 to 7.5, the CD spectrum reverts and regains a native-like far-UV CD spectrum (Fig. 5A, red line).  
625 This suggests that the acidic pH and not MCO is the major contributor to the conformational change  
626 of the secondary structure of TcdB seen in the far-UV CD (Fig. 5A, blue line). Unfortunately, raising  
627 the pH of TcdA to neutral after MCO detoxification was not suitable for CD analysis, as it led to the  
628 loss of overall CD spectrum, likely caused by precipitation.

629 To further study whether it is low pH, oxidative damage or the combination of both that is the  
630 main contributing factor to the changes in secondary structure seen for TcdA, we followed the  
631 progression of the far-UV CD spectrum over time intervals of 3 min. (Supplementary Fig. S9). Two  
632 sets of experiments were run, the first with TcdA at pH 4.5 only, and the second with TcdA at pH 4.5  
633 and MCO components. We found that already in the first spectrum at 0.1 min there is a slight  
634 difference between the MCO spectrum compared to the pH 4.5 only spectrum, with the MCO  
635 spectrum showing slightly reduced signal intensity. With further incubation, the MCO spectra show  
636 a gradual loss of the characteristic minima at 208 and 218 nm, and overall lower magnitude of molar  
637 ellipticity as we also see in Fig. 4A. This progression is plateauing around 15 min after which no  
638 further changes were detected even after 2 h. The changes of the TcdA pH 4.5 CD spectrum over  
639 time (data not shown) progresses both more slowly and is less extensive compared to the MCO  
640 spectrum, suggesting that the oxidative damage caused by MCO contributes to the change in  
641 secondary structure. Lastly, we followed the near-UV CD spectrum of MCO-detoxified TcdA over  
642 time with measurements made every 3 min during the MCO reaction. It is clear that some structural  
643 changes occur in the first 6 min, with a change of both minima at 275 and 282 nm (Supplementary  
644 Fig. S10). The decrease in the near-UV CD spectral intensity in the 275 and 282 nm region for MCO-  
645 detoxified TcdA and TcdB, suggest that tyrosine/tryptophan residues are affected by the MCO (Fig.  
646 4B and 5B). The features in this region, however, remain intact, which indicates that the modifications  
647 of the aromatic residue environment are not severe. However, the quality of the near-UV CD spectra  
648 makes it difficult to draw any definite conclusions. Nevertheless, from the CD data we can conclude  
649 that the majority of structural events caused by MCO are happening within the first 15 min of the  
650 reaction, as further incubation only showed insignificant changes in the CD spectra. Interestingly,  
651 neither TcdA nor TcdB showed sufficient reduction of cytotoxicity after 15 min of MCO. Even after  
652 30 min of MCO we only saw around a 400-fold reduction in cytotoxicity of TcdA (Supplementary

Fig. S1). To test whether the oxidative damage from 30 min of MCO treatment would continue to cause structural modifications over time even after the MCO reaction was quenched, we stored the sample for 48 h at 5 °C and re-tested for cytotoxicity. The level of cytotoxicity was identical to the level seen immediately after the 30 min MCO, indicating that no further structural modifications happen after the MCO reaction is quenched. A likely explanation could be that after the initial oxidation during the first 15 min, seen on both far-UV and near-UV CD (Supplementary Fig. S9 and S10), there are probably minor secondary events occurring which we cannot visually follow by CD. These secondary events are not causing significant structural changes and therefore are not visible in the CD analysis.

Epitope recognition studies of TcdA and TcdB with monoclonal antibodies show that the MCO treatment is not causing detrimental modifications of the epitopes. The mAb binding to MCO-detoxified TcdA and TcdB were on average reduced around 2-fold, relative to native TcdA and TcdB. However, when comparing to formaldehyde detoxification, we see a reduction of mAb binding of around 5-fold and 3-fold for TcdA and TcdB, respectively. These results support that our novel MCO system is more epitope conserving to TcdA and TcdB compared to conventional formaldehyde treatment. That oxidation-based detoxification is more epitope conserving than formaldehyde is in line with what was previously shown for Pertussis toxin [38].

#### **4.5 Toxoids are stable and irreversibly detoxified**

The stability and irreversibility of MCO-detoxified TcdA and TcdB were studied by far-UV CD analysis and cytotoxicity testing after 4 weeks of storage at either -20 °C, 4 °C or 25 °C. At all storage conditions, there is a decrease in CD spectral intensity for both native and MCO-detoxified TcdA and TcdB after storage (Fig. 3A-F). This is likely due to protein precipitation during storage,

as the same is seen for the native toxin samples and therefore cannot be attributed to MCO. The shape of the CD spectra for MCO-detoxified TcdA and TcdB is not changed during the storage at any temperature when compared to its corresponding CD spectra at day 0, suggesting that no further structural modifications are happening during long-term storage. Interestingly, it seems that during storage at room temperature (25 °C), the MCO-detoxified samples of both TcdA and TcdB are more resistant to precipitation compared to the native toxins (Fig. 3C and 3F). By contrast, we see the same degree of precipitation for the toxins before and after MCO at -20 °C and 4 °C. Importantly, we see no reversion of cytotoxicity after 4 weeks of storage at any temperature, whereas reversion of cytotoxicity is a well-known issue for formaldehyde-detoxified toxins [33,34].

#### 4.6 Efficacy of oxidatively modified TcdA and TcdB vaccine in mice

Our bivalent vaccine consisting of MCO-detoxified TcdA and TcdB was compared to a similar vaccine consisting of formaldehyde-detoxified toxins, by assessing their ability to protect immunized mice against a lethal *C. difficile* oral challenge and eliciting toxin-specific antibodies. Both vaccines were able to fully protect all mice against the infection (Fig. 7A) as well as concomitant disease symptoms such as diarrhea and weight loss (Fig. 7B). All unvaccinated mice developed disease symptoms and almost 40% were moribund by day 3 and were euthanized. Hence, the efficacy of the MCO vaccine was sufficient and comparable to the formaldehyde vaccine in protecting all mice from disease symptoms. Induction of serum IgG against native TcdA and TcdB was measured by ELISA (Fig. 7C and 7D) with no statistically significant differences between the mean IgG responses elicited by the MCO-detoxified or formaldehyde-detoxified vaccine, suggesting that the two methods are comparable with regards to immunogenicity. However, mice immunized with the MCO-detoxified vaccine had lower serum levels of toxin-neutralizing antibodies compared to the group receiving the

699 formaldehyde-detoxified vaccine. The mean anti-TcdA neutralizing antibody titer in the MCO  
700 vaccine group was only around half of the formaldehyde vaccine group (Fig. 7E) and we failed to  
701 detect anti-TcdB neutralizing antibodies in the MCO vaccine group, while low levels of neutralizing  
702 activity against TcdB was detected in the sera from the formaldehyde vaccine group (Fig. 7F). The  
703 results suggest that the MCO-detoxified vaccine is slightly less efficient in eliciting neutralizing  
704 antibodies against TcdA and TcdB compared to the formaldehyde-detoxified vaccine.

705         Neutralizing antibodies are crucial in CDI prevention, as they recognize and bind key epitopes  
706 on the toxins that prevent them from entering the host cells and causing disease symptoms [78–81].  
707 Our epitope recognition studies showed significantly higher binding of various mAbs to the MCO-  
708 detoxified toxins compared to formaldehyde-detoxified ones, indicating that the epitopes were closer  
709 to the native state (Table 3 and 4). This is supported by previous studies showing that oxidation-based  
710 detoxification of toxins, bacteria and viruses are significantly more epitope-conserving than  
711 formaldehyde [38,76,77]. We, therefore, believe that this issue is caused by some other factor(s) than  
712 direct modifications of key epitopes by MCO, and further optimization of our MCO-detoxification  
713 method is likely needed. Neutralizing antibodies against TcdB have been shown in several hamster  
714 studies to develop much more slowly than for TcdA, and require up to four immunizations over three  
715 months to reach the same levels [20,21,82]. The difficulty of stimulating anti-TcdB neutralizing  
716 antibodies, in general, might have contributed to the lower levels of neutralizing TcdB antibodies we  
717 detected, which were also very low in the formaldehyde vaccine group.

718         In conclusion, using mild  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ -catalyzed oxidation in combination with pH-dependent  
719 structural modulation we demonstrate efficient detoxification of TcdA and TcdB. The detoxification  
720 resulted in a significant reduction in toxicity yet maintaining the toxoids of TcdA and TcdB  
721 structurally preserved. Furthermore, our method resulted in the development of immunogenic toxoids  
722 highly recognizable by an array of monoclonal antibodies against TcdA and TcdB and capable of



723 protecting mice against CDI. Thus, the method may very well be suitable for the creation of safe  
724 toxoid-based antigens and a potential replacement for formaldehyde detoxification in future vaccine  
725 development.

## 726 **Author Contributions**

727 **AA** designed, executed, supervised and participated in all experiments, collected and analyzed all  
728 data, performed toxin purification, CD, DSF, MCO and epitope recognition experiments (Figs. 1 - 7  
729 and Tables 1 - 4), made all the figures and tables and wrote the manuscript. **MKT** designed, executed  
730 and supervised the MCO experiments (Table 2) and CD studies (Fig. 4 and 5), participated in making  
731 Fig. 1, revised and contributed intellectually to the manuscript. **SSMM** designed, performed and  
732 collected data for the stability experiments (Fig. 6). **SJN** designed the animal study and performed  
733 serum ELISA and TNA studies and collected data for Fig. 7. **ABH** contributed to the development of  
734 the methods used in the production and purification of TcdA and TcdB. **IMM** contributed with  
735 helpful intellectual suggestions at a number of meetings and by reading and editing the manuscript.  
736 **KAK** contributed by helping with ELISA studies and *in vivo* experiments and has revised and  
737 contributed to the manuscript with intellectual content and final revision. **MJB** conceived the idea of  
738 using copper ions for the metal-catalyzed oxidation and designed, supervised and funded the MCO  
739 and CD experiments, revised and contributed to the manuscript with intellectual content. **RJ**  
740 conceived the idea for oxidation-based detoxification of TcdA and TcdB, funded and administered  
741 the study, designed and supervised all experiments, revised and contributed intellectually to the  
742 manuscript. All authors have read and approved the final version to be published.

743

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750

751 **Declarations of interest**

752 We declare no conflict of interest

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983 **Supplementary Information**

984 **Detoxification of Toxin A and Toxin B by copper ion-catalyzed**  
985 **oxidation in production of a toxoid-based vaccine against *Clostridioides***  
986 ***difficile***

987

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1002

1003   **Contents**

1004   **Fig. S1.** *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO using with varying incubation

1005   times.

1006   **Fig. S2.** *In vitro* cytotoxicity on Vero cells of MCO control samples with TcdA.

1007   **Fig. S3.** *In vitro* cytotoxicity on Vero cells of MCO control samples with TcdB.

1008   **Fig. S4.** *C. difficile* toxin yield in various growth media tested on Vero cells.

1009   **Fig. S5.** *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO using with different metal

1010   ions.

1011   **Fig. S6.** *In vitro* cytotoxicity on Vero cells of native and MCO-detoxified TcdA.

1012   **Fig. S7.** *In vitro* cytotoxicity on Vero cells of native and MCO-detoxified TcdB.

1013   **Fig. S8.** *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO at varying temperatures.

1014   **Fig. S9.** Far-UV circular dichroism analysis of the structural effect of MCO detoxification on TcdA.

1015   **Fig. S10.** Near-UV circular dichroism analysis of the structural effect of MCO detoxification on

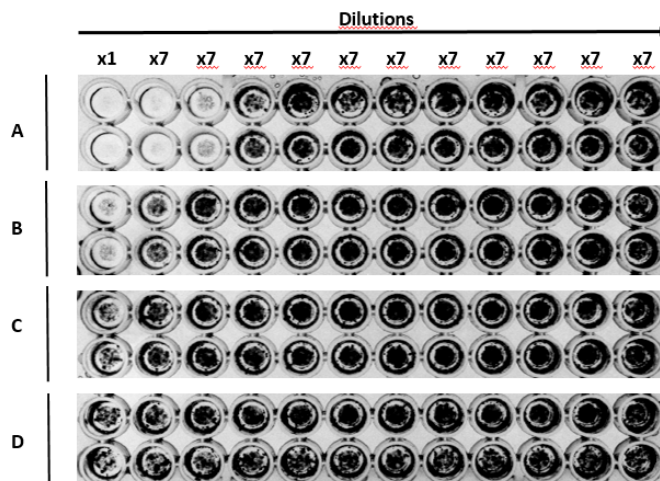
1016   TcdA.

1017   **Table S1.** Molar ratios and experimental conditions used for the MCO pilot study of TcdA.

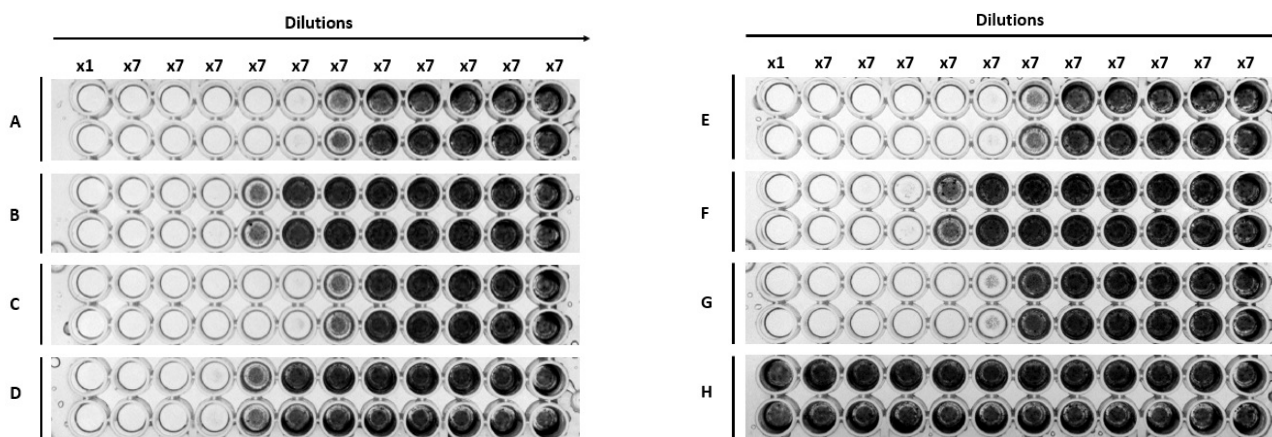
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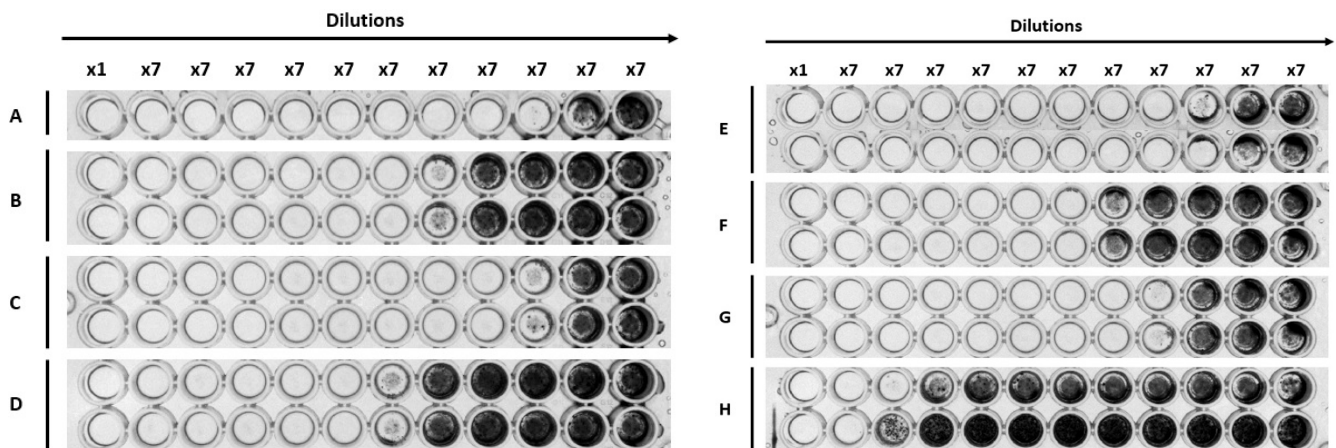


**Fig. S1. *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO using different incubation times.** Microtiter plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival (black wells). All samples were tested as duplicates and consisted of 2  $\mu$ g MCO-detoxified TcdA added to the first column and serially diluted horizontally. All samples were incubated at 37°C. **A)** 30 min **B)** 60 min **C)** 90 min **D)** 120 min.

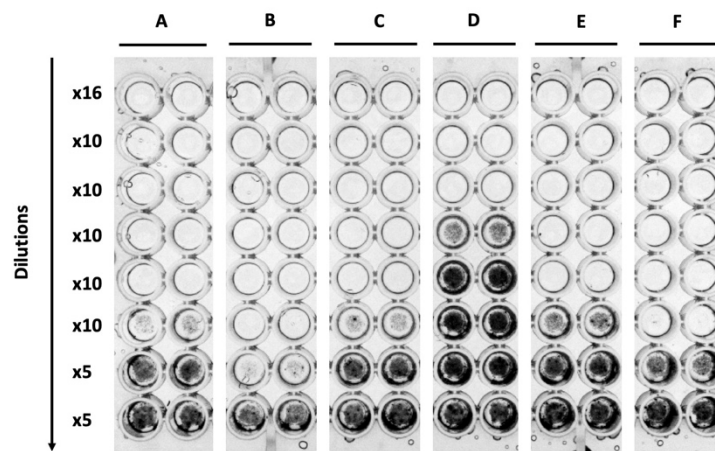


**Fig. S2. *In vitro* cytotoxicity on Vero cells of MCO control samples with TcdA.** Plates were stained as described in Fig S1. All samples were tested as duplicates and consisted of 1.9  $\mu$ g TcdA added to first column and serially diluted horizontally. All samples were incubated for 2 h at 37 °C. **A)** Native TcdA in pH 7.5 **B)** Native TcdA in pH 4.5 **C)** TcdA + H<sub>2</sub>O<sub>2</sub> in pH 7.5 **D)** TcdA + H<sub>2</sub>O<sub>2</sub> in pH 4.5 **E)** TcdA + CuCl<sub>2</sub> in pH 7.5 **F)** TcdA + CuCl<sub>2</sub> in pH 4.5 **G)** TcdA + H<sub>2</sub>O<sub>2</sub> + CuCl<sub>2</sub> in pH 7.5 (MCO) **H)** TcdA + H<sub>2</sub>O<sub>2</sub> + CuCl<sub>2</sub> in pH 4.5 (MCO).

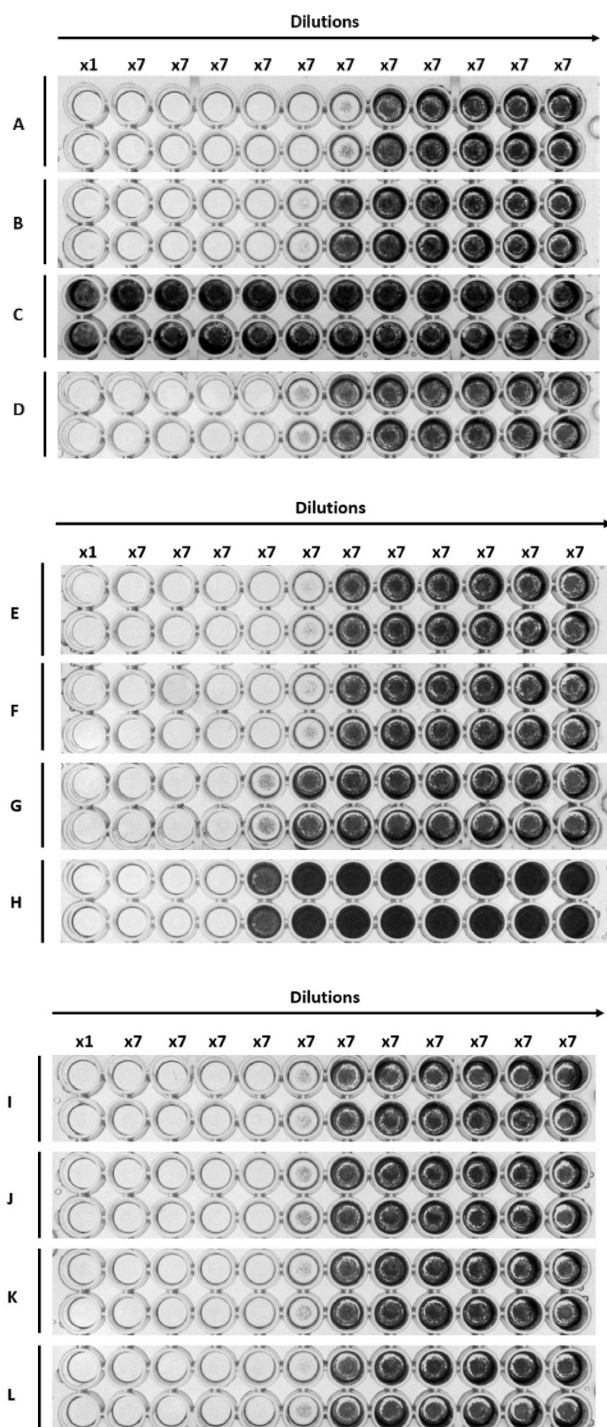




**Fig. S3. *In vitro* cytotoxicity on Vero cells of MCO control samples with TcdB.** Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 1.75 µg TcdB added to first column and serially diluted horizontally. All samples were incubated for 2 h at 37 °C. **A)** Native TcdB in pH 7.5 **B)** Native TcdB in pH 4.5 **C)** TcdB + H<sub>2</sub>O<sub>2</sub> in pH 7.5 **D)** TcdB + H<sub>2</sub>O<sub>2</sub> in pH 4.5 **E)** TcdB + CuCl<sub>2</sub> in pH 7.5 **F)** TcdB + CuCl<sub>2</sub> in pH 4.5 **G)** TcdB + H<sub>2</sub>O<sub>2</sub> + CuCl<sub>2</sub> in pH 7.5 (MCO) **H)** TcdB + H<sub>2</sub>O<sub>2</sub> + CuCl<sub>2</sub> in pH 4.5 (MCO).



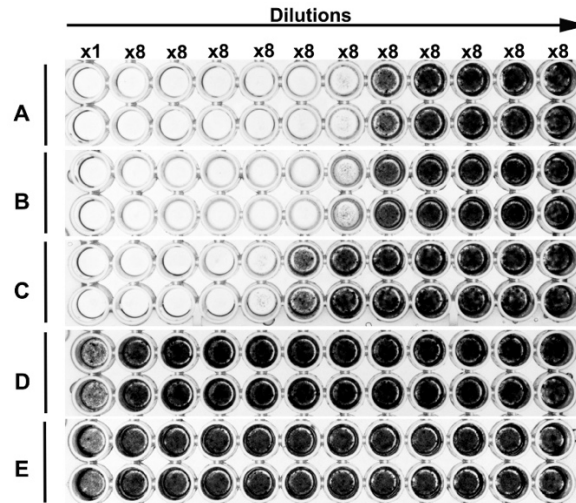
**Fig. S4. *C. difficile* toxin yield in various growth media tested on Vero cells.** Samples were taken from each culture after 48 hours of growth, centrifuged and filtered. Ten µL was added to the first row (x16) and serially diluted vertically. Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 20 g/L yeast extract, 1 g/L sodium thioglycolate and 30 g/L of either **A)** NZ-Soy **B)** NZ-Soy BL4 **C)** NZ-Soy BL7 **D)** Phytone peptone **E)** BHI **F)** Tryptone.



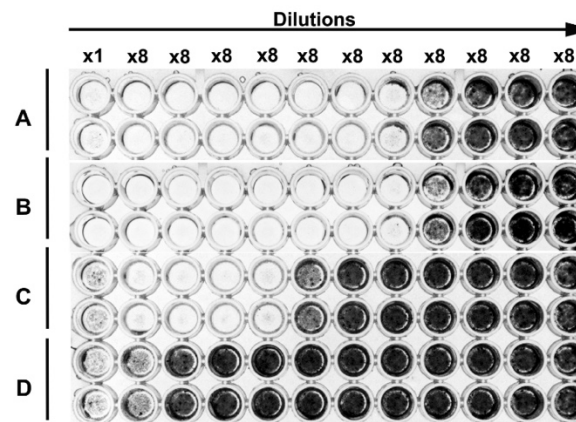
**Fig. S5. *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO using different metal ions.** Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 3.2  $\mu$ g MCO-detoxified TcdA added to first column, and serially diluted horizontally. All samples were incubated for 2 h at 37 °C prior to cytotoxicity testing. All MCO samples were in pH 4.5. **A)** TcdA in pH 7.5 **B)** TcdA in pH 4.5 **C)** TcdA MCO + CuCl<sub>2</sub> **D)** TcdA MCO + MgCl<sub>2</sub> **E)** TcdA MCO + CoCl<sub>2</sub> **F)** TcdA MCO + MnCl<sub>2</sub> **G)** TcdA MCO + Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> **H)** TcdA MCO + FeSO<sub>4</sub> **I)** TcdA MCO + CaCl<sub>2</sub> **J)** TcdA MCO + LiCl **K)** TcdA MCO + NiCl<sub>2</sub> **L)** TcdA MCO + AgNO<sub>3</sub>.

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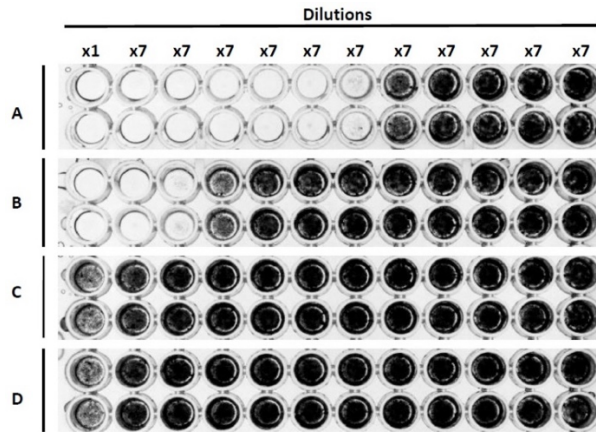
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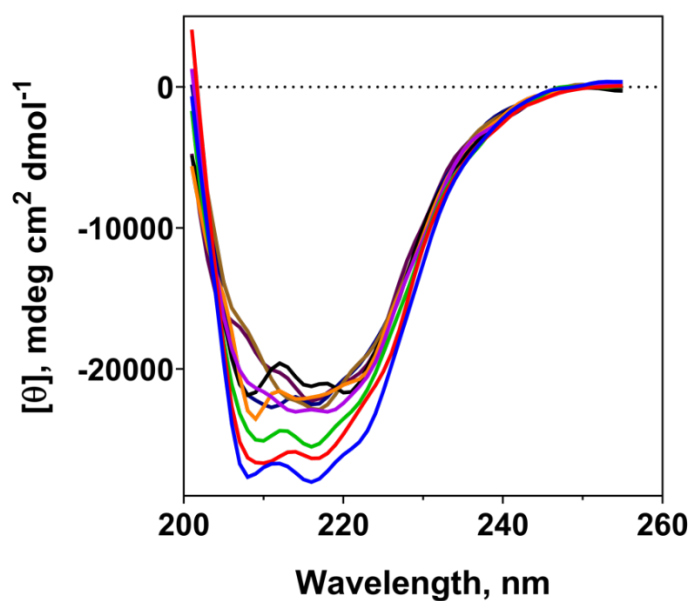
**Fig. S6. *In vitro* cytotoxicity on Vero cells of native and MCO-detoxified TcdA.** Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 3.7  $\mu$ g native or MCO-detoxified TcdA added to the first column and serially diluted horizontally. All samples were incubated for 2 h at 37 °C. **A)** Native TcdA in pH 7.5 **B)** MCO-detoxified TcdA in pH 7.5 **C)** Native TcdA in pH 4.5 **D)** MCO-detoxified TcdA in pH 4.5 **E)** MCO-detoxified TcdA in pH 4.5 re-adjusted to pH 7.5.



**Fig. S7. *In vitro* cytotoxicity on Vero cells of native and MCO-detoxified TcdB.** Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 2.2  $\mu$ g native or MCO-detoxified TcdB added to the first column and serially diluted horizontally. All samples were incubated for 2 h at 37 °C. **A)** Native TcdB in pH 7.5 **B)** MCO-detoxified TcdB in pH 7.5, **C)** Native TcdB in pH 4.5 **D)** MCO-detoxified TcdB in pH 4.5 re-adjusted to pH 7.5.



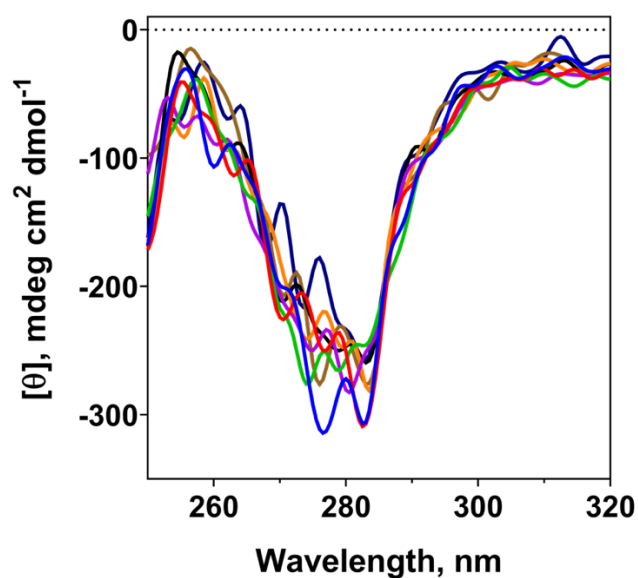
**Fig. S8. *In vitro* cytotoxicity on Vero cells of TcdA detoxified by MCO at varying temperatures.** Plates were stained with crystal violet after 48 h incubation and photographed to visualize cell survival in each well with black stain. All samples were tested as duplicates and consisted of 3.2  $\mu$ g TcdA added to the first column and serially diluted horizontally. All samples were incubated for 90 min. **A)** Native TcdA in pH 7.5, **B)** MCO-detoxified TcdA 25 °C, **C)** MCO-detoxified TcdA 30 °C, **D)** MCO-detoxified TcdA 37 °C.



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1083 **Fig. S9. Far-UV circular dichroism analysis of the structural effect of MCO detoxification on TcdA.** All samples  
 1084 are shown as an average of duplicate measurements with the buffer spectrum (blank) subtracted. The sample consisted of  
 1085 0.65  $\mu\text{M}$  TcdA. Far-UV spectra ranging from 200 – 255 nm. Blue: Native TcdA pH 4.5 0.1 min, red: TcdA MCO 0.1  
 1086 min, green: TcdA MCO 3 min, purple: TcdA MCO 6 min, orange: TcdA MCO 9 min, black: TcdA MCO 12 min, brown:  
 1087 TcdA MCO 15 min, dark blue: TcdA MCO 18 min, magenta: TcdA MCO 21 min.

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**Fig. S10. Near-UV circular dichroism analysis of the structural effect of MCO detoxification on TcdA.** All samples are shown as an average of duplicate measurements with the buffer spectrum (blank) subtracted. Sample consisted of 3.25  $\mu$ M TcdA. Near-UV spectra ranging from 250 – 320 nm. Blue: TcdA MCO 0.1 min, red: TcdA MCO 3 min, green: TcdA MCO 6 min, purple: TcdA MCO 9 min, orange: TcdA MCO 12 min, black: TcdA MCO 15 min, brown: TcdA MCO 18 min, dark blue: TcdA MCO 21 min.

1096 **Table S1. Molar ratios and experimental conditions used for the MCO pilot study on TcdA.**

Samples	Final concentrations in reaction mixture (µM)			Molar ratios	pH
	TcdA	CuCl <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	(TcdA:Cu <sup>2+</sup> :H <sub>2</sub> O <sub>2</sub> )	(tested at all pH)
Condition 1	0.5	15	50	1:30:100	4, 4.5, 5, 7.5*
Condition 2	0.5	15	250	1:30:500	4, 4.5, 5, 7.5
Condition 3	0.5	15	500	1:30:1000	4, 4.5, 5, 7.5
Condition 4	0.5	30	50	1:60:100	4, 4.5, 5, 7.5
Condition 5	0.5	30	500	1:60:1000	4, 4.5, 5, 7.5
Condition 6	0.5	37.5	250	1:75:500	4, 4.5, 5, 7.5
Condition 7	0.5	37.5	500	1:75:1000	4, 4.5, 5, 7.5
Condition 8	0.5	37.5	1000	1:75:2000	4, 4.5, 5, 7.5

\* = All four pH values were tested for each condition.

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