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# Protective efficacy of recombinant bacterin vaccine against botulism in cattle

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ABSTRACT

Botulism is a paralytic disease caused by the intoxication of neurotoxins produced by *Clostridium botulinum*. Among the seven immunologically distinct serotypes of neurotoxins (BoNTs A – G), serotypes C and D, or a chimeric fusion termed C/D or D/C, are responsible for animal botulism. The most effective way to prevent botulism in cattle is through vaccination; however, the commercially available vaccines produced by detoxification of native neurotoxins are time-consuming and hazardous. To overcome these drawbacks, a non-toxic recombinant vaccine was developed as an alternative. In this study, the recombinant protein vaccine was produced using an *Escherichia coli* cell-based system. The formaldehyde-inactivated *E. coli* is able to induce 7.45 ± 1.77 and 6.6 ± 1.28 IU/mL neutralizing mean titers against BoNTs C and D in cattle, respectively, determined by mouse neutralization bioassay, and was deemed protective by the Brazilian legislation. Moreover, when the levels of anti-BoNT/C and D were compared with those achieved by the recombinant purified vaccines, no significant statistical difference was observed. Cattle vaccinated with the commercial vaccine developed 1.33 and 3.33 IU/mL neutralizing mean titers against BoNT serotypes C and D, respectively. To the best of our knowledge, this study is the first report on recombinant *E. coli* bacterin vaccine against botulism. The vaccine was safe and effective in generating protective antibodies and, thus, represents an industry-friendly alternative for the prevention of cattle botulism.



## 1. Introduction

Botulism is a foodborne, life-threatening neurological intoxication caused by botulinum neurotoxins (BoNTs) mainly produced by the anaerobic spore-forming bacteria *Clostridium botulinum*. This bacterium is ubiquitous in nature and grows under optimal conditions to produce the neurotoxin [1]. Upon ingestion, BoNTs inhibit the release of acetylcholine at the neuromuscular junctions, resulting in flaccid paralysis. BoNT consists of a light chain (LC) and a heavy chain (HC) joined by a single disulfide bond. LC is the neurotoxin's catalytic domain, whereas HC comprises the translocation domain in its amino-terminal half ($H_N$) and the binding domain in its carboxy-terminal half ($H_C$) [2]. Among these protein regions, $H_C$ is the main research target and is known to contain protective antigenic properties. Of the seven serotypes immunologically distinct for BoNTs (A–G), serotypes C and D, or chimeric fusions termed/CD and/DC, are the main responsible serotypes for botulism in cattle [3,4].

Brazil is the world's largest cattle meat exporter and has the second-largest cattle herd in the world. With more than 220 million bovines, large doses of anti-botulinum vaccines are used on a yearly basis [5]. Vaccination is the most effective method to prevent the death of cattle by BoNT poisoning [6,7]. Several foodborne or waterborne botulism outbreaks have been reported in livestock with lethality rates ranging from 95 to 100% and resulting in great economic losses [7–11].

Commercial vaccines currently available for animal botulism are formalin-inactivated BoNTs. To ensure quality control, samples of commercial vaccines containing *C. botulinum* types C and D toxoids are regularly assessed by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) [12]. Although effective [13], the production of these toxoids is time-consuming and hazardous before and during detoxification. Hence, recombinant vaccines are

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used to overcome these limitations as only part of the BoNT is expressed, resulting in no toxicity, and the expression can be tightly regulated to yield high levels of antigen [14,15]. Previous studies have reported that recombinant BoNTs vaccines can effectively induce high levels of neutralizing antibodies and therefore protect against botulism in many animal species [16–18].

Our study was focused on developing and testing novel non-purified recombinant vaccines as an alternative to overcome the aforementioned drawbacks inherent to toxoid production [16,17,19]. These easily manufactured vaccines are produced by either inactivating recombinant *E. coli* BL21 (DE3) with formaldehyde (recombinant bacterin) or by obtaining recombinant *E. coli* BL21 (DE3) cell lysate supernatant after expression of the C-terminal fragments of BoNT serotypes C and D and were previously developed and evaluated in Guinea pigs [17]. We believe this to be a safe, simpler, faster, and more industry-friendly process than the BoNT detoxification process. The aim of this study was to assess whether recombinant *E. coli* bacterin is capable of inducing protective immunity in cattle and pass MAPA's quality control tests.

## 2. Materials and methods

### 2.1. Production and characterization of the recombinant proteins

Synthetic coding sequences for $h_cc$ and $h_cd$ using *E. coli* codon (Epoch life Science, Missouri, USA) were cloned into the pET28a expression vector and used to transform *E. coli* BL21 (DE3) Star™ (Thermo Scientific, Massachusetts, USA). Protein expression was induced with 0.5 mM IPTG for 16 h at 30 °C. Cells were harvested (7,000 × *g*, 15 min, 4 °C) and suspended in lysis buffer (0.2 M $NaH_2PO_4$, 0.5 M NaCl, 10 mM imidazole) with lysozyme (50 mg/mL) for 1 h under slight agitation at room temperature followed by sonication in ice bath (6 × 30 s, 60 kHz). Lysed supernatants containing soluble HcBoNTs/C and D proteins were collected by centrifugation (7,000 × *g*, 15 min, 4 °C), purified by nickel-affinity chromatography using ÄKTAprime (Amersham Biosciences, UK) and quantified using BCA kit (Thermo Scientific, Massachusetts, USA); part of the supernatant was stored unpurified. Recombinant *E. coli* bacterin was also produced as described previously with slight modifications [17]. After 16 h expression, *E. coli* cells were harvested by centrifugation (7,000 × *g*, 15 min, 4 °C), washed twice with PBS (7,000 × g, 15 min, 4 °C) and incubated with 0.2% (v/v) formaldehyde (200 RPM, 24 h, 37 °C). Residual formaldehyde was removed by washing bacteria three times with sterile PBS (7,000 × g, 15 min, 4 °C). Inactivation was confirmed by plating 100 μL of formaldehyde-treated cells on solid LB (18 h, 37 °C). The non-purified recombinant protein used in vaccine formulations was quantified by densitometry of expected band exhibited on western blot with mAb anti-His6x and compared with standard curve using TotalLab Quant™ analysis software (not showed). SDS-PAGE was performed to assess the purity and integrity of purified recombinant HcBoNTs and check whether a corresponding band is present in *E. coli* cell extract and cell lysate supernatant. Recombinant protein expression and antigenicity were confirmed by western blot with 1 IU/mL of standard anti-sera against BoNT serotypes C and D (NIBSC).

### 2.2. Vaccine formulation and safety

Vaccine formulations were prepared as previously described with minor modifications [17]. Bivalent recombinant vaccines containing 200 μg of both $H_C$BoNT/C and D per dose were formulated from the purified recombinant proteins, cell lysate supernatant or formaldehyde-inactivated *E. coli* adsorbed onto 15% Al $(OH)_3$ (4.09% w/v) for 18 h at room temperature as adjuvant, in a final volume of 5 mL. Vaccine sterility was evaluated by inoculating 0.5 mL vaccine formulation in 20 mL thioglycolate broth under anaerobic conditions and thioglycolate and Sabouraud broth under aerobic conditions at 37 °C for the first two and at 25 °C for the latter. Growth was determined daily for 21 days using spectrophotometry. Innocuity was assessed by inoculating two cows with twice the vaccine dose (400 μg) of each vaccine formulation and observing for side effects for 72 h. These protocols are as specified by the MAPA according to ministerial directive n. 23/2002 [12].

### 2.3. Cattle vaccination

About 30 18-month old Red Angus cows raised on pasture were randomly segregated into five groups of six animals each. The animals were provided with food and water *ad libitum* and were handled equally. All animals had no detectable antibody against BoNT serotypes C and D prior to the start of the experiment. Vaccines were administered on days 0 and 28; blood was taken from the jugular vein at days 0, 28, and 56, and serum samples obtained by centrifugation (3,000 × *g*, 15 min) and stored at −20 °C until use.

Groups 1, 2, and 3 were vaccinated subcutaneously with purified recombinant proteins, *E. coli* cell lysate supernatant (non-purified vaccine), or formaldehyde-inactivated *E. coli* (recombinant bacterin), respectively, in a final volume of 5 mL per dose containing 200 μg of each antigen. Group 4 was inoculated subcutaneously with 5 mL of commercial toxoid (ResGuard Multi, Biovet, Brazil), according to manufacturer's instructions, while Group 5 (negative control) received 5 mL of sterile NaCl solution (0.9% w/v) and aluminum hydroxide.

### 2.4. Evaluation of humoral immune response by indirect ELISA

Detection of anti-$H_C$BoNT/C and D specific IgG antibodies in bovine serum (days 0, 28, and 56) was performed by indirect ELISA. Thus, 96-well microplates (Nunc-Immuno Micro Well MaxiSorp) were coated with purified $H_C$BoNT/C or D (100 ng/well) in 100 μL of carbonate-bicarbonate buffer pH 9.6 (TCB) for 18 h at 4 °C. The plates were washed three times with PBS-Tween (0.1%) buffer and incubated with 200 μL/well blocking solution (5% skimmed milk powder in PBS-T) for 1 h at 37 °C. The plates were then washed and incubated with serum from the individual animals (Fig. 3A and B) and with pooled sera of each group (Fig. 4) diluted 1:200 in blocking solution (100 μL/well) for 1 h at 37 °C. After further washing, the plates were incubated (100 μL/well) with anti-bovine IgG peroxidase-conjugated (Sigma–Aldrich) diluted 1:5000 in PBS-T for 1 h at 37 °C. Finally, the plates were washed and incubated with substrate solution (0.1 M phosphate-citrate, pH 4.0) containing 0.4 mg/mL o-phenylenediamine and 0.1% (v/v) $H_2O_2$ for 15 min in the dark at room temperature. The absorbance was measured at 450 nm in a microtiter plate reader (Biochrom EZ read 400). The plates were washed with 200 μL PBS-T three times between each step and five times before the last step.

### 2.5. Potency test

Cattle sera were individually used in the mouse neutralization bioassay as described by MAPA directive n° 23 [12]. This protocol is based on the European Pharmacopoeia and on the Code of Federal Regulations Title 9 (CFR 9, USA) for measuring BoNTs/C and D antitoxins. Briefly, 1 mL of each toxin produced by LANAGRO (Pedro Leopoldo, MG, Brazil) and standardized using antibodies provided by NIBSC (Potters Bar, UK) was incubated at 37 °C for 1 h with 1 mL of 2-fold serial dilutions of each serum from 1:1 to 1:32. Then, two Swiss Webster mice weighing 18–22 g were

intravenously inoculated through the coccygeal vein with 0.2 mL of each sample per dilution and observed for 72 h for survival and euthanized if necessary. The procedure was repeated with intermediary dilutions of each serum to identify the lower protective dilution. The survival information was used to calculate the results in international units per milliliter (IU/mL).

### 2.6. Statistics analysis

Student's *t*-test was used to assess the statistically significant difference among the different vaccine groups using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). Significance was established at $p < 0.05$. Graphs were drawn using the same software.

### 2.7. Ethics statement

This study was carried out in accordance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and the Ethics Committee in Animal Experimentation of the Federal University of Pelotas (CEEA-UFPel). In terms of the latter, the project was approved and completed under permit N° 0102549/2017.

## 3. Results

### 3.1. Expression and characterization of HcBoNTs/C and D

*E. coli* BL21 (DE3) Star™ transformed with either pET28a/$h_cc$ or pET28a/$h_cd$ was able to express both recombinant proteins (~50 kDa) in a soluble form, as shown in inactivated *E. coli* cell extract, cell lysate supernatant and purified vaccines formulations (Fig. 1A). Western blot with standard sera against BoNTs serotypes C or D (NIBSC) demonstrated that all recombinant proteins evaluated are antigenic (Fig. 1B). These results suggest that both recombinant proteins HcBoNT/C and HcBoNT/D can be recognized by sera against the native form of BoNT serotypes C and D, respectively, suggesting they might be able to induce antibodies capable of recognizing the native toxins as well.

### 3.2. E. coli recombinant formulations are safe for animal use

In the sterility test, no growth was seen after 21-days, indicating that there was no contamination by any fungi or bacteria in the formulation. All cattle were observed by a veterinary surgeon during the experiment and, as well as in the innocuity test, no local or systemic adverse reactions were noticed in any animal. This is in accordance with the previous results obtained by our group, in which recombinant formulations composed of either *E. coli* bacterins or *E. coli* cell lysate supernatants containing $H_C$BoNT/C and D were shown to be non-toxic in guinea pigs [17]. These results indicate that either *E. coli* bacterins (inactivated) or *E. coli* cell lysate supernatant (non-purified) vaccines mixed with aluminum hydroxide as adjuvant are safe to use in cattle.

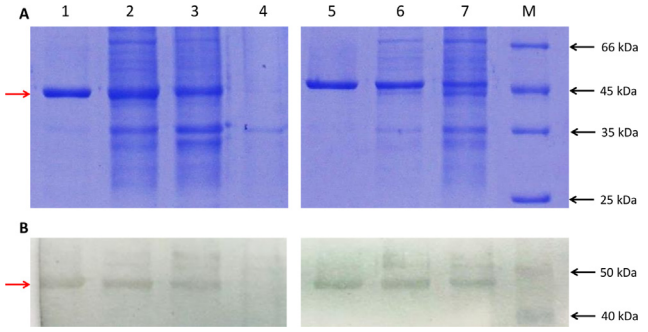


**Fig. 1.** SDS-PAGE 12% (A) and Western blot analysis with standard anti-BoNT serotypes C or D (B) of recombinant proteins used in vaccine formulations. 1: purified $H_C$BoNT/C; 2: *E. coli* BL21 (DE3) Star™-pET28a/$h_cc$ cell lysate supernatant; 3: formaldehyde-inactivated *E. coli* BL21 (DE3) Star™-pET28a/$h_cc$; 4: wild type *E. coli* BL21 (DE3) Star™; 5: purified $H_C$BoNT/D; 6: *E. coli* BL21 (DE3) Star™-pET28a/$h_cd$ cell lysate supernatant; 7: formaldehyde-inactivated *E. coli* BL21 (DE3) Star™-pET28a/$h_cd$; M: Pierce™ Unstained Protein MW Marker (Thermo Fisher–Panel A) or Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher–Panel B).

### 3.3. Induction of anti-$H_C$BoNTs/C and D IgG in vaccinated cattle

Total anti-HcBoNTs C and D IgG antibodies were detected by indirect ELISA in the pooled sera of each group at days 28 and 56 post-vaccination and compared with the pre-immune sera. Recombinant antigens (Groups 1–3) showed a significant increase (***$p < 0.001$) in the concentration of total anti-HcBoNT/C and D IgG antibodies in comparison to that of the previous time point (Fig. 2A and B, respectively), while the commercial toxoid (Group 4) showed only a slight increase in antibody concentration at day 28 when compared to that of the pre-immune sera (*$p = 0.05$), and no significant statistical difference was observed between days 28 and 56 (Fig. 2).

ELISA was used to evaluate the individual response of each animal on day 56. The results showed a uniform antibody concentration pattern for both anti-HcBoNTs/C and D antibodies in animals of each group (Fig. 3B and Fig. 4B, respectively). Anti-HcBoNTs/C and D total IgG antibody levels generated in response to immunizations in each vaccine group were compared by average absorbance obtained in bovine serum at day 56 (Fig. 3A and Fig. 4A). There was no statistical difference in the concentration of specific anti-HcBoNTs/C and D antibodies between vaccine Groups 2 and 3, composed of inactivated (*E. coli* bacterins) and non-purified (*E. coli* cell lysate supernatants) antigens, respectively. The magnitude of the immune response generated by the animals vaccinated with the purified recombinant antigens (Group 1) was significantly higher (***$p < 0.001$) than that generated by animals vaccinated with inactivated and non-purified recombinant antigens (Groups 2 and 3, respectively) or by the group that received the commercial toxoid (Group 4). Furthermore, the response of Groups 2 and 3 was statistically the same, although significantly higher than that of Group 4 (Fig. 3A and Fig. 4A).

### 3.4. Recombinant vaccines induce high titers of neutralizing antitoxins in cattle

Neutralizing antibody mean titers in each cattle group on day 56 post-immunization were determined by mouse neutralization bioassay (Table 1). The recombinant vaccines were capable of inducing 8.35, 7.45, and 7.45 IU/mL of anti-BoNT/C and 7.66, 6.60, and 6.60 IU/mL of anti-BoNT/D neutralizing mean titers when animals were vaccinated with purified, recombinant *E. coli* bacterin or *E. coli* cell lysate supernatant formulations, respectively. Animals vaccinated with the commercial vaccine developed 1.33 and 3.33 IU/mL neutralizing mean titers against BoNT serotypes C and D, respectively. Animals in the negative control group had no detectable levels of neutralizing antibodies against either toxin.

All recombinant vaccines were able to surpass the minimum mean titer of antitoxin required by MAPA (5 IU/mL and 2 IU/mL for BoNTs C and D, respectively), while the commercial vaccine showed significantly lower antibody mean titers than those requested by the Brazilian legislation for both toxins. All vaccine formulation tested in the present study would be considered and approved by MAPA's quality control tests. Moreover, our results are in accordance with those obtained by Moreira et al. [20], sug-

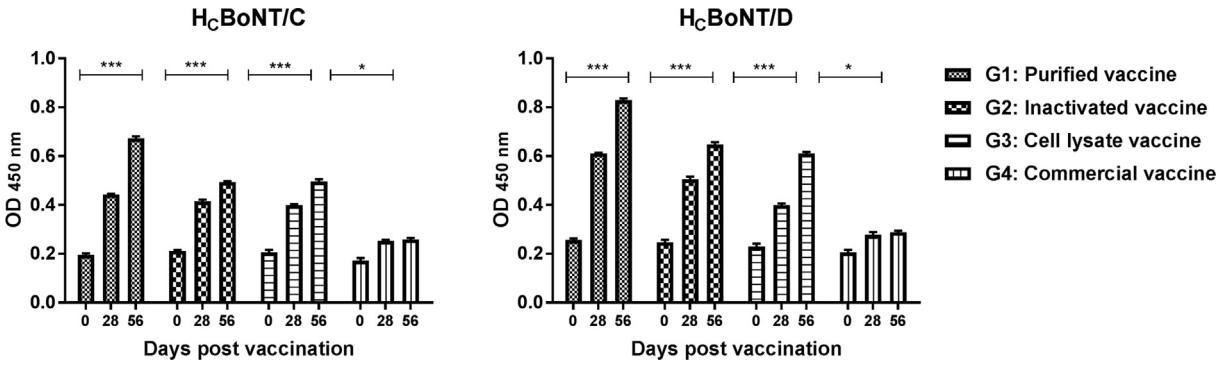


**Fig. 2.** Total IgG antibody response against HcBoNT/C and D in the pooled sera of each vaccine group before vaccination and at days 28 and 56 post-vaccination as measured by indirect ELISA. (A) Total IgG antibody titers against HcBoNT serotype C; (B) Total IgG antibody titers against HcBoNT serotype D. (* p = 0.05; *** p < 0.001).

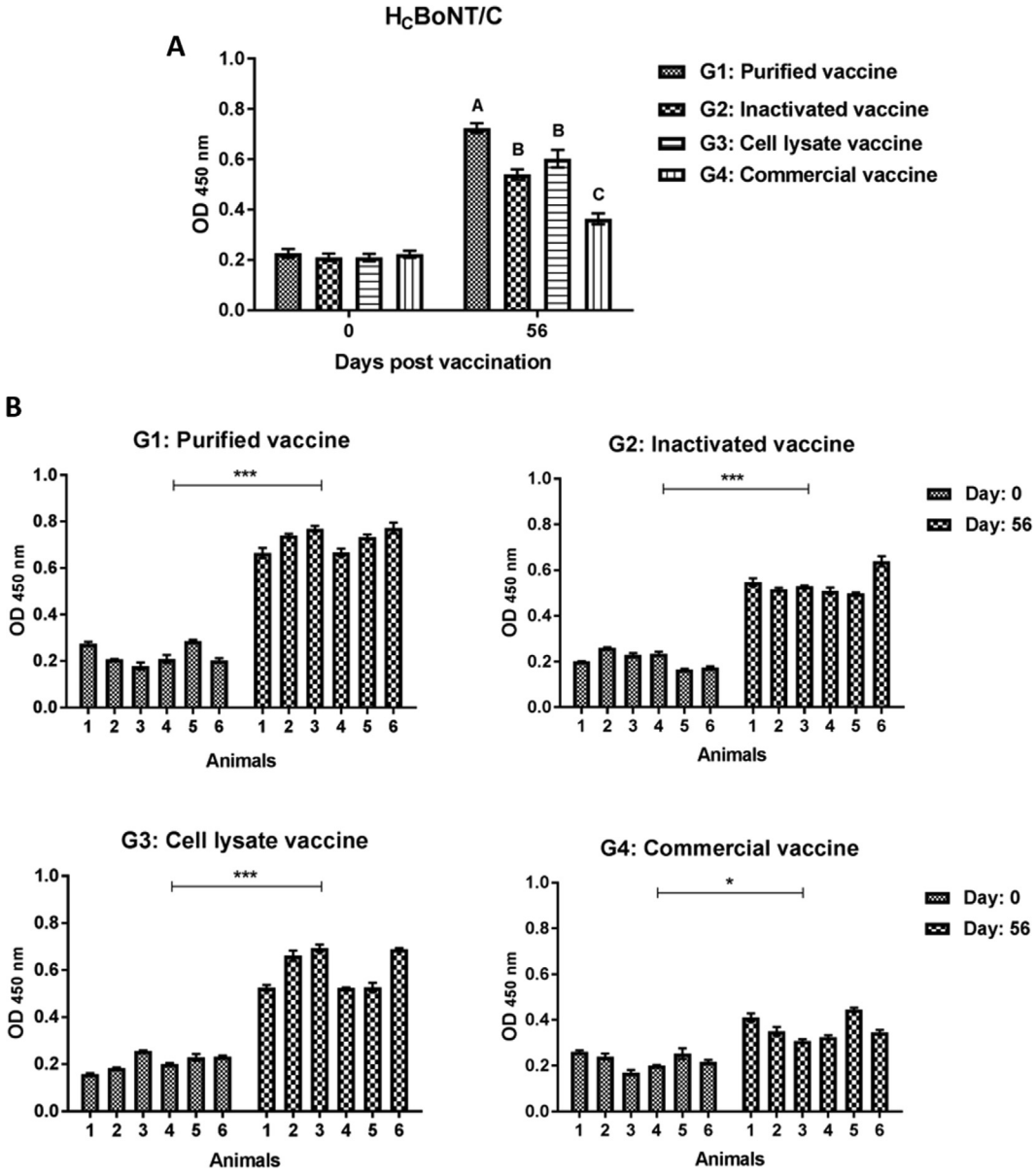


**Fig. 3.** Total cattle IgG antibody response against HcBoNT/C before vaccination and 28 days after the second vaccination (day 56) as measured by indirect ELISA using HcBoNT/C as a captured antigen. (A) Total IgG antibody mean titer of pooled sera from each group, the different capital letters (A–C) indicate a statistical difference between the groups (p < 0.001). (B) The titer was obtained for each animal individually per group against HcBoNT/C at days 0 (pre-vaccination) and day 56 post-vaccination (*p = 0.03; *** p < 0.001).

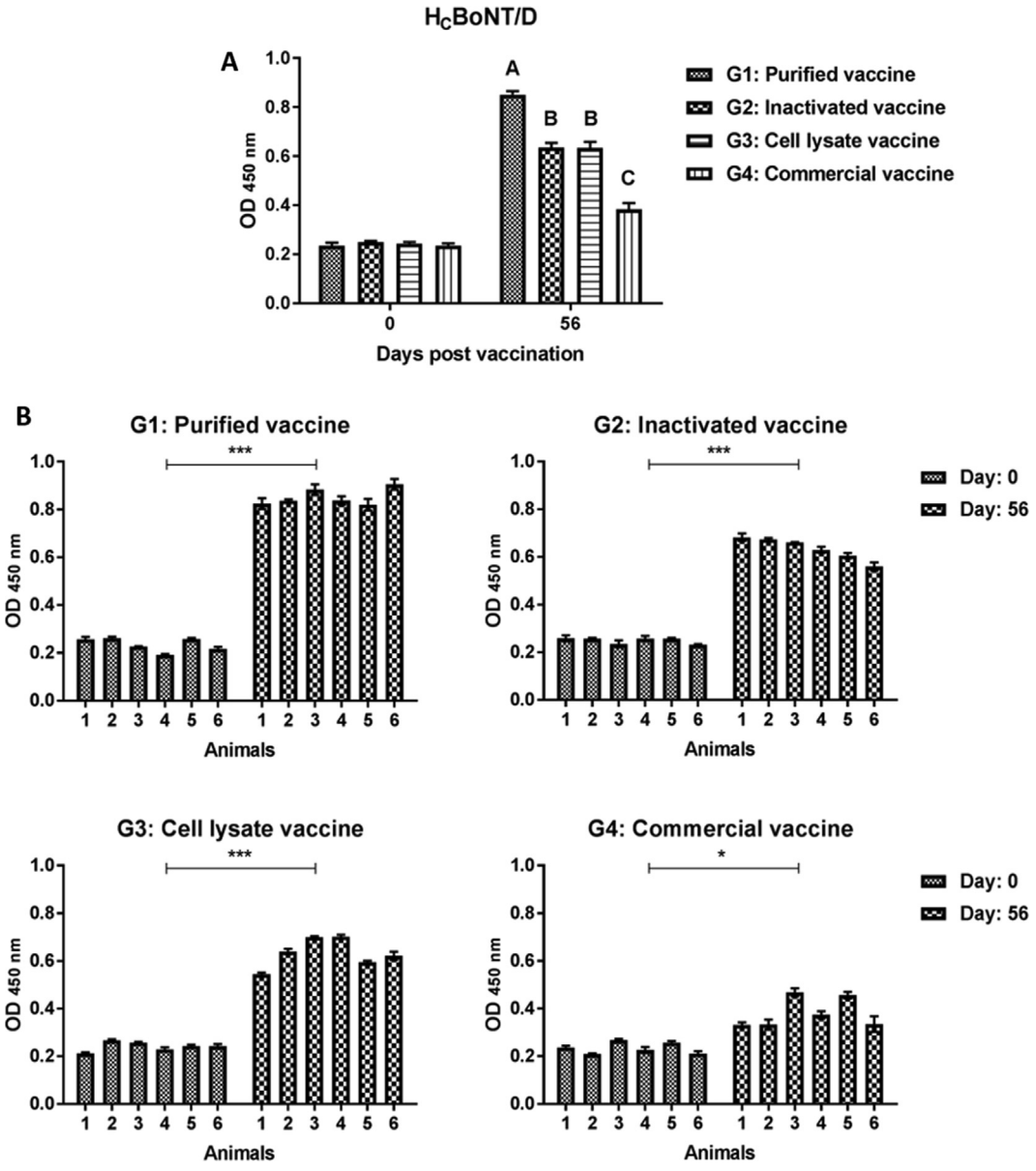


**Fig. 4.** Total IgG antibody response against HcBoNT/D of cattle before vaccination and 28 days after the second vaccination, measured by indirect ELISA using HcBoNT/D as a capture antigen. (A) Total IgG antibody mean titer of each group, different capital letters (A–C) indicate a statistical difference between groups ($p < 0.001$). (B) The titer was obtained for each animal, individually per group against HcBoNT/D at days 0 (pre-vaccination) and day 56 post-vaccination (*$p = 0.03$; *** $p < 0.001$).

**Table 1**
Anti-toxin mean titers against BoNT serotypes C and D detected in cattle on the potency test after immunization, compared by Tukey's test ($p < 0.001$). Letters were used to point out mean titers that were statistically equal or different. Small letters (a–b) were used to compare BoNT/C mean titers and capital letters (A–B) were used to compare BoNT/D mean titers. Means that do not share a letter are statistically different. *Standard deviation.

| Vaccine formulation | Anti-toxin Mean Titer (IU/mL) | |
|---|---|---|
| | BoNT/C | BoNT/D |
| Purified | 8.35 $^{a}$ (±1.05*) | 7.66 $^{A}$ (±0.72) |
| Recombinant *E. coli* bacterin | 7.45 $^{a}$ (±0.60) | 6.60 $^{A}$ (±0.65) |
| *E. coli* cell lysate supernatant | 7.45 $^{a}$ (±0.60) | 6.60 $^{A}$ (±0.65) |
| Commercial toxoid | 3.33$^{b}$ (±2.58) | 1.33$^{B}$ (±1.03) |

gesting antitoxin titers obtained in laboratory animals are significantly higher than those in ruminants for each vaccine formulation [20–23].

Individual cattle antitoxin titers against BoNTs serotypes C and D as obtained by seroneutralization potency test are shown in Fig. 5 panels A and B, respectively. It is noteworthy that two animals in each commercial vaccine group developed no detectable level of neutralizing antibodies against either BoNT, representing a 66.6% (4/6) seroconversion rate, whereas all animals in the groups vaccinated with 200 μg of each recombinant vaccine, despite the formulation, surpassed the minimum level required for approval, achieving 100% seroconversion rate. The lack of response in two of 6 animals in the commercial vaccine group in the present study is in accordance to results described by Oliveira et al. [13]. This study evaluated six commercial clostridial vaccines in cattle and showed only 40,6% to 78,1% of animals produced detectable levels neutralizing antibodies.

## 4. Discussion

The current toxoid vaccine used for protection against botulism in cattle consists of BoNT production by growing *C. botuli-*

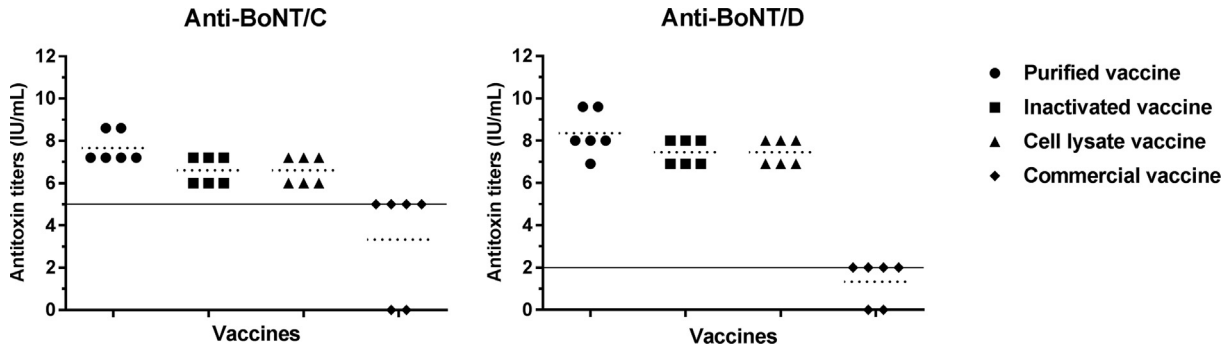


**Fig. 5.** Titers of the neutralizing antibodies for individual animals against BoNT serotypes C and D as determined by the seroneutralization assay. The straight lines represent the required minimum level of neutralizing antibodies against each BoNT as determined by MAPA. The dotted lines show the average antibody titers of each group. (A) Neutralizing antibody titers for each vaccine group against BoNT serotype C; (B) Neutralizing antibody titers for each vaccine group against BoNT serotype D.

*num* in fermenters using complex media. The whole culture is inactivated with formaldehyde, a time-consuming process that can lead to reduced immunogenicity and poses a potential hazard to laboratory workers during the manipulation of the active BoNT, known as the most potent biological toxin in nature [24,25]. Vaccination is the best way to prevent botulism poisoning given the impossibility of eradicating *C. botulinum* spores from the environment, which obliges producers to keep their herds immunized [26].

Nowadays, the efficacy and quality control for commercial toxoids against BoNT serotypes C and D are systematically guaranteed by the MAPA whereas minimum required levels of neutralizing antibodies in pooled sera of vaccinated guinea pigs are assessed by the mouse neutralization bioassay (5 and 2 IU/mL for serotypes C and D, respectively) [12]. On the other hand, Silva et al. [21] showed neutralizing antibody titers against *C. botulinum* type C toxin are higher in guinea pigs than those in cattle, in concordance with results described by Oliveita et al. [13], who showed that the humoral response induced by commercial clostridial vaccines, previously approved in official tests, failed to achieve the minimum neutralizing antibody titer against BoNT serotype C in cattle. These previous results are in accordance to the ones obtained in the present work.

In many countries, including Brazil, cattle botulism has been associated with highly lethal outbreaks and large economic losses [6,10,27]. The most recent outbreak has been reported in Midwestern Brazil in 1700 steers fed with corn silage contaminated by *C. botulinum* serotype C, affecting 1100 animals and resulting in 99.1% lethality rate. Such a high rate has been attributed to the lack of vaccination, resulting in the death of 1090 animals within four days [7].

In this study, in order to verify the results obtained when using guinea pigs as the animal model [17], cattle (target species) were immunized either *E. coli* bacterins containing HcBoNT/C and D or *E. coli* cell lysate supernatants against botulism serotypes C and D. This novel production process proposed by our research group is simpler, safer and more industry-friendly, since it uses non-pathogenic *E. coli* BL21 (DE3) to express the Hc domain of BoNTs C and D, which are non-toxic and dismiss high-cost purification steps such as affinity chromatography or a long time formaldehyde-inactivation. To the best of our knowledge, this is the first report in cattle showing the individual anti-BoNTs/C and D response against recombinant *E. coli* bacterins.

Western blotting was performed to show that standard immune sera against each serotype can recognize HcBoNTs/C and D in animals vaccinated with either inactivated or non-purified vaccine formulations (Fig. 1). These results suggest both the recombinant *E. coli* cell lysate supernatant and the formaldehyde-inactivated recombinant *E. coli* can be used as immunogens to protect against botulism.

Although this study does not intend to correlate ELISA anti-HcBoNTs titers and neutralizing antibody titers as determined by the potency test, our data demonstrate that both ELISA (Figs. 3 and 4) and seroneutralization assay (Table 1) are capable of showing statistically significant difference among animals that receive vaccines containing recombinant HcBoNT/C and D formulations (Groups 1–3) and commercial vaccine (Group 4). Likewise, a homogeneous pattern on immune response among the animals evaluated individually is shown both by indirect ELISA (Fig. 3b and Fig. 4b) and mouse neutralization bioassay (Fig. 5).

The results presented in Fig. 2 show that no significant difference was observed ($p = 0.05$) in the anti-BoNTs/C and D antibodies titers when comparing samples from day 28 and 56 of animals vaccinated with commercial toxoid (Group 4). However, when sera of animals vaccinated with either recombinant formulation, a significant increase in antibody titers can be observed in these time points. This increase after the second dose was a further advantage of using recombinant production methods. These results suggest recombinant toxins can induce a stronger immunity than the currently available commercial toxoid, as supported by data shown in Fig. 5.

Furthermore, the higher titers induced by recombinant bacterin vaccines against BoNT/C and D (7.45 and 6.60 IU/mL of anti-BoNTs/C and D, respectively) than those induced by commercial toxoid (3.33 and 1.33 IU/mL of anti-BoNTs/C and D, respectively) are in accordance to results presented by Moreira et al. [17] in guinea pigs, in which formaldehyde-inactivated recombinant *E. coli* containing 200 μg of $H_C$BoNT serotypes C and D were able to induce 2-fold higher neutralizing antibodies (12 and 20 IU/mL against BoNTs/C and D, respectively) than that induced by the commercial toxoid (6 IU/mL against BoNT/C and 10 IU/mL against BoNT/D).

Moreover, when the antibody levels of the recombinant purified vaccine were compared with those of the recombinant non-purified vaccines (*E. coli* bacterin and *E. coli* cell lysate supernatant) in cattle, no significant statistical difference was observed (Table 1), similarly to those shown by Moreira et al. [17] in guinea pigs. This demonstrate that the efficacy of low-cost vaccines proposed by our group is similar to those obtained through purified recombinant antigens. Furthermore, we showed that both recombinant *E. coli* bacterin and cell lysate vaccines induced 7.45 ± 1.77 and 6.6 ± 1. 28 IU/mL neutralizing titers against BoNTs C and D, respectively, in cattle, and have been deemed effective according to the MAPA standards.

Similarly, *E. coli* BL21 (DE3) cell lysate supernatant containing 200 μg of $H_C$BoNT serotypes C and D were able to induce higher levels of neutralizing antibodies in cattle (8.65 and 10.25 IU/mL

against BoNT/C and D, respectively) and buffaloes (6.1 and 6.2 IU/mL against BoNT/C and D, respectively) than those achieved by commercial toxoid in cattle (4.0 and 2.62 IU/mL of anti-BoNT/C and D, respectively) and buffaloes (2.62 and 2.5 IU/mL of anti-BoNT/C and D, respectively) [15,28].

Recently, our research group has demonstrated that formaldehyde-inactivated recombinant *E. coli* vaccines can effectively induce high titers of *Clostridium perfringens* alpha (CPA) and epsilon (ETX) toxins. Ferreira et al. [29] showed that recombinant *E. coli* bacterin containing 200 μg of rCPA was able to induce 13.82 and 4.0 IU/mL anti-toxin CPA titers in rabbits and sheep, respectively. Likewise, recombinant *E. coli* bacterin containing 200 μg of rETX was evaluated by Ferreira et al. [19] achieving up to 10 IU/mL of anti-ETX neutralizing antibody in rabbits as well as anti-ETX neutralizing antibodies mean titers in sheep and cattle of 7.04 and 4.7 IU/mL, respectively.

Taken together, our results indicate that the production method herein proposed can deliver an efficient, safe, cost-effective and industry-friendly vaccine against botulism in cattle. This statement is supported by the fact that even after dismissing expensive, time-consuming steps such as protein purification, high levels of neutralizing antibodies were elicited by recombinant *E. coli* bacterins or *E. coli* cell lysate vaccines, comparable to those induced by purification proteins, and significantly higher than those induced by commercial native toxoid. These results represent a significant improvement in the current botulism vaccine technology that could be very beneficial not only to the industrial workers but also to cattle breeders and the economy.

## CRediT authorship contribution statement

**Clóvis Moreira Jr:** Conceptualization Investigation, Data curation, Methodology, Formal analysis, Writing - original draft. **Marcos R.A. Ferreira:** Conceptualization, Investigation, Data curation, Methodology, Formal analysis, Writing - original draft. **Paula F. Finger:** Investigation, Data curation. **Carolina G. Magalhães:** Investigation, Data curation. **Carlos E.P. Cunha:** Methodology, Formal analysis, Writing - original draft. **Rafael R. Rodrigues:** Investigation, Data curation. **Denis Y. Otaka:** Investigation, Data curation, Methodology, Formal analysis. **Cleideanny C. Galvão:** Investigation, Data curation. **Felipe M. Salvarani:** Conceptualization, Methodology, Formal analysis, Methodology, Formal analysis, Supervision, Writing - review & editing. **Ângela N. Moreira:** Conceptualization, Supervision, Writing - review & editing. **Fabricio R. Conceição:** Methodology, Formal analysis, Supervision, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author Contributions

Designed experiments: CMJ, MRAF, FMS, ANM, FRC; Conducted experiments: CMJ, MRAF, PFF, CGM, FMS, RR, DYO, CCG; Analyzed data: CMJ, CEPC, DYO, FMS, MRAF, FRC; Wrote and revised the manuscript: CMJ, CEPC, ANM, FRC; Oversaw and oriented experiments: FMS, ANM, FRC.

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