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Evaluation of the expression and immunogenicity of four versions of recombinant *Clostridium perfringens* beta toxin designed by bioinformatics tools

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ABSTRACT

Beta toxins (CPB) produced by *Clostridium perfringens* type B and C cause various diseases in animals, and the use of toxoids is an important prophylactic measure against such diseases. Promising recombinant toxoids have been developed recently. However, both soluble and insoluble proteins expressed in *Escherichia coli* can interfere with the production and immunogenicity of these antigens. In this context, bioinformatics tools have been used to design new versions of the beta toxin, and levels of expression and solubility were evaluated in different strains of *E. coli*. The immunogenicity in sheep was assessed using the molecule with the greatest potential that was selected on analyzing these results. *In silico* analyzes, greater mRNA stability (−169.70 kcal/mol), solubility (−0.755), and better tertiary structure (−0.12) were shown by rCPB-C. None of the strains of *E. coli* expressed rFH8-CPB, but a high level of expression and solubility was shown by rCPB-C. Higher levels of total and neutralizing anti-CPB antibodies were observed in sheep inoculated with bacterins containing rCPB-C. Thus, this study suggests that due to higher productivity of rCPB-C in *E. coli* and immunogenicity, it is considered as the most promising molecule for the production of a recombinant vaccine against diseases caused by the beta toxin produced by *C. perfringens* type B and C.

1. Introduction

Clostridium perfringens is a gram-positive, ubiquitous, spore-forming, and anaerobe bacillus, which causes lethal infections such as hemorrhagic necrotic enteritis and enterotoxemia affecting swine, cattle, sheep, and goats [1,2]. *C. perfringens* type B and C produce beta toxin (CPB) of 34.86 kDa (322 aa) as the main virulence factor of these toxin types. The action mechanism is characterized by the pore formation in the plasma membrane leading to cell death [3,4]. CPB causes injuries to the intestine and is absorbed into the circulation, causing injuries to other organs [5]. Proteolytic enzymes, particularly trypsin, break down CPB that makes it lethal to infant mammals because of the presence of trypsin inhibitors in the colostrum [6].

Vaccination against *C. perfringens* represents a prophylactic measure that guarantees significant protection levels and generates neutralizing antibodies. For production, the toxoids currently available are obtained from the cultivation of *C. perfringens* and inactivation of the toxins [7]. However, despite the degree of protection induced, these vaccines are produced from non-pure supernatant of *C. perfringens* cultures in which other toxins and proteins are present, which leads to a higher degree of antigen diversity in the vaccine [8]. The high variability between different batches requires continuous selection of strains that exhibit greater

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production of toxins. This increases the complexity of the production process and does not result in an effective increase in yield. In addition, the cultivation of *C. perfringens*, which requires the use of a complex culture medium, represents a biosafety risk, since the pathogen is dangerous for humans [9].

The recombinant proteins expressed in *Escherichia coli* can be used in recombinant vaccines for controlling and preventing diseases caused by *C. perfringens* [9–11]. This recombinant vaccine has a rapid and simplified production process, biosafe, antigen stability, high yield, control of all stages of growth and expression of proteins, and ease of genetic manipulation [12,13]. However, inclusion bodies that are an aggregate of insoluble antigens that may have reduced immunogenicity are formed in this process, as due to conformational changes, epitopes are inaccessible to immune receptors [9,14].

The main strategies for the production of recombinant CPB (rCPB) described in the literature were the expression of holotoxin [10], non-toxic mutants [3], chimeric proteins [15,16], and expression of the C-terminal domain [16]. This study has proposed that animals can be immunized with inactivated cells of recombinant *E. coli* containing antigens of *Clostridium* spp., eliminating the stages of lysis, purification, and refolding of recombinant antigens [17–20]. Previous studies have shown that rCPB is highly insoluble, therefore, it is important to evaluate the level of expression and solubility to refine antigens [9–11]. Thus, this study aimed to evaluate four recombinant versions of CPB of *C. perfringens*: CPB^{1-322} (rCPB), rSUMO-CPB, rFH8-CPB, and C-terminal $domain^{143-311}$ (rCPB-C) *in silico*, *in vitro,* and *in vivo*.

2. Materials and methods

2.1. Ethical aspects

All animal experiments were performed as per the guidelines of the National Council for Animal Experimentation Control (CONCEA), and the Animal Experimentation Ethics Committee of the Federal University of Pelotas (Process 23110.002529/2018–53) approved the experiments.

2.2. In silico analysis

Protein sequences were analyzed for their physicochemical properties, including GRAVY (large hydropathic mean), half-life, molecular mass, instability index, aliphatic index, isoelectric point (pI), amino acid composition and the total number of residues positives and negatives through the online tool ProtParam (http://expasy.org/tools/protparam.html) [21]. The i-Tasser program (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [22–24], prediction tool by threading, was used to perform protein modeling.

The online platform m-fold (http://unafold.rna.albany.edu/cgi-bin/mfold-3.4.cgi) [25] was used to evaluate the secondary structure of the RNA. Based on the structural stability and Gibbs free energy (ΔG), translation efficiency was analyzed. VaxiJen v2.0 was used to predict protein antigenicity with a threshold of 0.4 [26], available at http://www.ddg-pharmfac.net/vaxijen/.

2.3. Cloning of rCPB versions in E. coli expression vector

The amplification of the CPB coding sequence was performed from the vector previously constructed by Moreira et al. [9], using primers: *cpb*-forward: 3′ CGCGGATCCAATGATATAGGTAAA 5′ e *cpb*-reverse: 3′ TGTGAATTCCTAAATAGCTGTTACTTTG 5′, and cloned into the ChampionTM pET-SUMO vector according to the manufacturer's instructions (Thermo Fisher Scientific). FastBio synthesized the vector pET28a-*fh8-cpb*. Epoch Life Science™ built and synthesized the vector pET28a-*cpb-c*.

2.4. In vitro analysis - evaluation of expression of rCPB versions

Expression was evaluated in *E. coli* strains (DE3) Star (Star), *E. coli* codon-plus (DE3) Ril (Ril), *E. coli* BL21 (DE3) C41 (C41), *E. coli* BL21 (DE3) C43 (C43), *E. coli* BL21 (DE3) (DE3), *E. coli* BL21 (DE3) Salt Induction (SI), *E. coli* BL21 (DE3) Rosetta (Rosetta), *E. coli* BL21 (DE3) plysS (pLysS), *E. coli* BL21 (DE3) plysE (pLysE), *E. coli* BL21 (DE3) pT-Trx (pT-Trx), *E. coli* BL21 (DE3) RP (RP). All of these strains were transformed with the above-mentioned plasmids. The strains were cultivated in 100 mL Luria-Bertani broth (LB) supplemented with 100 μg/mL kanamycin, ampicillin, and/or chloramphenicol 34 μg/mL, following the specifications of each strain. The cultures were incubated in a shaker (16 h, 37 °C, 200 rpm). In the logarithmic growth phase ($D.O._{600nm}$ = 0.6–0.8) with 0.5 mM isopropril-β-D-1-thiogalactopyranosidium (IPTG), the protein expression was induced for 3 h. Every 1 h, 1 mL aliquots of the cultures were collected, and the samples were centrifuged (13,000 g, 2 min). The aliquots were adjusted DO_{600nm} to 1. The pellet was suspended in ultrapure water (80 μL) and 5 × sample buffer (20 μL - 250 mM Tris pH 6.8; 1% SDS; 50% glycerol; 5% β-mercaptoethanol; 0.15 g bromophenol blue) was added and boiled (100 °C, 10 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with anti-His6 × mAb (Sigma, USA) were performed for expression analysis.

Protein solubility was evaluated by centrifuging the culture (7000 g, 4 °C, 10 min) and suspending the pellet in a wash buffer (200 mM NaH_2PO_4, 0.5 M NaCl and 5 mM imidazole, pH 8.0), supplemented with lysozyme (1 μg/mL) and incubated (1 h, 37 °C, 200 rpm) for subsequent cell lysis by sonication and subsequent centrifugation (10,000 g, 4 °C, 10 min), resulting in the supernatant. The pellet was washed and suspended in a wash buffer. SDS-PAGE 12% and Western blot were used to evaluate the proteins obtained in the supernatant and pellet (inclusion bodies).

2.5. Vaccine formulations

According to the method developed by Ferreira et al. [17], vaccine formulations were produced. Briefly, a thermal shock was given to transform the DE3 strain with the plasmids and the expression occurred as previously described. Subsequently, the cultures were centrifuged (7000 g, 10 min, 4 °C), suspended in phosphate-buffered saline (PBS), and inactivated with 0.4% formaldehyde (24 h, 37 °C, 200 rpm). Then, the inactivation efficiency was evaluated by plating an aliquot of 100 μL of the culture on LB agar plates and incubated at 37 °C, 16 h. After inactivation, the cultures were subsequently washed twice with PBS to remove residual formaldehyde and subsequently stored at 4 °C in PBS plus thimerosal (0.01%).

2.6. In vivo analysis - vaccination of sheep

A total of 200 μg of each antigen, rCPB, rCPB-C, and commercial toxoid (Covexin® 9, Schering Plow) were injected into 21 adult Texel sheep, divided into three groups (n = 7). Before initiating the experiment, day 0 sera from the animals (negative control) were titrated. All animals were vaccinated on days 0 and 28. Blood samples were collected by puncturing the jugular vein on days 0, 28, and 56 [17,18] and then centrifuged (3000 g, 15 min) immediately after collection and the serum was stored at −20 °C until use.

2.7. Evaluation of anti-CPB antibody levels by indirect ELISA

The levels of anti-rCPB and anti-rCPB-C antibodies were

evaluated on days 0, 28, and 56 in sheep, with a total of 200 ng/well of the antigens in carbonate-bicarbonate buffer pH 9.6 (18 h, 4 °C) were coated in microplates (Nunc-Immuno Micro Well MaxiSorp). Between each step, the plates were washed three times with PBS-T. Post-sensitization incubations were performed for 1 h at 37 °C. The plates were blocked with 5% skimmed milk powder in PBS-T, and the serum samples were diluted in PBS-T (1:50) and added to the plate (duplicate). The IgG HRP anti-mouse conjugate was diluted 1: 2500 and added to the plate. Subsequently, orthophenylenediamine (OPD); citrate-phosphate buffer (0.2 M, pH 4.0, and 0.02% H_2O_2) was added and incubated for 15 min in the darkroom. The reading was performed using a microplate reader (Biochrom EZ Read 400) calibrated at an absorbance of 450 nm.

2.8. Titer evaluation of neutralizing anti-CPB antibodies by serum neutralization

In mice, seroneutralization was used to titrate the sheep serum pools in each group. Neutralizing antibodies (CPB antitoxins) were titrated according to European Pharmacopeia. Soon, the sera were diluted from 1: 2 to 1:32 in PBS and incubated with a standard toxin (37 °C for 30 min). A total of 0.2 mL of the homogenate was intravenously inoculated in five Swiss Webster mice (18–22 g) and observed for 72 h. Then, the results were measured as 50% neutralization (IC_{50}) titer in international units per mL (IU/mL) [9,11,17].

2.9. Statistical analysis

GraphPad Prism Version 7.0 software (GraphPad Software, San Diego, CA, USA) was used for data analysis. Variables between different groups were compared using a two-way analysis of variance (ANOVA).

3. Results

3.1. In silico analysis - physical and chemical parameters

ProtParam analysis (Table 1) was done to evaluate the molecular mass of the CPB (36.19 kDa), CPB-C (21.66 kDa), FH8-CPB (45.12 kDa), and SUMO- CPB (48.69 kDa). The range of pI of all CPB versions was between 5.7 and 6.4, which is favorable when proteins are expressed in the cytoplasm [27].

The estimated half-life of the proteins was greater than 10 h in the cytosol of *E. coli*. The half-life takes into account the total time required for a protein to disappear after being synthesized in the cell, it was estimated at 30 h for mammalian reticulocytes, > 20 h for yeast, > 10 h for *E. coli*. The instability index CPB (36.81), CPB-C (42.86), FH8-CPB (32.73), and SUMO-CPB (40.69) indicated that proteins with an index value of less than 40 are stable, and with a value above 40 are unstable. The best stability was shown by FH8-CPB and CPB. The instability index provides an estimate of the stability of your protein in a test tube and it can be a determining factor for its effective production in heterologous expression systems. Statistical analyzes reveal that there are certain dipeptides of significantly different occurrence in unstable proteins when compared to stable proteins. Using values of weight attributed to different dipeptides, it is possible to calculate the instability index (<or> 40) [28].

Table 1
Physico-chemical parameters of the beta toxin versions of *Clostridium perfringens*.

Parameters	CPB	CPB-C	FH8-CPB	SUMO-CPB
Amino acids (n)	320	195	404	432
Molecular mass (kDa)	36,19	21,66	45,12	48,69
Isoelectric point (pI)	5,94	6,44	6,08	5,76
Negative waste (Asp + Glu)	37	19	50	57
Positive waste (Arg + Lys)	31	17	44	47
Half life (h) (*Escherichia coli*)	>10 h	>10 h	>10 h	>10 h
Instability index	36,81	42,86	32,73	40,69
Aliphatic index	71,25	56,46	73,32	70,16
Average hydropathicity (GRAVY)	−0,604	−0,755	−0,593	−0,655

The best thermostability was, respectively, 73.32 (FH8-CPB) and 71.25 (CPB), followed by SUMO-CPB (70.16) and CPB-C (56.46). The aliphatic index is an indicator of greater thermostability and also it indicater of solubility in a cell when the protein is overexpressed [29]. The GRAVY index showed that all versions of CPB presented negative values indicating water-soluble. Hydrophobic/non-polar residues receive more positive values while polar/ionic residues receive negative values [30].

3.2. In silico analysis - prediction of tertiary structure, antigenicity and mRNA structure

VaxiJen v2.0 was used to assess the antigenicity of each selected protein. An antigenicity index above 0.7 (threshold = 0.4) was shown by all molecules; therefore, all arrangements were predicted to be antigenic. The best structural model predicted in i-Tasser was estimated to be in the range of −5 to 2. Greater structural similarity to protein was shown by CBP and CPB-C *in vivo*, with C- score of -0.06 and - 0.12, respectively (Fig. 1).

The thermodynamic characteristics of mRNA secondary structures indicated that CPB-C (ΔG: −169.70 kcal/mol) showed the more stability, followed by FH8-CPB (ΔG: −255 kcal/mol), SUMO-CPB (ΔG: −268.50 kcal/mol), and CPB (ΔG: −288.60 kcal/mol). The m-fold program predicts the most energy-stable mRNA molecules, providing a set of possible structures in an energy interval and indicating reliability. In general, the minimum free energy value is an adequate indicator of level of expression. Some authors consider that higher values may indicate a lower expression due to the greater stability of the secondary structure [31,32], but this proportion differs between recombinant proteins. Thus, greater mRNA stability may indicate high expression and vice versa [32].

The results of the stability and antigenicity of the CPB versions are shown in Table 2.

3.3. In vitro analysis - evaluation of expression and solubility

Three versions of CPB with expected molecular mass, rCPB (36.19 kDa), rCPB-C (21.66 kDa), and rSUMO-CPB (48.69 kDa) were expressed by different strains of *E. coli*, whose analysis of solubility is shown in Fig. 2.

Of 11 strains evaluated, 7 strains expressed the rCPB protein. Only Ril, RP, and SI expressed rSUMO-CPB. While Star, DE3, Ril, and pT-Trx expressed rCPB-C at high levels. The rCPB-C showed greater solubility among the evaluated recombinant versions, being detected predominantly in the supernatant of the cultivation of Star, DE3, and pT-Trx. The remaining toxins were detected in at least two strains, but less than rCPB-C. The rFH8-CPB expression was not detected in any of the strains evaluated. Strain DE3 was selected for vaccine production based on the expression level, solubility, and the non-use of strain-specific antibiotics.

3.4. In vivo analysis - immunogenicity in sheep

The main criteria for antigen selection was the expression level, solubility, type of strain, and *in silico* analyzes. In addition, the rCPB and rCPB-C molecules expressed in the DE3 strain were selected.

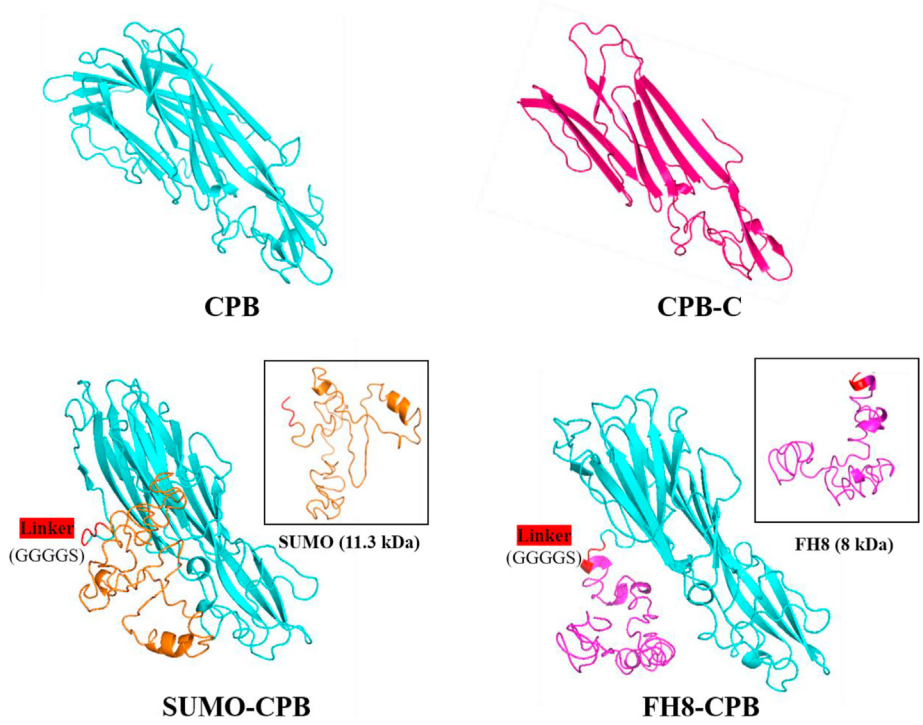

Fig. 1. Prediction of tertiary structure by i-Tasser, each domain is efficiently separated by a flexible linker (GGGGS).

Table 2
Prediction of stability and antigenicity of CPB, CPB-C, FH8-CPB, and SUMO-CPB.

Construction	i-Tasser (C-Score)	VaxiJen (*threshold* = 0.4)	RNAfold ΔG (Kcal/mol)
CPB	- 0,06	0,7164	−288,60
CPB-C	- 0,12	0,7734	−169,70
FH8-CPB	−2.42	0,7144	−255
SUMO-CPB	−2,48	0,7361	−268,50

In sheep, antibody levels were higher in animals vaccinated with rCPB-C ($p < 0.001$). On day 0, the presence of serum anti-CPB immunoglobulins (Fig. 3) was evaluated by ELISA. The neutralizing antibody titers in the serum of sheep vaccinated with rCPB, rCPB-C, and commercial toxoids are shown in Table 3.

4. Discussion

New strategies have been developed to immunize animals against *Clostridium* spp., which is an alternative method to overcome vaccine-related challenges [33–35]. The current production process of these formulations is difficult and dangerous for the professionals directly involved in handling the pathogens, such as *C. perfringens* [36]. The process used in this work has the potential to overcome most problems related to the production of commercial immunogens and minimize, or even eliminate, limitations related to modern vaccines composed of insoluble recombinant proteins. The use of inactivated *E. coli* cells allows simple and non-toxic production of recombinant antigens and eliminates the steps of lysis, purification, and refolding.

The expression of rCPB in the form of inclusion bodies hinders the large-scale production. The structure and function of proteins are modulated by a Small Ubiquitin-like Modifier (SUMO), a molecule with 100 residues, and a molecular mass of 11.5 kDa by covalent modification of target proteins in eukaryotes [37–40]. Fh8, an 8 kDa calcium-binding protein, is extracted from the parasite Fasciola hepatica [41]. Increased expression and solubility of fused proteins are promoted by Fh8 [42] and SUMO [40,43–45] *in vitro*. rCPB-C has shown an immunoprotective role; when evaluated fused with the toxin Iota (ITX) it protected the mice from the lethal challenge with the native ITX and CPB toxins [16]. The CPB-$C^{143-311}$ domain is closely related to cell receptor binding and pore formation, which means that this domain contains most of the epitopes recognized by neutralizing antibodies. However, rCPB-C was not assessed individually.

In silico studies of physicochemical characteristics such as aliphatic index, GRAVY, and instability index, are the important properties in the expression of recombinant proteins, allowing the refinement of antigens and reduction of *in vivo* experiments [46]. In this study, CPB and FH8-CPB were more stable (<40), as recommended by Gasteiger et al. [21]. The aliphatic index indicated CPB-C with the lowest thermostability (56.6 °C), but higher than the *E. coli* growth temperature (37 °C). Analysis of mRNA secondary structure showed that CPB-C had the more stability (ΔG = −169.70 kcal/mol). *In vitro*, rCPB-C showed greater expression and considerable levels of rCPB and rSUMO-CPB were observed. FH8-CPB was the second molecule with greater stability of the secondary structure of the mRNA; on the other hand, none of the strains evaluated was able to express this molecule. Rosano and Ceccarelli [47] and Singha et al. [48] demonstrated that in addition to mRNA stability, toxicity, insolubility, and codons of the gene impact the expression by modifying the protein production in *E. coli* and affecting its productivity.

Although *in silico* analyzes showed that all versions of rCPB were soluble, *in vitro* only rCPB-C showed considerable solubility in

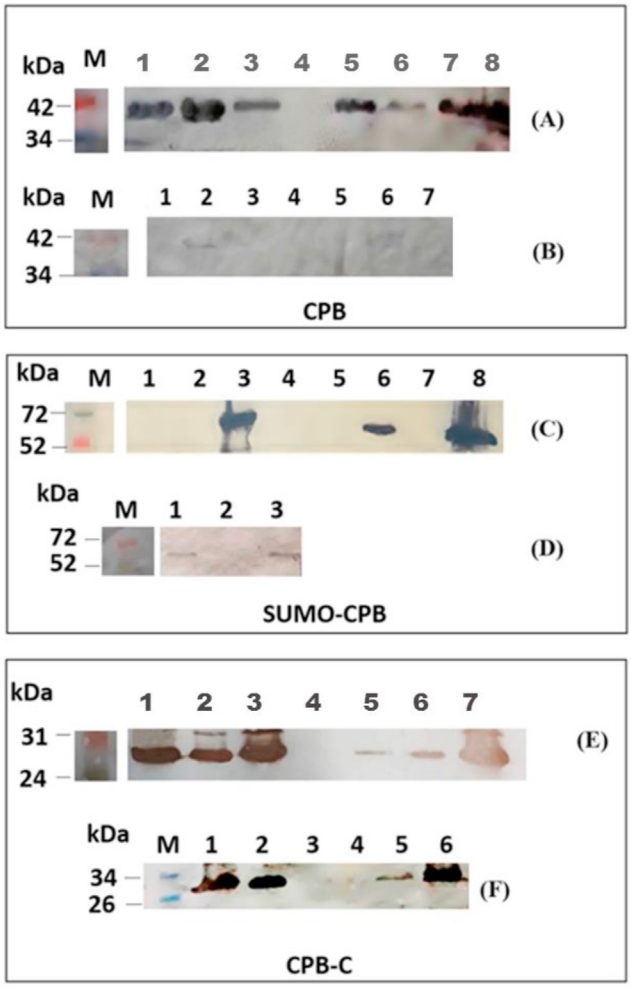

Fig. 2. Western blot of rCPB, rCPB-C and rSUMO-CPB. (**A**, **C**, **E**) - Total cell extract after expression in *Escherichia coli* strains. M - Marker; 1 - Star; 2 - BL21 (DE3); 3 - Ril; 4 - Rosetta; 5 - C43; 6 - RP; 7 - pT-Trx; 8 - Sl. (**B**, **D** and **F**) - Lysis supernatant (soluble proteins). Only strains that expressed the respective CPB version. (**B** and **F**) - M - Marker; 1 - Star; 2 - DE3; 3 -Ril; 4 - C43; 5 - RP; 6 - pT-Trx; 7 - Sl. (**D**) - M - Marker; 1 - *E. coli* Ril; 2 -RP; 3 - Sl.

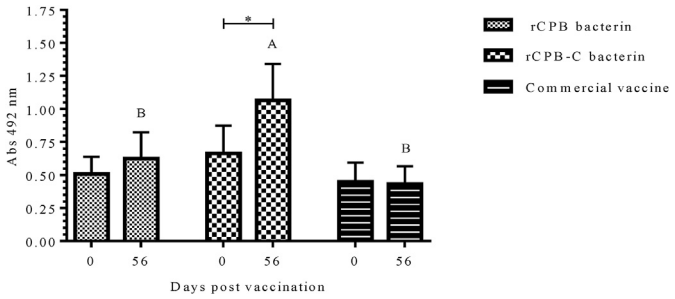

Fig. 3. Levels of total serum immunoglobulins determined by indirect ELISA in the sheep vaccinated with rCPB, rCPB-C and commercial vaccine (Covexin 9®), on day 0 (pre-vaccination) and 56 post-vaccination (*p < 0.001). Different capital letters (A–B) indicate a statistical difference between the groups (p < 0.001).

relation to the other proteins. Previous studies have also found the expression of totally insoluble rCPB [4–6]. According to Arabshahi et al. [49], the heterologous expression of low molecular weight proteins such as rCPB-C, decrease the probability of formation of the inclusion body and consequently favor its soluble expression,

Table 3
Serum antibodies neutralizing anti-CPB antibodies of sheep vaccinated with rCPB, rCPB-C, and commercial toxoid bacterins, determined by seroneutralization assay.

Vaccines	Antitoxin CPB (IU/mL)		
	Day 0	Day 28	Day 56
Bacterin rCPB-C$^{143-311}$	ND	14,4	14,4
Bacterin rCPB	ND	<10[a]	10
Toxoid commercial	ND	ND	10

[a] Of the five mice used for seroneutralization, 2 died close to 72 h of evaluation, ND = not detected.

according to our results.

Due to high antigenicity, high similarity, and reliability of the different versions of CPB and CPB-C to native protein, allow considering its application in vaccinology. Native CPB causes the lethality of *C. perfringens* and an immunogen for the target species [14]. Anti-CPB antibody titers of 14 UI/mL [10], 10 UI/mL [15], 20,4 UI/mL [11], and 25 UI/mL [9] were found in rabbits. However, when immunizing against species of the genus *Clostridium* spp., the humoral response generated in rabbits is usually more pronounced than that observed in the target species, and as such, results found in rabbits do not guarantee the same immunoprotective effect in the target species [9–11,50–53]. Therefore, conducting vaccine studies with target species should be considered when designing longevity experiments [9].

Several studies have shown the potential of rCPB in the immunization of farm animals. Jiang et al. [54] injected purified rCPB separately into cattle, resulting in 33.70 IU/mL of antitoxin. In other studies, the mean titer of neutralizing anti-CPB antibodies in pigs vaccinated with purified rCPB was 14.5 IU/mL [11] and 13,71 UI/mL in sheep, goats, and cattle [9]. Similarly, Zeng et al. showed that while generating a protective immune response in pregnant sows and cows, the recombinant antigen contains the alpha, beta, and beta 2 protein sequences [55]. However, both studies addressed the purified form of rCPB.

Here, the humoral response generated by inactivated *E. coli* cells containing rCPB and rCPB-C was evaluated. After 28 days of immunization, the bacterin containing rCPB generated 10 IU/mL, similar to that obtained by Langroudi et al. [15]. Neutralizing serum anti-CPB antibodies stimulated by the bacterin were a lower immune stimulus, since the stability of rCPB can have been affected, but its protective epitopes were retained. In addition, the beta toxin is unstable and can quickly lose its activity [10]. On the other hand, sheep vaccinated with the bacterium rCPB-C resulted in 14 IU/mL of neutralizing serum antibodies in the vaccinated animals 28 days after the first immunization, similar to that obtained by purified rCPB [9–11]. Accordingly, the total serum immunoglobulins levels in sheep vaccinated with rCPB-C were also higher than the other immunogens.

Although the commercial vaccine did not induce greater production of antibodies, it is essential to consider that the potency of the vaccine may have shown variability, since the commercial vaccine is produced with the native CPB toxin, which may lead to less specific antibody stimulation when evaluated only as a control in the ELISA test. In addition, according to the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) directive n° 23, the animals vaccinated with the commercial toxoid reached the minimum (10 IU/mL) regulating the trade of vaccines against *C. perfringens* in Brazil; in this case, the antibody titer induced by the commercial vaccine was detected only 28 days after the administration of the second dose.

Studies have shown that the ideal time interval between the first vaccination and the booster can be of great importance for generating a humoral response. Ziang et al. found that 28 days after

the first vaccination with rCPB is effective; however, 28 days later, the levels of neutralizing antibodies start decreasing in all immunized groups [54]. According to Bernáth et al. the peak of antibodies resulting from the second vaccination may appear later, if a booster with a longer interval between doses is used [56]. Here, the neutralizing serum anti-CPB antibody levels in sheep immunized even with the bacterium rCPB-C remained stable. However, further studies are required to optimize the revaccination intervals with rCPB-C to achieve the booster effect and a longer protection period in vaccinated animals.

5. Conclusion

In conclusion, the strategies used to increase solubility and productivity, added to *in silico* analyzes, showed that the use of the $CPB^{143-311}$ C terminal domain (rCPB-C) is more promising than the use of integral protein (rCPB). In summary, rCPB-C bacterin induces higher neutralizing antibody titers. rCPB-C has higher productivity, solubility, and immunogenicity. Thus, a simple and robust alternative for the production of a recombinant vaccine is proposed for the prevention against *Clostridium perfringens* type B and C.

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Declaration of competing interest

The authors declare that they have no competing interests.

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