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Non-toxigenic strain of *Clostridioides difficile* Z31 reduces the occurrence of *C. difficile* infection (CDI) in one-day-old piglets on a commercial pig farm

C.A. Oliveira Júnior[a], R.O.S. Silva[a,*], A.P. Lage[a], F.M. Coura[a], C.P. Ramos[a], A.A. Alfieri[b], R.M.C. Guedes[a], F.C.F. Lobato[a]

[a] Veterinary School, Universidade Federal de Minas Gerais (UFMG), Av. Antônio Carlos, 6627, Belo Horizonte, MG, CEP 31.270-901, Brazil
[b] Universidade Estadual de Londrina (UEL), Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Londrina, PR, CEP 86.057-970, Brazil

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ABSTRACT

Neonatal porcine diarrhea (NPD) is a current problem on pig farms and is caused by several enteropathogens. Among them, *Clostridioides difficile* stands out due to its importance in piglets and zoonotic potential. A non-toxigenic strain of *C. difficile* (NTCD), named Z31, was previously tested in hamster and piglet experimental models as a strategy to prevent *C. difficile* infection (CDI). To evaluate the capacity of the strain Z31 to prevent CDI and NPD in one-day-old piglets on a commercial farm, 90 piglets from 16 litters received 1×10^6 spores of Z31 while 84 animals from the same litters served as controls. Animals were clinically evaluated, and fecal samples were collected 24 h after administration and submitted to A/B toxin detection and isolation of *C. difficile*. Stool samples were also submitted to rotavirus, *Escherichia coli*, and *Clostridium perfringens* detection. Administration of Z31 reduced the incidence of CDI in treated animals (7.8%) when compared to the control group (25.0%; $P = 0.003$). In animals that developed CDI, the intensity of diarrhea was lower in those that received Z31 than in the control group. Neonatal porcine diarrhea was reduced in treated animals when compared to untreated animals ($P < 0.001$). The present study suggests that Z31 can potentially be used to prevent CDI in piglets on commercial farms.

1. Introduction

Neonatal porcine diarrhea (NPD) is a recognized challenge on pig farms, causing several production losses (Larsson et al., 2016). Many agents, such as rotavirus, enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens*, and *Clostridioides* (prev. *Clostridium*) *difficile* can cause this problem (Lippke et al., 2011; Cruz Junior et al., 2013; Silva et al., 2013a; Malik et al., 2014; Luppi, 2017). In addition to the importance of *C. difficile* as a cause of NPD worldwide, evidence of the zoonotic potential of this agent has been reported in previous years, with some studies claiming pigs as potential reservoirs of this bacterium (Hensgens et al., 2012; Keessen et al., 2013; Moono et al., 2016; Knight et al., 2017).

Despite the known impact of *C. difficile* infection (CDI) in piglets, there are no specific commercial products to prevent the disease in swine (Mizrahi et al., 2014; Mills et al., 2018). A promising alternative is the use of non-toxigenic strains of *C. difficile* (NTCD), which have already been reported to prevent or reduce clinical signs of CDI in laboratory rodents, piglets, and also humans (Borriello and Barclay, 1985; Sambol et al., 2002; Songer et al., 2007; Villano et al., 2012; Nagaro et al., 2013; Gerding et al., 2015, 2018; Arruda et al., 2016). Although NTCD strains do not produce A and B toxins, the main virulence factors responsible for CDI, these strains are able to colonize the intestinal tract, inhibiting the colonization of toxigenic *C. difficile* strains, thereby preventing the occurrence of the disease (Mills et al., 2018). Recently, using a fully characterized (phenotype and genotype) NTCD strain, named Z31, we were able to prevent CDI in experimental models of hamsters and piglets (Oliveira Júnior et al., 2016, 2018a, 2018b; Pereira et al., 2016). In order to understand the impact of Z31 on pig farms, the present work studied the efficacy of Z31 in preventing CDI on a commercial pig farm with a known history of NPD.

2. Material and methods

2.1. Strain and spore production

The complete genome of NTCD strain Z31 is available at https://www.ncbi.nlm.nih.gov/bioproject/302266 and a few important phenotypic characteristics have also been described elsewhere (Oliveira Júnior et al., 2016, 2018a). For spore production, a previously

* Corresponding author.
E-mail address: rodrigo.otaviosilva@gmail.com (R.O.S. Silva).

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described protocol was used with some modifications (Oliveira Júnior et al., 2018a). Briefly, *C. difficile* Z31 was plated onto Müeller Hinton agar (Thermo Fisher Scientific Oxoid™, Waltham, MA, USA) supplemented with 5% horse blood and 0.1% sodium taurocholate (HiMedia, Mumbai, India), and incubated for two days under anaerobic conditions at 37 °C. Colonies were harvested and diluted in phosphate-buffered saline and the turbidity was adjusted to McFarland scale 1 (approximately 3.0×10^8 CFU/mL). Next, 500 µL of this solution was transferred to 4.5 mL of a pre-reduced Brain Heart Infusion broth (BHI; Thermo Fisher Scientific Oxoid™, USA) and incubated in an anaerobic chamber (Thermo Fisher Scientific Oxoid™, USA) for 24 h. The entire volume of this first pre-inoculum was then inoculated into 45 mL of pre-reduced BHI and kept under anaerobic conditions. After 24 h, the entire culture was then transferred to 450 mL pre-reduced BHI, which was kept under anaerobic conditions for five days, followed by a five-day-period under aerobic conditions at room temperature (25 °C ± 2 °C). The broth was then centrifuged at 3000 × g for 30 min at 4 °C (Hettich ROTINA 420R, Tuttlingen, Germany) followed by resuspension of spores in 5 mL of 0.9% saline solution. This step was repeated five times. The percentage of spores was evaluated using the Wirtz-Conklin staining technique (Hamouda et al., 2002) and observing them under an optical microscope (Nikon, Tokyo, Japan), as well as counting the spores on plates, as previously described (Oliveira Júnior et al., 2018a). One day prior to animal administration, the spore solution was diluted with 0.9% saline solution to achieve a concentration of 1×10^6 spores/mL (Oliveira Júnior et al., 2018b).

2.2. Farm management and previous occurrence of C. difficile infection

The experimental methods were approved by the institutional ethics committee (CEUA-UFMG) under the protocol 013/2014. The experiment was performed in a 600 sow-farm, with a one-site, all-in, all-out per barn swine production system, located in the municipality of Pará de Minas, Minas Gerais state, Brazil. At birth, piglets were dried with paper towels, their umbilical cords cut, navel treated with 6.0% iodine, and covered with Vidasec® powder (Bayer, São Paulo, Brazil) before suckling maternal colostrum. The veterinarian responsible for this farm described a high occurrence of diarrhea in piglets, mostly in animals aged 1–3 days, and also reported a CDI diagnosis in the farm in the previous year. To investigate the occurrence of CDI in the farm, 49 fecal samples of one-day-old piglets from 12 different litters were randomly collected from three out of four farrowing rooms a week before the beginning of the experiment. All fecal samples were classified as diarrheic or non-diarrheic, submitted to A/B toxin detection, isolation of *C. difficile*, *C. perfringens*, and ETEC, and rotavirus detection (Fig. 1).

2.3. A/B toxin detection and C. difficile isolation

A commercial enzyme immunoassay (Tox A/B II, Techlab, Blacksburg, VA, USA) was used to detect A/B toxins in the stool samples. The presence or absence of A/B toxins was used to determine the occurrence of CDI. For *C. difficile* isolation, feces were diluted in equal volumes of ethyl alcohol (96%), kept at 37 °C for 30 min and then plated onto Cycloserine Cefoxitin Fructose Agar (CCFA, Himedia) supplemented with 7% horse blood and 0.1% sodium taurocholate. After incubation under anaerobic conditions (37 °C for 72 h), three flat, irregular and with ground-glass appearance colonies from each plate were subjected to thermal extraction of DNA (98 °C for 20 min) and a previously described protocol of Multiplex polymerase chain reaction (PCR) to detect the housekeeping gene *tpi* and toxin genes *tcdA*, *tcdB*, and *cdtB* (Silva et al., 2011). One toxigenic strain per litter was also subjected to Multilocus Sequence Typing (MLST) as previously reported (Griffiths et al., 2010).

2.4. Isolation of C. perfringens and E. coli, and rotavirus detection

For *C. perfringens* culture, feces were plated onto Sulfite Polymyxin Sulfadiazine agar (SPS; Thermo Fisher Scientific Oxoid™) and incubated under anaerobic conditions (37 °C for 24 h). Three rounded sulfite-reducing colonies from each plate were submitted to PCR (Keyburn et al., 2008; Silva et al., 2013b). For *E. coli* identification, fecal samples were plated onto MacConkey agar (Prodimol Biotechnology, Belo Horizonte, Brazil) and incubated at 37 °C for 24 h under aerobic conditions. Three lactose-fermenting colonies were collected and submitted to biochemical tests employing the EPM-MILI-Simmons Citrate Enterobacteriaceae identification test (Starr, 1986). Next, isolates confirmed as *E. coli* were submitted to a PCR method that was previously described to investigate the ETEC virulence factors associated with NPD, i.e., fimbrial adhesins F4 (K88), F5 (K99), F6 (987 P), F41, and the toxins STaP, STb, and LT (Macêdo et al., 2007). Strains were classified as ETEC if positive for the presence of at least one toxin gene, regardless of the presence or absence of fimbrial genes (Dubreuil et al., 2016). The presence of rotavirus A, B, and C was tested using a previously reported real-time-PCR method (Almeida et al., 2018).

2.5. Experimental design

After confirmation of CDI in the farm, 16 litters from three different farrowing rooms (F1, F2, F3) were used in an experiment to evaluate the capacity of Z31 to prevent CDI in one-day-old piglets. A total of 90 piglets received Z31, while 84 animals were used as controls (Table 1). After all farm standard birth procedures (previously described) were followed and before the suckling of colostrum, half of the newborn piglets from each litter were inoculated orally with 1.0 mL of a solution containing 1×10^6 spores of strain Z31, using a disposable syringe without a needle. For each sow, piglets of odd birth order (1, 3, 5, and so on) received Z31, while piglets of an even birth order did not receive Z31 and were used as negative controls. This protocol of alternating inoculation of Z31 was chosen in order to reduce the possible litter effect on the occurrence of CDI in a naturally-infected farm. All animals from the same litter were kept together, but no further alterations of farm management were necessary. The dosage in UFC/mL was the same as used in piglets in a previous trial experimental model (Oliveira Júnior et al., 2018b). Twenty-four hours following this procedure, fecal samples of all animals were collected by rectal stimulation and scored according to aspect (0 – normal, 1 – softened, 2 – pudding-like, 3 – watery). All samples were submitted to A/B toxin detection, isolation of *C. difficile*, *C. perfringens*, and *E. coli*, and rotavirus detection as previously described (Fig. 1).

2.6. Statistical analysis

The Mann-Whitney U test was used to analyze data involving diarrhea scores. Occurrence of pathogens in both groups and association to diarrhea were analyzed by Fisher exact test. Correlation between toxin detection and toxigenic culture of *C. difficile* was assessed by Spearman's rank correlation coefficient. Tests were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) with a significance level of 0.05.

3. Results

The total spore production of Z31 in each 500 mL bottle ranged from 8.2×10^7-5.9×10^8, with a median value of 1.3×10^8 spores and a median concentration of 2.6×10^6 spores/mL. The spore percentage varied from 80 to 90% using the Wirtz-Conklin staining technique and more than 95% by bacterial counting in blood agar.

Stool samples collected before the experiment revealed that 83.7% of the animals exhibited diarrhea and the presence of three enteropathogens, *C. difficile*, *C. perfringens* type A beta2-positive, and

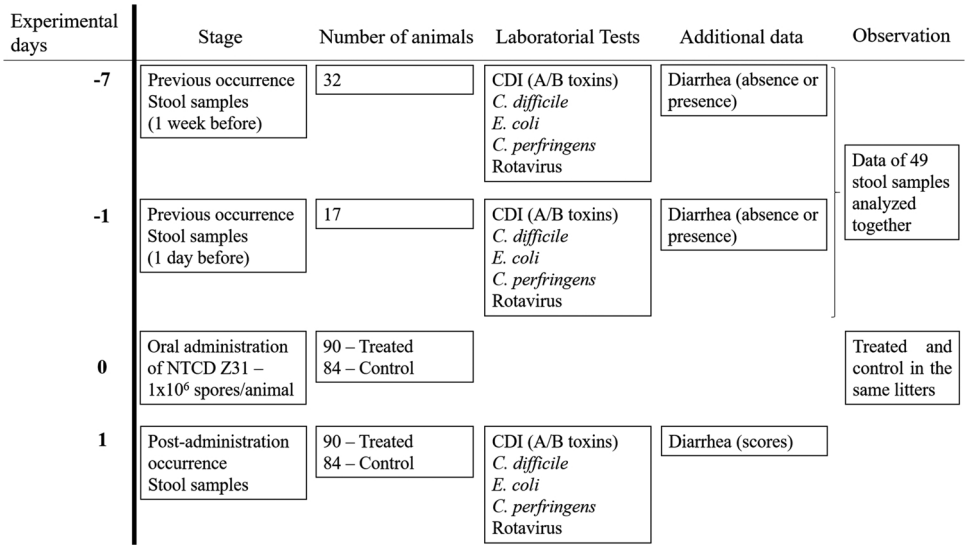

Fig. 1. Flow chart of experimental procedures performed in the farm before and after the administration of the non-toxigenic strain of *Clostridioides difficile* (NTCD) Z31 in the piglets 1 day-old.

ETEC. NTCD strains were not detected. *C. difficile* A/B toxins were found in the feces of 59.2% animals, 93.1% of which were also positive for toxigenic *C. difficile*. Both A/B toxins and toxigenic *C. difficile* were associated with the presence of diarrhea ($P = 0.004$). On the other hand, *C. perfringens* type A and ETEC were found in the feces of 22.4% and 18.4% of sampled animals, respectively; however, they were not associated with diarrhea ($P = 0.999$).

Fecal samples collected one day after administration of Z31 indicated that 56.3% of animals had some level of NPD, with an occurrence of 46.6% in treated versus 66.6% in control groups ($P = 0.009$). The occurrence of diarrhea found in both groups at this time point (after experimental procedures) was lower than the previous occurrence ($P < 0.05$). In addition, diarrhea scores in control animals were higher than in treated animals (Fig. 2). At this time point, *C. difficile*, *C. perfringens* type A beta2-positive, and ETEC were also found as enteropathogens (Table 2). The highest diarrhea scores were found in animals with CDI and, interestingly, animals positive for ETEC and *C. perfringens* type A had diarrhea scores higher than uninfected animals, but lower than animals with CDI ($P < 0.05$; Fig. 2).

The CDI results of the farm and each farrowing room, after administration of Z31, are summarized in Table 3. The occurrence of CDI in treated animals was 7.8% versus 25.0% in the control group ($P = 0.003$). The results of both groups are significantly lower than the previous incidence ($P < 0.05$). In farrowing rooms, the occurrence of CDI was significantly lower in control groups of F1 ($P = 0.026$) and F3 ($P = 0.048$). In addition, administration of NTCD strain Z31 was able to prevent the disease in all animals in rooms F2 and F3, in which the occurrence in the control group was up to 15.0%. In F1, feces collected from some treated animals were positive for A/B toxins, but the occurrence was significantly reduced, and diarrhea was milder when compared to the corresponding control group (Fig. 3).

The microbiological data after administration of NTCD strain Z31 is shown in Table 2. All toxigenic *C. difficile* isolates were considered sequence type (ST) 11 and a reduction was observed in the fecal spreading of toxigenic *C. difficile* in the treated group ($P = 0.003$), especially in F1 and F3. Interestingly, a positive correlation between detection of A/B toxins and toxigenic culture was found (Spearman $\rho = 0.859$, $P < 0.001$). The occurrence of *C. perfringens* type A beta2-positive (23.6%) and ETEC (10.9%), after administration of Z31, was similar to the occurrence calculated before administration of Z31. On the other hand, occurrence of *C. perfringens* type A was higher in the control group when compared to treated animals ($P = 0.032$; Table 2). Coinfection of two or more pathogens were found in six cases (Supplemental Material 1). The diarrhea scores in these animals were similar to those with CDI and toxigenic *C. difficile* was present in five out of six cases. NTCD strains were isolated in 73.3% of the piglets from treated group and in 20.2% of the piglets from control group.

4. Discussion

4.1. Spore production

In a previous study, the dosage of 1×10^6 spores per animal was successfully used to prevent CDI in an experimental model using piglets (Oliveira Júnior et al., 2018b), and therefore was defined as the standard dose in the present study. As such, the number of spores produced in each 500 mL culture bottle in this study was sufficient for use in 100 newborn piglets (Oliveira Júnior et al., 2018a, 2018b). The techniques used to estimate the spore percentage exhibited different outcomes, most likely due to the Wirtz-Conklin technique being more subjective

Table 1
Experimental design used to test a non-toxigenic strain of *C. difficile* (isolate Z31, strain type 3, ribotype 009) against natural *C. difficile* infection (CDI) in piglets located in three farrowing rooms (F1, F2, and F3) on a commercial farm previously diagnosed with CDI. An alternating inoculation of Z31 was applied such that piglets of odd birth order (1, 3, 5, and so on) received Z31, while piglets of even birth order did not receive the strain (negative controls).

Groups	F1		F2		F3		Farm	
	Animals	Litters	Animals	Litters	Animals	Litters	Animals	Litters
Treated*	30	5	30	5	30	6	90	16
Control	24		32		28		84	
Total	54		62		58		174	

* Animals received 10^6 spores of non-toxigenic strain of *C. difficile* (NTCD) Z31 orally.

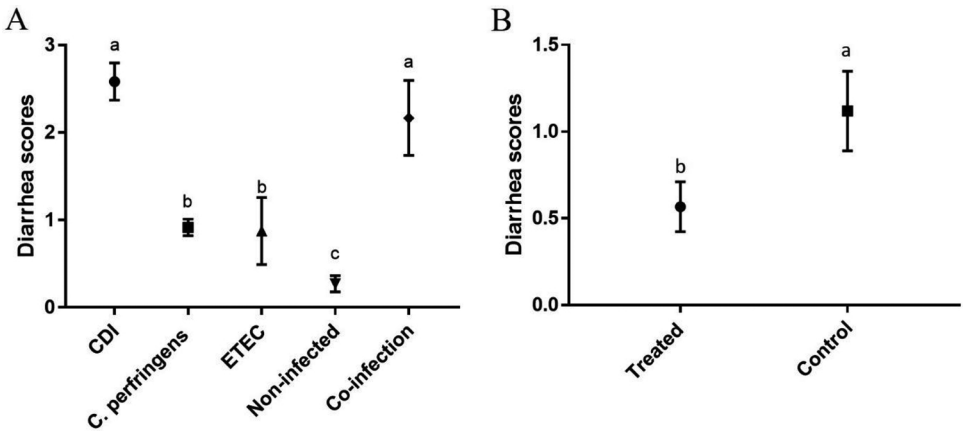

Fig. 2. Scores of diarrhea in 1-day old piglets one day after administration of Z31. In (A) the scores are represented according to infectious agent. In (B) are represented scores of diarrhea of the group treated with non-toxigenic strain of *Clostridioides difficile* (NTCD) Z31 versus control. Different letters means statistical differences by Mann-Whitney test ($p < 0.05$). CDI: *C. difficile* infection (positive for A/B toxins); ETEC: enterotoxigenic *Escherichia coli.*

and the possibility of staining non-viable cells (Hamouda et al., 2002). Nonetheless, spore percentages higher than 90%, found in bacterial counting, were similar to previous studies with Z31, revealing its ability to sporulate without additional steps (Oliveira Júnior et al., 2016, 2018a, 2018b). This high spore production is in agreement with previous results using strain Z31 and would be a highly desirable feature of the strain if it became a commercial product (Songer et al., 2007; Oliveira Júnior et al., 2018a).

4.2. Previous occurrence of NPD and CDI in the farm

Stool sampling a week before the administration of Z31 demonstrated that more than 50% of one-day-old piglets were colonized with *C. difficile*. This finding is in agreement with previous studies that found this pathogen to be commonly associated with this age group (Songer, 2004; Weese et al., 2010). In addition, detection of toxigenic *C. difficile* was highly correlated with A/B toxin detection and all animals in which A/B toxins were detected showed some level of diarrhea, pointing to a high occurrence of CDI (Songer, 2004; Oliveira Júnior et al., 2018b). The morbidity of CDI in pig farms is commonly around 20%, but it can be higher than 50% with cases of diarrhea also being more evident, as was observed in the present study (Songer, 2004; Lippke et al., 2011; Cruz Junior et al., 2013; Silva et al., 2013c). Although *C. perfringens* type A and ETEC were also detected in the present study, only toxigenic *C. difficile* and its toxins were associated to diarrhea, thereby making it the main enteropathogen in one-day-old piglets on this farm.

4.3. Clostridium difficile infection after administration of Z31

The oral administration of Z31 in piglets reduced the occurrence of CDI in this commercial farm, and these results are in agreement with previous studies in which Z31 prevented the occurrence of CDI in hamster and piglet experimental models (Oliveira Júnior et al., 2016, 2018b). The only other report of the use of an NTCD in a commercial farm found a slight reduction in A/B toxin detection in piglets, suggesting a benefit of this strategy in reducing CDI occurrence (Songer

Table 3
Occurrence of *C. difficile* infection (CDI) accessed by detection of A/B toxins in feces of animals which received the non-toxigenic strain of *C. difficile* Z31 and control animals housed in three different farrowing rooms (F1, F2 and F3) on a pig farm. Different letters in columns means statistical differences by Fisher exact test ($P < 0.05$).

Groups	F1 (%)	F2 (%)	F3 (%)	Farm (%)
Treated*	23.3[b]	0.0	0.0[b]	7.8[b]
Control	54.2[a]	12.5	14.3[a]	25.0[a]
Total	37.0	6.5	6.9	16.1

* Received 10^6 spores of non-toxigenic strain of *C. difficile* (NTCD) Z31 orally.

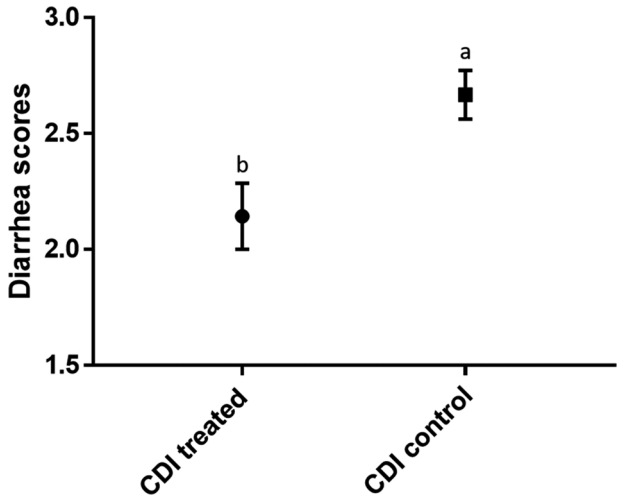

Fig. 3. Scores of diarrhea in piglets from treated and control groups that developed *C. difficile* infection (CDI). Different letters mean statistical differences by Mann-Whitney test ($p < 0.05$).

Table 2
Occurrence of toxigenic *C. difficile* (TCD), *C. perfringens* type A (CP), and enterotoxigenic *Escherichia coli* (ETEC) in animals treated with the *C. difficile* non-toxigenic strain Z31 and control animals from three different farrowing rooms (F1, F2, and F3) on a pig farm. Different letters in columns means statistical differences by Fisher exact test ($p < 0.05$).

Groups	F1			F2			F3			Farm		
	TCD	CP	ETEC	TCD	CP	ETEC	TCD	CP	ETEC	TCD	CP	ETEC
Treated*	20.0[b]	30.0	0.0	3.3	10.0	10.0	0.0[b]	10.0[b]	20.0	7.8[b]	16.7[b]	10.0
Control	54.2[a]	16.7	12.5	18.8	31.3	9.4	17.9[a]	42.9[a]	14.3	28.6[a]	31.0[a]	11.9
Total	35.2	24.1	5.6	11.3	21.0	9.7	8.6	25.9	17.2	17.8	23.6	10.9

* Received 10^6 spores of non-toxigenic strain of *C. difficile* (NTCD) Z31 orally.

et al., 2007). The competitive exclusion, based on the adherence ability of each strain, is hypothesized to be the main mechanism of action of NTCD in the prevention of CDI (Songer et al., 2007; Natarajan et al., 2013; Gerding et al., 2018). This mechanism may explain the reduction in diarrhea intensity of piglets that developed CDI after receiving Z31 in the present study.

The administration of Z31 also reduced the fecal shedding of toxigenic *C. difficile* in treated animals. It is also important to note that all *C. difficile* toxigenic strains isolated in the present study were classified as ST11, which is commonly related to ribotypes 078 and 126, and highly reported in swine worldwide (Keel et al., 2007; Schneeberg et al., 2013; Oliveira Junior et al., 2018c). Sequence type 11 has also been suggested as the zoonotic strain responsible for increasing community-associated CDI in humans (Suo et al., 2017; Wu et al., 2017). It is also important to remember that, in previous studies, Z31 was used to prevent CDI in piglets challenged with a *C. difficile* infection from ribotype 014/020 (ST2), another very common toxigenic type in piglets and humans (Knight et al., 2015; Martin et al., 2016; Oliveira Júnior et al., 2018b). Taken together, these results suggest that Z31 is capable of preventing CDI in piglets challenged with the most common sequence types associated with the disease in humans and piglets. Therefore, the use of NTCD strains might be an interesting preventive strategy considering the One Health concept (Squire and Riley, 2013).

Of note, the incidence of CDI in control animals was lower than the incidence tested before the experiment. Considering the isolation of NTCD in some animals from control group, it could be hypothesized that non-inoculated piglets receive some indirect benefit due to ingestion of spores eliminated into the environment by treated animals once they were all bred together. This indirect benefit of an NTCD was not observed by Songer et al. (2007), who spread NTCD spores on the perineum and teats of sows before delivery, but did not observe differences in A/B toxin detection in newborn piglets. Although it is not a consensus, this indirect benefit should be considered, and further studies should evaluate the direct and indirect long-term effect of continuous NTCD administration in a commercial farm.

4.4. Neonatal porcine diarrhea after administration of Z31

The piglets with CDI, mainly those that did not receive Z31, showed the highest scores of diarrhea, and the results are in accordance with some studies suggesting that *C. difficile* is one of the main causes of NPD (Songer and Anderson, 2006; Cruz Junior et al., 2013; Silva et al., 2013a, 2013c; McElroy et al., 2016). In ETEC isolates, the most common virulence genes found were STaP and adhesin F5 (previously K99), which are commonly reported in ETEC isolates from feces of piglets with diarrhea caused by this agent (Gyles and Fairbrother, 2010). On the other hand, the association of *C. perfringens* type A with diarrhea was surprising. Although the *cpb2* gene (encoding beta2 toxin) was found in all isolates, there is no consensus of its role or of any virulence factor in inducing diarrhea in swine, making it impossible to differentiate commensal *C. perfringens* strains from pathogenic isolates (Cruz Junior et al., 2013; Farzan et al., 2013; Allaart et al., 2014). Interestingly, all piglets with coinfections showed moderate to severe diarrhea, in agreement with other reports, showing that association among pathogens can worsen the clinical signs (de la Fé Rodríguez et al., 2012; Wang et al., 2013).

The reduction in isolation of *C. perfringens* in animals that received Z31 deserves attention. Coinfection of *C. difficile* and *C. perfringens* type A or C has already been described in dogs and foals (Uzal et al., 2012; Diniz et al., 2018). In both cases, the authors hypothesized a possible synergism between these species. In this scenario, it is possible that the reduction in the incidence of CDI conferred by Z31 might have directly or indirectly diminished the colonization by *C. perfringens*.

As previously reported, *C. difficile* was the main cause of NPD in this farm. However, administration of Z31 was able to reduce diarrhea caused by this agent and, consequently, demonstrate a general reduction of NPD in the farm. To the best of our knowledge, this is the first report that an NTCD efficiently and significantly reduced CDI, producing a positive impact on NPD in a commercial establishment (Songer et al., 2007; Arruda et al., 2016). Given that NPD is one of the major problems affecting this swine category, the use of preventive strategies to reduce diarrhea is essential and has potential impacts in the productive chain (Larsson et al., 2016; Ruiz et al., 2016; Oliveira Júnior et al., 2018b).

5. Conclusion

The non-toxigenic strain of *C. difficile* Z31 was able to reduce the occurrence of CDI and NPD in one-day-old piglets on a commercial farm. The present work confirms the potential use of NTCD strains as a preventive method against CDI.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.026.

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