Evaluation of growth and sporulation of a non-toxigenic strain of *Clostridioides difficile* (Z31) and its shelf viability

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Abstract

The oral administration of non-toxigenic strains of *Clostridioides difficile* (NTCD) is currently showing promising results for the prevention of *Clostridioides difficile* infection (CDI) in humans and animals, and is being considered as a possible commercial product to be used in the near future. The aim of this work was to evaluate five culture media for the growth and sporulation of one NTCD (Z31) and evaluate the viability of a lyophilized spore solution of NTCD Z31 stored at 4 °C or at 25 °C for 2 years. Reinforced clostridial medium (RCM) and brain heart infusion broth (BHI) provided the highest production of NTCD Z31 spores. In the first 6 months of the storage of the lyophilized solution, a reduction in spore count of approximately 0.3 Log₁₀ CFU/mL was observed; however, no further significant reduction in spore count was observed up to 24 months. No difference in spore concentration was found between the two storage temperatures from 6 to 24 months of storage. The present work showed BHI and RCM to be the best choices for the growth and sporulation of NTCD Z31 and suggested that the spores of NTCD Z31 are stable for up to 2 years under both temperature conditions.

Keywords Probiotic · Culture media · Long-term viability · Spores

Introduction

Clostridioides (previously *Clostridium*) *difficile* is a strict anaerobic, sporulated, and Gram-positive bacterium that can be classified as toxigenic or non-toxigenic based on the production of toxins A, B, and binary [1, 2]. *C. difficile* is responsible for pseudomembranous colitis in humans as well as diarrhea in animals, such as pigs, horses, and dogs [3, 4]. Indeed, this pathogen is considered one of the most important causes of neonatal diarrhea in piglets [5, 6] and studies performed in Brazil showed that 35% to 53% of herds were affected, with 16% of neonatal piglets positive for *C. difficile* toxins [7–9]. Despite the importance of *C. difficile* in swine, current control measures for *C. difficile* infection (CDI) are based only on

hygiene and disinfection of facilities, and there are no preventive products available to control the disease [3, 6].

Several studies are currently underway aimed at controlling CDI in humans and domestic animals through immunization (active and passive) and the use of antibiotics or probiotics [10–12]. In 1983, Wilson & Sheagren [13] reported that previous colonization by a non-toxigenic strain of *C. difficile* (NTCD) increased the survival rate of hamsters challenged with a toxigenic strain. Following this report, many studies in hamsters, mice, and humans confirmed the protective effect of oral administration of NTCD against CDI [14–19]. In piglets, administration of NTCD in neonates was also demonstrated to be an effective strategy against CDI, as it reduced toxin detection and the appearance of macroscopic and microscopic lesions in these animals [12, 20].

In Brazil, an NTCD strain (named Z31) isolated from a healthy dog presented promising results for CDI prevention. Whole genome sequencing demonstrated the presence of important genes related to colonization and sporulation [21], and studies in an experimental hamster model showed that the strain could prevent CDI in all of the animals tested [22]. Despite these promising results, the commercial utilization of NTCD to prevent CDI requires the significant in vitro

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production of these candidate strains as well as the maintenance of an acceptable shelf viability of their commercial form. Therefore, the aim of this work was to evaluate five culture media for the growth and sporulation of NTCD Z31 and to evaluate the temporal viability of a lyophilized spore solution of NTCD Z31 maintained in two different temperatures for 2 years.

Material and methods

Strain

The NTCD Z31 strain was isolated from a healthy dog in February 2009, in the city of Belo Horizonte (state of Minas Gerais, Brazil). It was confirmed to be non-toxicogenic by PCR owing to the absence of the genes *tcdA*, *tcdB*, and *cdtB* [7] and it was classified as ribotype 009, the most common ribotype among the non-toxicogenic strains in several countries [23, 24]. The complete sequence of the NTCD Z31 genome showed some desirable genes for virulence factors not related to toxin production, such as intestinal colonization (*slpA*) and biofilm formation (*fliC* and *fliD*), which are likely to be important from the perspective of competitive exclusion [21]. This strain also showed a higher spore production than other NTCDs previously isolated and it prevented CDI in 100% of exposed hamsters [22].

Culture media

Five different broth media were used. Two were made according to the recommendations of the manufacturers: brain heart infusion broth (BHI, Oxoid, USA) and reinforced clostridial medium (RCM, Difco, USA). The third medium was BHI supplemented with 0.1% L-cysteine (Merck, USA) and 0.5% yeast extract (Difco, USA) (BHIS) [25]. The fourth medium contained only 1.5% tryptose (Difco, USA) and 1.0% yeast extract (Difco, USA) (TY). Finally, a sporulation broth was used which contained 1.5% tryptose (Difco, USA), 1.1% sodium hydrogen phosphate (Na_2HPO_4 , Synth, Brazil), 0.3% starch (Merck, USA), 0.3% yeast extract (Difco, USA), 0.1% Na-thioglycollate (Synth, Brazil), and 0.01% magnesium sulfate (MgSO_4 , Synth, Brazil) (SB) [26].

Measurement of *C. difficile* growth and spore production

NTCD Z31 was primarily cultured on Müller Hinton agar (Oxoid, UK) supplemented with 5% horse blood and 0.1% sodium taurocholate (Himedia, India) (AST) in an anaerobic chamber (Thermo Fisher Scientific, USA) (80% N_2 , 10% CO_2 , 10% H_2) for 2 days. The colonies were diluted in

phosphate-buffered saline (PBS – 1x, pH 7.4), until the solution reached the tube 1 of the McFarland scale ($\sim 3.0 \times 10^8$ UFC/mL). Subsequently, 5.0 μL of this solution was inoculated in 5.0 mL of each pre-reduced culture medium previously described (1:1000 ratio) and incubated under anaerobic conditions, at 37 °C for 5 days [25]. After this period, cultures were maintained in aerobic conditions at 25 °C $\pm$ 2 °C until day 30. The total numbers of cells and spores were counted on days 3, 5, 10, 15, and 30. Assays were performed in sextuplicate for each medium at each time point [25].

To determine the growth of Z31, the solution from each tube was homogenized, serially diluted in PBS, plated on AST, and incubated under anaerobic conditions for 2 days. To measure spore production, the same procedure was followed with the addition of a single heat treatment (20 min, 70 °C) to eliminate the vegetative cells prior to the serial dilution [22, 25]. Plates containing 30 to 300 colonies were used to determine the amount of Z31 from each culture in colony forming units per milliliter (CFU/mL). The frequency of sporulation was calculated by dividing the spore concentration by the total CFU/mL. In addition, a pool of each broth at each time point was analyzed using the Wirtz-Conklin staining technique to visualize spores [27].

Lyophilization and measurement of long-term viability

The spore solution of *C. difficile* was produced in BHI according to a previously described method with some adaptations [28]. Briefly, two steps of pre-inoculation were performed; each step contained 10% of the total volume of the next inoculum and was incubated for 24 h under anaerobic conditions. *C. difficile* was grown in bottles containing 500 mL of BHI, which were incubated under anaerobic condition for 120 h, then aerobic conditions for 120 h. After this period, the solution was centrifuged (3000 $\times g$, 30 min, 4 °C) (Hettich, Germany) and the pellet was subjected to shock with ethyl alcohol (1:2) at 37 °C for 30 min. The solution was centrifuged again, and the pellet was suspended with the same volume of PBS. This procedure was repeated eight times. The solution was then subjected to a heat treatment (20 min, 70 °C) to eliminate the vegetative cells. Three aliquots were tested using the Wirtz-Conklin staining technique [27] to determine the proportion of spores using an optic microscopic (Nikon, Japan).

The final solution was used to count the number of spores on AST plates, as previously mentioned, and diluted to a final concentration of 10^8 spores/mL. One milliliter of this solution was aliquoted into tubes, which were frozen (24 h, –80 °C), lyophilized in a freeze dryer (Enterprise, Terroni, Brazil), and maintained at two different temperatures (4 °C and 25 °C) for 2 years. The number of spores was counted after the dilution of the lyophilized strains in 1 mL of PBS following the same procedures for spore counting described above. The analyses

were done in triplicate 1 day after the counting procedure and were performed monthly for 12 months and then again at 18 and 24 months after lyophilization.

Statistical analysis

The spore counts were converted to a base 10 logarithm and analyzed by ANOVA and Student's *t* test. A *p* value of < 0.05 was considered to be statistically significant. Linear and non-linear regressions were constructed for the data related to long-term viability. The analysis was performed in GraphPad Prism 7 (GraphPad Software, USA).

Results

Growth and sporulation

The growth of NTCD Z31 varied from 3.86×10^4 CFU/mL (TY, 3 days) and 7.15×10^6 CFU/mL (RCM, 5 days) for the media tested (data not shown). Spore production of NTCD Z31 varied

from 2.24×10^4 CFU/mL (TY, 3 days) to 6.57×10^6 CFU/mL (BHI, 5 days) (Fig. 1A). At day 3, culture in RCM provided the highest growth and spore production. From day 5 on, culture in RCM and BHI showed similar results, followed by culture in SB, BHIS, and then TY (Fig. 1A). The frequency of sporulation at 72 and 120 h was between 68.1% and 80.5% for the culture media tested, except in BHIS, which featured a sporulation rate under 50.0% at both time points. After storage in aerobic conditions, all culture media showed sporulation rates above 98.0% at days 10, 15, and 30 (Fig. 1B).

Long-term viability

After spore production, solutions containing 10^8 spores of NTCD Z31 were lyophilized, stored at 4 °C (cooled) or 25 °C (room temperature), and tested for their long-term viability during the course of 24 months. A slight decrease in the spore count was observed in both storage temperatures in the first 12 months of the experiment; however, this decrease was followed by the stable maintenance of spore counts for up to 24 months (Fig. 2A). Statistical differences between the two

Fig. 1 Sporulation of NTCD Z31 in five different culture media. All cultures were incubated in anaerobic conditions at 37 °C until day 5, after which, were kept in aerobic conditions at 25 °C until the end of experimentation. (A) Spore counts of NTCD Z31 for each growth medium (Log 10 CFU/mL). (B) Frequency of sporulation of NTCD Z31 for each growth medium. Different letters at a same time of evaluation indicate statistical differences between the sporulation rates of NTCD Z31 in each culture media at that moment (*p* < 0.05)

Fig. 2 Spore counts of lyophilized NTCD Z31 stored over a 24-month period at 4 °C or 25 °C. (A) Spore counts and the standard deviation of samples at 4 °C or 25 °C. (B) Non-linear regression model of the original data

storage temperatures were only observed in the fourth and fifth months, with higher concentrations of spores being found in samples maintained at room temperature. No differences were found from 6 months until the end of experiment.

Linear regression performed using the data from the first 12 months of counting estimated that the spore concentrations of NTCD Z31 obtained after 24 months of storage were 1.66×10^7 CFU/mL for the cooled samples and 1.55×10^7 CFU/mL for the room temperature samples, with R^2 values of 81.0% and 94.4%, respectively (data not shown). A non-linear regression using all data obtained in this experiment was also performed (Fig. 2B), because the spore concentration showed relative stability in the last months of measurements. The estimated spore concentration of NTCD Z31 from this model during the stable period was 4.16×10^7 CFU/mL for cooled storage and 3.85×10^7 CFU/mL for room temperature storage (R^2 of 90.58% and 90.67%, respectively). These results were statistically similar to the actual concentrations found in the final measurement of the experiment (4.17×10^7 CFU/mL for cooled storage and 3.92×10^7 CFU/mL for room temperature storage).

Discussion

Growth and sporulation

CDI is an important disease in both humans and domestic animals and the use of bacterial probiotics could be a viable alternative to prevent this disease [12]. NTCD Z31 is a possible candidate to be used for the prevention of CDI, because it was able to prevent disease in 100% of treated hamsters [22]. Despite these results, it is essential to understand the in vitro growth and sporulation characteristics of the strain NTCD Z31 to assess the viability of its commercial use. The quantification of total cells and spores in present study was performed based on colony-forming units (CFU), similar to other studies [25, 29, 30]. Experiments with toxigenic strains of *C. difficile* found spore concentrations between 10^6 and 10^7 CFU/mL after 2 days of incubation and over 10^7 CFU/mL after 3 and 5 days of incubation [25, 30]. Although these values are higher than those found in the present study, comparisons between different studies must be done cautiously, as even small differences in methods may cause significant alterations in the growth and sporulation of the strains [31]. Despite this, the average of the spore concentrations found in two culture media tested (RCM and BHI) were higher than the suggested concentration to prevent CDI in swine (2.0×10^6 spores/mL), which could represent viable productivity if this value could be maintained in large scale [12].

The best results for the growth and sporulation of NTCD Z31 in present study were observed in RCM and BHI media. RCM has been used for the isolation of *C. difficile* and contains the components and nutrients necessary to support growth of *Clostridia* and other anaerobic bacteria [32]. Although culture in RCM provided adequate conditions for growth and spore production during this experiment, the addition of agar in the formula hinders the separation of spores from culture media, which could be a limitation when larger volumes are used. For this application, culture in BHI appears to be the best option among the tested media, because it showed similar results to culture in RCM and is an inexpensive culture media.

Many studies that evaluated *C. difficile* growth and sporulation used BHIS [25, 33]; however, in the present study, culture in this medium produced a concentration of spores more than ten times lower than that produced in the culture with BHI. The addition of L-cysteine in BHIS is the main difference of this broth in comparison to BHI. Although it is not essential for growth, cysteine is one of the main sources of carbon and energy when added to a culture medium, and it is consumed at the beginning of the growth phase [34]. Cysteine also leads to changes in *C. difficile* metabolism, such as reductions in the production of toxins, short-chain fatty acids (SCFAs), and enzymes, including 3-hydroxybutyryl-CoA dehydrogenase [35]. This enzyme is responsible for

solventogenesis in *Clostridium acetobutyricum*, a process that converts SCFAs to ketones and alcohols. Solventogenesis is linked to sporulation in *C. acetobutyricum*, with mutants unable to convert butyric acid to butanol, resulting in poor sporulation [36]. Similarly, this may have led to the low sporulation rate of NTCD Z31 when cultured in BHIS media (Fig. 1B). It is noteworthy that inclusion of cysteine in anaerobic culture media is fairly common, because it is a reducing agent that improves the growth of anaerobic bacteria when conditions are not optimal [37, 38]. Interestingly, some culture media indicated for sporulation use cysteine, even in higher concentrations when compared to present study [39]. Nevertheless, there are many differences among the sporulation patterns of each strain, which make generalizations difficult and reinforce the importance of specific studies focusing on the growth and sporulation of a particular strain [25].

With the exception of BHIS, the sporulation ratio in present study was higher than 68% after 3 and/or 5 days of culture. Previous studies evaluating different strains of *C. difficile* found sporulation rates between 1% and 60% after 2 or 3 days of culture, with some evidence that endemic and hypervirulent strains are associated with enhanced sporulation rates [29, 30, 33, 40]. It is known that spore production increases with incubation time, and recent studies have shown that even non-endemic strains can sporulate at high rates [25, 33, 41]. In this context, NTCD Z31 seems to be a strain with a relatively high sporulation rate, confirming a prior study where NTCD Z31 was the most efficient strain in spore production when compared to four NTCD isolated from different animals [22]. This is an important feature to consider for its possible use as a commercial preventive method, because the administration of spores, and not vegetative cells, has been shown to prevent the disease in piglets [12, 20].

It is worth noting that the sporulation rate found in the present study was higher than 98% after aerobic storage, and the spore concentration was maintained from day 5 until the end of experiment in all culture media. Oxygen present in the atmosphere is toxic to anaerobic microorganisms, such as *C. difficile*. Under aerobic conditions, vegetative cells undergo apoptosis or begin the sporulation process [38]. Despite this, obtaining a solution with a high proportion of spores is difficult in cultures of *Clostridium*, leading to the requirement of additional steps such as heat treatment, alcohol shock, sonication, enzymatic treatment, centrifugation, or a combination of these methods [28, 42, 43]. The NTCD Z31 strain showed a high sporulation rate without any additional steps, which is a promising feature for its potential on a commercial scale.

Long-term preservation

C. difficile spores are commonly stored cooled or frozen and the spore concentration in this storage condition is usually

maintained or slightly decreased between 30 and 56 days of storage [44–46]. In the first step of present study, the spore concentration kept stable in solutions maintained at 25 °C for 30 days. The choice of the two storage temperatures in this study was made considering these previous results and the difficulty of maintaining a cold chain for commercial product delivery in many regions. Additionally, although previous studies have stored spores in a spore solution, the option of lyophilization was selected because this method is one of the most conservative for evaluating long-term viability and it is often applied for biotechnological or commercial use. Furthermore, lyophilization is a safe, stable, and suitable method commonly used for the long-time preservation of bacteria [47–49].

In the first 6 months of storage, a slight decrease in spore concentration was observed at both temperatures. It is known that some spores are capable of germinating in adverse conditions owing to defects in exosporium and spore coat, or spontaneous activation of genetic factors linked to germination [50]. In that case, the decrease in spore count could be explained by apoptosis of vegetative cells from these defective spores. Another explanation is linked to dormancy of spores, which could be accelerated at lower temperatures [51]. This hypothesis could explain differences observed in the fourth and fifth months of counting, when the spores maintained at room temperature showed higher concentrations than those maintained at 4 °C. Results obtained in the last 18 months of experimentation showed maintenance of spore counts for the duration of the experiment.

It should be highlighted that both forms of storage provided the same final concentration of spores. Importantly, this shows that cold chain maintenance, which increases costs and is not practical in many situations, is not necessary for the storage of viable NTCD Z31 [52]. In addition, previous studies using NTCD strains to prevent CDI in piglets have suggested a dosage of approximately 10^6 spores per animal [12, 20]. Following these recommendations, the number of NTCD Z31 spores found in a single bottle after 2 years of storage (around 4.0×10^7 CFU/mL) would be able to prevent CDI in 40 animal; however, further studies on target species will be necessary to confirm this dosage.

Conclusion

The NTCD Z31 strain showed a high sporulation rate when cultured in BHI and RCM, and these spores were highly stable after 2 years of storage under both cooled and room temperature conditions. Additional studies evaluating the ability of NTCD Z31 to prevent disease in target species are needed to corroborate its potential use for the prevention of CDI.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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