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PROPERTIES OF THE *BACILLUS CEREUS* STRAIN USED IN PROBIOTIC CenBiot

Carlos Gil-Turnes*, Andrea Freitas dos Santos, Flávia Weykamp da Cruz, Alegani Vieira Monteiro

Centro de Biotecnologia (CenBiot), Universidade Federal de Pelotas, Pelotas, RS, Brazil.

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SHORT COMMUNICATION

ABSTRACT

Bacillus cereus CenBiot fulfilled the requirements to be used as probiotic. The spores showed D_{80} of 14 hs, inhibited *Escherichia coli* and *Yersinia pseudotuberculosis* after 24 hs in associative culture, were innocuous for suckling and adult mice and were not inhibited by antibiotics at low concentrations.

Key words: probiotic, *Bacillus cereus*

Probiotics are "viable mono or mixed cultures of microorganisms which, applied to animal or man, beneficially affect the host by improving the properties of the indigenous flora" (10). They must promote growth, improve feed conversion and inhibit enteropathogens, without causing any undesirable effect. In addition, they must survive the stress produced during manufacturing, storage and administration at farm conditions.

The search for new probiotic strains has increased in recent years due to the necessity to find economic and effective substitutes for antibiotics used as feed additives. Several strains of *Lactobacillus*, *Pediococcus*, *Bacteroides*, *Bifidobacterium*, *Bacillus*, *Streptococcus* and *Escherichia coli*, alone or consortiated, have been used as probiotics (8), giving controversial results. Among the various species of probiotics, those belonging to the genus *Bacillus* have the advantage that, due to their capacity to sporulate, they survive at ambient temperatures as well as during desiccation by methods that involve moderate heating, such as spray dryers, avoiding the use of lyophilization or other expensive technologies (10). This property also makes possible the administration of spores mixed with powdered vehicles instead of gels or liquids used with non sporulated bacteria. Although several strains of different species of *Bacillus* are being used for this purpose, information concerning their properties was seldom reported.

Probiotic CenBiot, prepared with a strain of *Bacillus cereus* at Centro de Biotecnologia of Universidade Federal de Pelotas, Rio Grande do Sul, Brazil, was tested in commercial farms showing beneficial effects in the control of diarrhoea and feed conversion in pigs (21). The effects of some factors that could affect the survival of *Bacillus cereus* in the intestinal tract and during manufacturing, such as interaction with enteropathogens, resistance to heat and to variation of pH, are reported in this work.

The strain was classified as *Bacillus cereus* due to its morphology, Gram staining, capacity to form

*Corresponding author. Mailing address: Centro de Biotecnologia (CenBiot), Universidade Federal de Pelotas, Campus Universitário, CEP 96010-900, Pelotas, RS, Brasil. Fax: (+5553) 275-7550. E-mail: gil@ufpel.tche.br

thin wall spores in aerobic conditions, growth in glucose broth under anaerobic conditions, production of lecithinase and acetyl-methyl-carbinol and absence of urease (20). To obtain spore suspensions, cultures were heated at 80°C for 15 minutes, centrifuged, and after three successive washings with ultrapure water, resuspended in ultrapure sterile water pH 7.2.

To test the effect of heat, aliquots of 300µl of spore suspensions were heated in a water bath at 50°C for 4 days, 80°C for 60 min, 85°C for 60 min and 90°C for 40 min. Samples were collected every 24 hours, 10 min, 10 min and 2 min, respectively, and immediately chilled in iced water. Decimal dilutions in saline were plated on sheep blood agar, incubated at 37°C for 24 hours and the Colony Forming Units (CFU) counted. D values were calculated following Frank and Campbell (7). At 50°C the counts increased 3.7 times after 4 days, probably due to heat activation. D values were 14.28 hours at 80°C, 10.7 min at 85°C and 1 min at 90°C. Results are the means of at least three experiments.

Trying to foresee how the pH of the gastrointestinal tract could affect viability, spore suspensions were adjusted to pH 1.0 with HCl and to pH 7.6 with NaOH and held at 37°C for 24 hours. Another suspension was incubated at pH 1.0 for four hours and then adjusted to pH 7.6 and incubated for the following 20 hours. Samples taken at 0, 2, 4, 6 and 24 hours, were processed as above to count CFU. The concentrations of viable spores after 24 hours remained almost unchanged in the suspensions at pH 7.6, but they decreased to 35 % of the initial value in those held at pH 1.0 for 4 hours and at pH 7.6 for another 20, and to 11 % in those at pH 1.0 (Fig. 1). Results are the means of at least three experiments. This decrease in viability may explain the necessity of supplying *Bacillus* based probiotics in high concentrations and on a daily basis (6). It has been shown that results obtained *in vitro* highly correlate with those obtained *in vivo*, thus allowing the use of the formers in the selection of probiotic candidates (10).

Eight-day-old mice were inoculated intragastrically with 1 x 10^9 spores and observed for 14 days. Adult mice were fed for 30 days with granulated mice feed containing Probiotic CenBiot at a concentration of 1 x 10^9 spores per kg. Undesirable reactions were not produced in suckling or in adult mice during this experiment, neither in 248 piglets in the suckling phase and 199 in the nursery phase used in field tests (21). Even considering that many strains

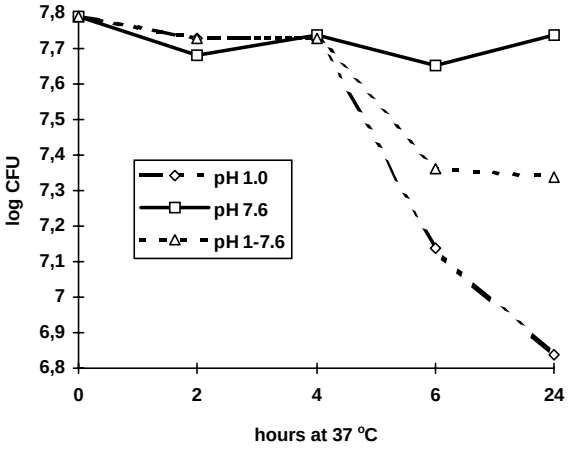

Figure 1 - Effect of pH on the viability of *B. cereus* CenBiot spores incubated at 37°C

of different species of *Bacillus* produce toxins harmful to man and animals, some are innocuous (5) or even beneficial (18), as was demonstrated for *B. cereus* CenBiot in the experiments here reported.

Yersinia pseudotuberculosis O III recovered from water buffaloes with diarrhoea, *Escherichia coli* K88ac recovered from piglets with diarrhoea, and *E. coli* O147 (O147:K89, 88ac:H19) from the Statens Serum Institut, Copenhagen, Denmark, were grown in BHI agar overnight and suspended in saline to A_{450} = 0.6. 500µl of each strain was inoculated into two 50 ml Erlenmeyer flasks containing 10 ml of BHI; to one flask 500µl of a suspension of *B. cereus* CenBiot with A_{450} = 0.6 was added. The flasks were incubated in a G24 Environmental Incubator Shaker (NBS) at 37°C and 250 rpm for 24 hours. Then, the cultures were decimally diluted in saline, plated on MacConkey agar and incubated at 37°C for 24 hours, when the CFU/ml were determined. The counts of *E. coli* K88ab and of *Y. pseudotuberculosis* dropped to 33.3 % and that of *E. coli* O147 to 30 % after growth in association with *B. cereus* CenBiot, when compared with the strains grown alone (Fig. 2). These results could be related to those of field experiments in which *Escherichia coli* K88ac was not recovered from pigs fed Probiotic CenBiot, while it was isolated from 20 % of diarrhoeic faeces collected from control animals (21). Apella *et al.* (1) used a similar procedure to show the inhibitory effect of two species of *Lactobacilli* on *Shigella sonnei*, and Hillman and Fox (11) also used associative cultures of *Lactobacillus* and enterotoxigenic *E. coli* to screen probiotic strains.

Plasmids were not obtained from suspensions of

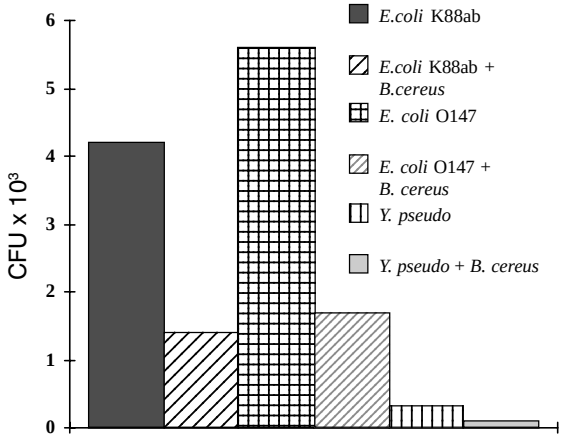

Figure 2 - Effect of *B. cereus* CenBiot on the growth of two *E. coli* strains and *Y. pseudotuberculosis*

B. cereus CenBiot by the extraction methods of Kado and Liu (3) and Birnboim and Doly (9). Plasmids encode several products related to the beneficial effects attributable to probiotics, such as the bacteriocins of *Lactobacillus lactis* (16) and lactococcal proteinases (4), suggesting that the probiotic effect showed by *B. cereus* CenBiot (21) is produced by other mechanisms not involving bacteriocins.

The effect that antibiotics commonly used in Brazil as food additives, or for therapeutic purposes, could produce on *B. cereus* CenBiot was also studied in this work. Sensitivity to Cloramphenicol (10, 40, 160 and 320µg / ml), Tetracycline (10, 40, 160 and 320µg / ml) and Kanamycin (1.25; 2.5; 5 and 10µg / ml) was tested by the minimal inhibitory concentrations method, and for Streptomycin (10µg), Erythromycin (15µg), Gentamycin (10µg), Cloramphenicol (30 µg) and Tetracycline (30µg) by the disk method (2). The strain was inhibited by all the antibiotics tested by the disk method, but not by Cloramphenicol, Tetracycline and Kanamycin at a concentration of 10µg / ml of medium. Several authors claim the utility of using probiotics in association with antibiotics, aiming to optimise the effects of both (12). Synergism between these additives, however, is not always produced. Tortuero *et al.* (19) reported that *Bacillus cereus* fed in association with Virginiamicin showed beneficial effects in piglets, while Roth and Kirchgessner (14) found that Olaquindox did not enhance the probiotic effect of *Streptococcus faecium*.

Spores were not affected by the temperatures used in feed processing. Their viability at 80ºC was 94.6 % after 10 minutes and 85.7 % after one hour, showing that the spores may be desiccated in spray dryers and pelletized (10) without appreciable loss of viability. Russel (15) showed that D values increased 10 fold every 10ºC fall in temperature; therefore, it may be expected that at temperatures at which the probiotic is stored, the loss in viability shall be inexpressive (17), as it was confirmed by our observations after several months of storage (data not shown). Heat activation was detected after heating at 50ºC during 96 hours. However, 80ºC seems to be the highest temperature to which the spores may be heated without substantial loss of viability, as can be deduced from the D_{85} of 10.7 min and D_{90} of 1 min. *B. cereus* CenBiot seems to be more resistant to heat than *Bacillus* C.I.P. 5832 used successfully as probiotic (13).

The results reported show that *B. cereus* CenBiot fulfils the requirements to be a suitable candidate for probiotic elaboration (10).

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RESUMO

Propriedades da cepa de *Bacillus cereus* utilizada no probiótico CenBiot

Bacillus cereus CenBiot possui as características necessárias para ser utilizada como probiótico. Os esporos apresentaram D_{80} de 14 hs, inibiram *Escherichia coli* e *Yersinia pseudotuberculosis* após cultivadas associativamente por 24 hs, foram inócuos para camundongos lactentes e adultos e não foram inibidos por antibióticos a baixas concentrações.

Palavras-chave: probiótico, *Bacillus cereus.*

REFERENCES

1. Apella, M.C.; González, S.N.; Macías, M.E.N.; Romero, N.; Oliver, G. *In vitro* studies on the inhibition of the growth of *Shigella sonnei* by *Lactobacillus acidophilus*. *J. Appl. Bacteriol.*, 73: 480-483, 1992.
2. Balows, A.; Hausler, W.J.; Herrmann, K.L.; Isenberg, H.D.; Shadomy, H.J. *Manual of Clinical Microbiology*, American Society for Microbiology, Washington, 1991. Chapters 110 and 111.
3. Bergquist, P.L. Incompatibility. In: *Plasmids: a practical approach*. K.G. Hardy, ed., IRL Press Limited, Oxford, England, 1987. Chapter 3.
4. Coffey, A.G.; Daly, C.; Fitzgerald, G. The impact of biotechnology on the dairy industry. *Biotechnol. Adv.*, 12: 625-633, 1994.
5. de Boer, A.S.; Priest, F.; Diderichsen, B. On the industrial use of *Bacillus licheniformis*: a review. *Appl. Microbiol. Biotechnol.*, 40: 595-598, 1994.
6. De Cupere, F.; Deprez, P.; Demeulenaere, D.; Muylle, E. Evaluation of the effect of 3 probiotics on experimental *Escherichia coli* enterotoxemia in weaned piglets. *J. Vet. Med. B*, 39: 277-284, 1992.
7. Frank, H.A.; Campbell, L.L. The influence of recovery media on thermal resistance values of spores of a putrefactive anaerobic bacterium. *Appl. Microbiol.*, 3: 300, 1955.
8. Fuller, R. Probiotics. *J. Appl. Bacteriol.* SS, 1S-7S, 1986.
9. Hardy, K.G. Purification of bacterial plasmids. In: *Plasmids: a practical approach*. K.G. Hardy, ed., IRL Press Limited, Oxford, England, 1987. Chapter 1.
10. Havenaar, R.; Brink, B.T.; Veld, J.H. Selection of strains for probiotic use. In: *Probiotics: the scientific basis*, R. Fuller ed., Chapman & Hall, London, 1992, p 209-224.
11. Hillman, K.; Fox, A. Effects of porcine fecal lactobacilli on the rate of growth of enterotoxigenic *Escherichia coli* O149:K88:K91. *Lett. Appl. Microbiol.*, 19: 497-500, 1994.
12. Kreuzer, M. Probiotic-antibiotic interactions in performance, intestinal fermentation and manure properties of piglets using a *Bacillus* (*B. licheniformis* / *B. subtilis*) preparation and Carbadox. *Agribiol. Res.*, 47: 13-23, 1994.
13. N'Guyen, T.H. Effects zootechniques et sanitaires du bioregulateur: Paciflor ND. *G.T.V.*, 6: 39-52, 1990.
14. Roth, F.X.; Kirschgessner, M. Zur nutritiven Wirksemkeit von *Streptococcus faecium* (Stamm M74) in der Ferkelaufzucht. *Landwirtschaftliche-Forschung*, 39: 198-205, 1986.
15. Russel, A.D. *The destruction of bacterial spores*. Academic Press, London, 1982.
16. Scherwitz-Harmon, K.M.; McKay, L.L. Restriction enzyme analysis of lactose and bacteriocin plasmids from *Streptococcus lactis* subsp. *diacetylactis* WM 4 and cloning of *Bcl*I fragments coding for bacteriocin production. *Appl. Environ. Microbiol.*, 53: 1171-1174, 1987.
17. Setlow, P. Mechanisms that contribute to the long term survival of spores of *Bacillus* species. *J. Appl. Bacteriol.* SS., 76: 49S-60S, 1994.
18. Smoragiewicz, W.; Bielecka, M.; Babuchowski, A.; Boutard, A.; Dubeau, H. Les probiotiques. *Can. J. Microbiol.*, 39:1089-1095, 1993.
19. Tortuero, F.; Rio Perez, J.; Martin, L.; Vinaras, R. *Bacillus cereus* y Virginiamicina en dietas para lechones. *Archiv. Zootechny*, 39: 67-75, 1990.
20. Wolf, J.; Baker, A.N. The genus *Bacillus*: aids to the identification of its species. *In:* B.M. Gibbs and D.A. Shapton, eds., *Identificaton methods for microbiologists*, Part B, Academic Press, London, 1968, p 93-109.
21. Zani, J.L.; da Cruz, F.W.; dos Santos, A.F.; Gil-Turnes, C. Effect of probiotic CenBiot on the control of diarrhoea and feed efficiency in pigs. *J. Appl. Microbiol.*, 84: 68-71, 1998.