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GROWTH OF ENTEROTOXIN PRODUCING *BACILLUS CEREUS* IN MEAT SUBSTRATE AT 10ºC AND 30ºC

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ABSTRACT

The behaviour of enterotoxin-producing *Bacillus cereus* in meat was investigated by inoculating spore suspensions of five cultures into meat substrate (pH 5.8) and incubating at 10ºC and 30ºC. The bacterial populations were evaluated after different times by plate counts in nutrient agar. All the cultures presented growth at 30ºC with the generation time varying from 28.8 to 36.0 minutes. Three cultures also presented growth at 10ºC with generation times between 10.16 and 28.38 h. Considering the results, it was concluded that meat kept at abusive temperatures would be subject to development of this microorganism.

Key words: *Bacillus cereus*, meat, growth

INTRODUCTION

Bacillus cereus is a spore-forming organism capable of developing at a wide range of temperatures, pH and water activity values (24; 25; 28). This bacterium, frequently associated with food borne diseases (5), can be found in the natural environment (soil, water and air) and isolated from various foods, including meat and meat products (15; 23).

Some of the enterotoxins produced by *B. cereus* cause food borne diarrhoea, characterised as the "diarrheic syndrome" (17; 21). This syndrome can be associated with the production of five different proteins: haemolysine BL enterotoxin (HBL) (3); non-haemolytic enterotoxin (NHE) (18); cytotoxin K (Cyt K) (17); enterotoxin T (BcET) (1) and enterotoxin FM (Ent FM) (2). The symptoms last for a period of from 12 to 24 hours and consist of abdominal pain, watery diarrhoea and nausea. It is believed that the enterotoxins are mostly produced in the small intestine of the individual (11).

Foods associated with outbreaks of *B. cereus* diarrhoea frequently include protein-rich foods (26) and researchers have emphasised the importance of the presence of the microorganism or its spores in meat and meat products for the occurrence of such outbreaks (22). Practices such as inadequate cooling after heat treatment and storage at inadequate temperatures during food processing are indicated as the main factors contributing to the spread of the microorganism in these foods (13; 22).

With the objective of investigating the growth of enterotoxin-producing *B. cereus* in processed meat, this study was carried out using cultures of the microorganism from different origins (food and environment) experimentally inoculated into meat substrate kept at 10°C and 30ºC.

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MATERIALS AND METHODS

Microorganism and its culture conditions

Four cultures of enterotoxin-producing *B. cereus* were used in this experiment, isolated from samples collected during the processing of a meat dish in an institutional restaurant. The cultures, isolated from the ambient air (BcA), equipment surface (BcE), seasoning (BcS) and processed meat (BcM), were confirmed and identified as *B. cereus* according to the methodology recommended by the Food and Drug Administration (9). In addition a reference diarrheic toxin-producing strain (*B. cereus* NCTC 11145) was used. After identification, the cultures were maintained in nutrient agar (Merck) at 4ºC. The toxigenic potential of the five cultures was confirmed using the Bacillus Diarrhoeal Enterotoxin Visual Immunoassay (kit BDE, vs MS2207 05/01; Tecra, Roseville New South Wales, Australia) according to the manufacturer's instructions.

Before use in the experiment, each of the stock *B. cereus* cultures was submitted to cell activation by inoculating into 10 mL BHI (Brain heart infusion broth; Difco; pH 7.4) at 30ºC. This procedure was repeated successively three times, transferring one loopful of culture every 18 h.

Spore suspensions

Spore suspensions of the cultures were prepared according to methodology described in the literature (5; 7; 20; 27). After cell activation in BHI broth, the cultures were inoculated into Petri dishes containing sporulation medium (nutrient agar supplemented with 0.05% manganese sulphate) and incubated at 30ºC for 4 days, when spore formation was confirmed by a microscopic examination. With the help of a Drigalski loop and the addition of 10 mL Milli Q water, the culture mass, with 85% or more sporulation, was aseptically removed from the surface of the culture medium. The total volume of the suspensions of each culture was transferred to a sterile tube and centrifuged at (5000 x *g*) (6ºC/20 min). The supernatant was discarded and the pellet re-suspended in Milli Q water and centrifuged again. This procedure was repeated five times and the resulting pellet re-suspended in Milli Q water and maintained at 4ºC.

Before inoculation into the meat substrate, the *B. cereus* spore suspensions were submitted to heat shock at 75ºC for 10 min and standardised with respect to the spore concentration (approximately 10^4 spores/mL). This standardisation was carried out by successive decimal dilutions in phosphate buffer (Butterfield's phosphate-buffered dilution water, pH 7.2), and inoculation and counting of the colony forming units (CFU) in Petri dishes containing nutrient agar, incubated at 30ºC for 24 h. The purity of the suspensions was verified by inoculation into a medium selective for *B. cereus* (MYP agar; Mannitol yolk polymixin agar; Difco).

Meat substrate, inoculation and counting of *B. cereus*

Growth of the *B. cereus* cultures was studied in triplicate, inoculating spore suspensions into meat substrate incubated at 10ºC and 30ºC. Approximately 1 kg beef (*semitendinosus* muscle), obtained on the local retail market, was sterilised under pressure in an autoclave (111ºC to 115ºC/1 h), cooled and stored at 4ºC for about 18 h. The pH of the cooked meat was determined using a pH-meter (Micronal; model B-374).

Meat samples (10 g) were weighed aseptically in sterile pouches for the stomacher (model 400; Stomacher Labsystem Circulator Bag 6141) and pre-incubated at 10ºC or 30ºC for about 1 h before inoculation. The samples were then inoculated with 1 mL of spore suspension (approximately 10^4 spores/mL), manually homogenised for 15 s and incubated at 10°C and 30ºC.

For the experiment at 10ºC, the inoculated samples were incubated for 7 days and analysed after 0, 1, 2, 3, 4, 5, 6 and 7 days of incubation. For the experiment at 30ºC, the samples were analysed after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. For the bacterial count, 90 mL of sterile phosphate buffer were added to the samples for homogenisation in the stomacher (Stomacher 400 Circulator; Seward) at 200 rpm for 1 min. The *B. cereus* populations were determined by way of appropriate

decimal dilutions and inoculations (0.1 mL) into duplicate nutrient agar plates, incubated at 35ºC for 20-24 h. The absence of contamination of the inoculated samples was verified by regularly using a selective medium for *B. cereus* (MYP agar; Difco). A negative control (non-inoculated sample) was used in each of the experiments to verify the absence of contaminants. These controls were analysed after the incubation period.

Generation time

The generation time (*g*) of the microorganism in the meat substrate was calculated from the angular coefficient of the regression straight line, resulting from the mean values of the bacterial counts verified in the three repetitions of the experiments, considering the exponential bacterial growth phase. The equation described by Madigan, Martinko, and Parker (19) was used (g = 0.301/angular coefficient).

Statistical analysis

The statistical analysis was carried out by calculating the means of the bacterial counts for the three repetitions with their respective standard deviations.

RESULTS AND DISCUSSION

From the results obtained in the present study it was possible to observe that the *B. cereus* spores germinated in the meat substrate at both 10ºC and 30ºC. All the *B. cereus* cultures grew at 30ºC, the bacterial populations increasing about 5 logarithmic units after 12 hours incubation at this temperature (Figure 1). At 10ºC, three cultures (BcA, BcE and BcS) showed growth, with a maximum increase of 4.3 logarithmic units after 168 h of incubation (Figure 2). In general, typical *B. cereus* strains are mesophiles, multiplying between 10ºC and 48ºC with an optimum range between 28ºC and 35ºC (10). According to the literature, at temperatures between 5ºC and 10ºC some psychrotrophic and mesophilic strains are capable of producing populations of up to 10^6 CFU/g and of producing toxin in foods (6; 8;12; 29). The pH of the meat substrate used in this study was 5.80. Studies have affirmed that *B. cereus* is capable of growing in substrates with pH values between 4.3 and 9.0 (16).

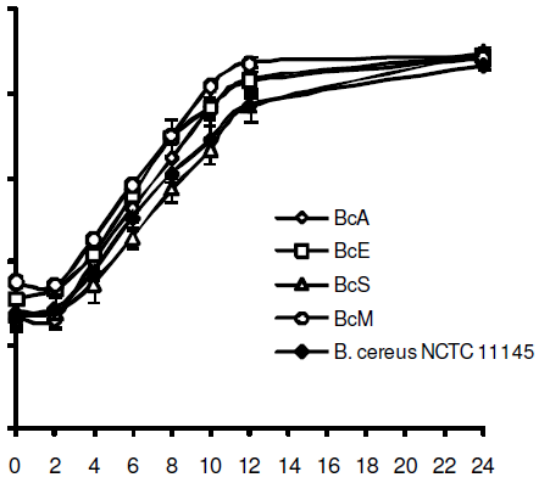

Figure 1. Growth of *B. cereus* in meat substrate at 30°C. The symbols represent the means (± standard deviation) of the three repetitions of each experiment. The standard deviation bars do not appear when the symbols are greater than the values of the standard deviation.

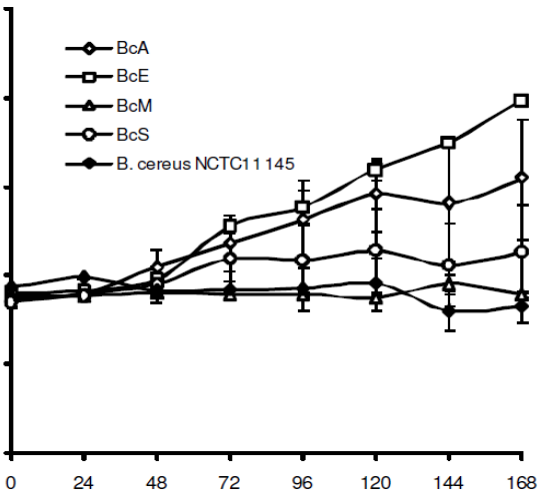

Figure 2. Growth of *B. cereus* in meat substrate at 10°C. The symbols represent the means (± standard deviation) of the three repetitions of each experiment. The standard deviation bars do not appear when the symbols are greater than the values of the standard deviation.

The bacterial generation time (*g*) at the different temperatures was calculated considering the results obtained (Table 1). At 30ºC the generation time of the microorganism varied from 0.48 to 0.60 h. Harmon and Kautter (13) studying ready to serve foods maintained at 26ºC, and Valero et al. (27) in a study in nutrient broth at 30ºC, reported longer generation times than those obtained in the present study, between 0.72 and 0.97 h and between 0.69 and 1.09 h, respectively. These variations were possibly determined by interactions between characteristics of the strain under test and extrinsic factors, such as the temperature, pH and substrate in which the microorganism was maintained. In studies on the behaviour of *B. cereus*, variations in the generation time, growth, toxin production and destruction of the microorganism or its spores have been reported (4; 14; 29).

At 10ºC the generation time oscillated between 10.16 and 28.38 h (Table 1). These values are greater than those reported in other experiments with *B. cereus* strains maintained at low temperatures in BHI broth. Benedict et al. (4) reported generation times between 5.17 and 7.04 h in an experiment using BHI broth incubated at 12ºC with emetic and diarrheic toxin producing *B. cereus* strains isolated from foods, and Borge et al. (5) reported generation times between 6.7 and 13.1 h with enterotoxigenic isolates obtained from dairy and meat products, incubated in BHI broth at 7ºC.

Table 1. Generation time (*g*) in hours, for *B. cereus* in meat substrate maintained at 10°C and 30°C

Culture	Generation time*	
	at 10°C	**at 30°C**
BcA	12.97 (0.999)	0.48 (0.999)
BcE	10.16 (0.970)	0.52 (0.988)
BcS	28.38 (0.890)	0.60 (0.994)
BcM	ng	0.50 (0.999)
B. cereus NCTC 11145	ng	0.58 (0.994)

* *g* (R^2)
ng: no growth observed

CONCLUSION

Protein rich foods such as meat and meat products have been associated with food borne outbreaks of diarrhoea caused by *B. cereus*. The results of the present study confirm that meat is an adequate substrate for the growth of enterotoxin producing *B. cereus* at 30ºC. In the experiment carried out at 10ºC, the growth of some of the cultures of the microorganism is relevant and should be considered in order to minimise the potential risk of spreading this microorganism in meat exposed to abusive temperatures. Spore germination and the population increase at 10ºC emphasise the importance of the time/temperature binomial during the processing of foods.

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