J. Dairy Sci. 100:1–9
https://doi.org/10.3168/jds.2017-12978

Effect of dairy product environment on the growth of *Bacillus cereus*

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ABSTRACT

pH is one of the most important parameters to manage bacterial replication in foodstuffs. In this study, the ability of 2 *Bacillus cereus* strains, one clinical human isolate (GPe2) and one isolate from a dairy products (D43), were investigated for the in vitro growth at different pH values (from 3.5 to 7.5) at 2 temperatures (15 and 37°C), showing their ability to grow from 5.5 to 7.5 and from 5.0 to 7.5, respectively. The ability of spores of these 2 microorganisms to germinate in different typologies of dairy products (unflavored yogurt, Taleggio cheese, mascarpone cheese, and raw and pasteurized milk) was also investigated by inoculating the spores and maintaining the products at 15°C. No growth was observed in yogurt, likely due to the combined effect of low pH (<5) and the presence of natural microflora. An inhibitory action of the natural microflora on the growth of *B. cereus* was also hypothesized for Taleggio cheese and raw milk, as these substrates were characterized by a high natural lactic acid bacteria population and permissive pH values (5.8/6.8 in Taleggio cheese, >7 in raw milk). In pasteurized milk and mascarpone cheese, where pH was not restrictive for *B. cereus* growth and where no significant natural microflora was present, growth occurred rapidly up to loads close to 7 log cfu/g.
Key words: pH, milk, cheese

INTRODUCTION

The *Bacillus cereus* group includes 8 species of ubiquitous gram-positive spore-forming bacteria, very similar from a genetic point of view but characterized by extremely specialized behaviors. This group includes *B. cereus sensu stricto*, which is frequently associated with severe food poisoning episodes thanks to its ability to produce toxins such as cereulide, cytotoxin K, hemolysin BL, and nonhemolytic enterotoxin. A total of 453 foodborne outbreaks were reported in the period 2007 to 2014 in the European Union with *B. cereus* identified as causative agent, with more than 6,600 human cases and a hospitalization rate equal to 5.3% (Kotiranta et al., 2000; EFSA, 2016).

Foods that may represent a risk for *B. cereus* include ready-to-eat products based on rice or pasta, dairy products, flavorings, pastry, and vegetables, and those included in the subcategory called refrigerated processed foods of extended durability (Wijnands et al., 2006).

Milk, as produced from healthy cows, could be considered free of bacteria, but farm and dairy environments may be a source of contamination especially during milking and cheese production. In particular, *B. cereus* was previously recognized as responsible for raw milk spoilage (Bartoszewicz et al., 2008); moreover, a concise risk assessment on *B. cereus* in the Netherlands predicted that almost 7% of pasteurized milk was characterized by loads of this pathogen above 5 log cfu·mL^{-1} (Notermans et al., 1997). Although vegetative cells of *B. cereus* are not able to survive to pasteurization, spores are resistant to heat treatments, highlighting the possibility of their persistence in pasteurized milk. *Bacillus cereus* was also found in several dairy products, with different prevalence from 2 to 52%, depending on the typology (Wong et al., 1988; Svensson et al., 2006; Spanu et al., 2016).

It is well known that pH is one of the most important parameters used to manage bacterial replication and nowadays low pH foods are widely produced and consumed as they guarantee bacterial stability. Dairy products are characterized by various substrate acidity levels, that may differently affect the growth of *B. cereus*, whose presence results from the contamination of milk or a postprocess contamination. Recently, the combined effect of anaerobiosis, low pH, and cold temperatures on the growth ability of 2 *B. cereus* strains was assessed, highlighting that at 10°C growth of psychrotrophic strains occurred at pH higher than or equal to 5.7 in anaerobiosis, whereas aerobic growth occurred when pH was above 5.4 (Guérin et al., 2016).

Also, the ability of *B. cereus* to determine food poisoning (e.g., diarrheal syndrome) is strictly related to its ability to resist to low pH, as the production of en-

Received April 3, 2017.
Accepted May 27, 2017.
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terotoxins is a consequence of the survival of *B. cereus* spores and vegetative cells ingested with food through the stomach, reaching the small intestine alive.

The aim of the present study was the assessment of growth ability of 2 strains of *B. cereus* (one isolated from a human infection and one from a dairy product) in broths at different pH (from 3.5 to 7.5). Moreover, the ability of these 2 strains to grow in various dairy products characterized by different pH and substrate conditions was also evaluated.

MATERIALS AND METHODS

Bacterial Strains and Characterization of the Isolates

In the present study, 2 *B. cereus* strains were used. The first strain was a human clinical isolate (GPe2), previously characterized for the ability to grow within the thermal range from 15 to 37°C and for virulence characteristics (Celandroni et al., 2016; Tirloni et al., 2017). The second strain (D43), previously isolated from a dairy product (Taleggio cheese) was identified by MALDI-TOF MS as previously described (Celandroni et al., 2016).

The D43 strain was characterized to identify its thermal range of growth. The strain stock was kept frozen at −80°C in Microbank Cryogenic vials (Pro-Lab Diagnostics UK, Merseyside, UK) until a loop of bacterial culture was transferred into nutrient broth tubes (Sigma 70122, St. Louis, MO) and incubated at 37°C for 24 h. Cells were harvested in exponential growth phase, defined as a relative change in optical density (**OD**) of 0.05 to 0.2 at 540 nm (6320D spectrophotometer, Jenway, Staffordshire, UK). Cell concentration of the suspension was calculated by counting under a phase-contrast microscopy (BA 310, Motic, Barcelona, Spain) and was diluted in sterile saline water (0.85% NaCl), to obtain a concentration of 2 log cfu·mL^{-1}. Afterward 0.1 mL of the suspension were plated in duplicate onto Nutrient Agar (Sigma 70148), and incubated at different temperatures (5, 7, 10, 15, 20, 37, 41, 45, 50, and 55°C) checked twice per day for colony formation for up to 15 d.

Determination of Growth Ability of B. cereus Strains in Broth at Different pH Values

The 2 strain suspensions were prepared from the frozen stocks as described in the Bacterial Strains and Characterization of the Isolates section; then, they were counted and diluted to reach a starting concentration of 2 log cfu·mL^{-1}. Aliquots of 0.1 mL of each strain suspension were inoculated into 10-mL tubes containing nutrient broth at different pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) obtained adjusting the initial value (7.5 ± 0.2) with HCl. At the moment of inoculation (**T0**), OD was measured and the tubes were then incubated at 2 different temperatures (15 and 37°C) in duplicate. At settled times [24, 48, 72, 96, 120 (**T5**), 144, 168, and 192 h from inoculation], OD was newly measured. Blank (not inoculated) broth series were also prepared for each pH value and incubated at the 2 storage temperatures.

Growth Potential of B. cereus GPe2 and D43 in Dairy Products

Harvesting of Dormant Spores. Spores of the 2 *B. cereus* strains were produced separately on fortified nutrient agar (Senesi et al., 1991) added with sporulation salts: NaCl (5.0 mg·mL^{-1}), $CaCl_2$, (0.1 mg·mL^{-1}), and $MgSO_4$, $7H_20$ (2.0 mg·mL^{-1}). Roux bottles with 150 mL of the above medium were inoculated with 2.0 mL of bacterial suspension (∼10^8 cfu·mL^{-1} in sterile distilled water). After incubation at 37°C for 20 d, spores were scraped from the surface of the medium with a sterile stirrer and washed 5 times by centrifugation (10,640 × *g* for 10 min/2°C) with ice-cold sterile distilled water. To remove residual vegetative cells, each spore suspension was heated at 80°C for 10 min in a water bath. Then, the 2 suspensions were quickly cooled in ice, rewashed with ice-cold sterile distilled water, and conserved at 2°C until inoculation that was within 3 d.

Challenge Tests with Inoculated Dairy Products. For challenge tests with GPe2 and D43 strains, 5 typologies of dairy products were considered: raw and pasteurized milk, mascarpone cheese (an Italian cream cheese coagulated by the addition of food grade organic acids), unflavored yogurt, and Taleggio cheese (a Protected Designation of Origin semisoft, washed-rind, smear-ripened Italian cheese).

Spore suspensions, obtained as described in the Harvesting of Dormant Spores section, were diluted in sterile saline to reach a final concentration of about 5 log cfu·mL^{-1} for each of the 2 *B. cereus* strains. These suspensions were immediately used for the inoculation of aliquots of each of the 5 products in duplicate: briefly, spore suspension was spread onto the surface of Taleggio cheese in 5 spots and immediately was homogeneously distributed using a spatula; for yogurt, milk, and mascarpone, thanks to their nonsolid matrix, the spore suspension was distributed using a spatula.

To minimize changes in product characteristics, the inoculum volume did not exceed 1% of the final weight. Blank samples, inoculated with the same volume of sterile saline solution were also prepared. The inocu-