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Effects of lowering water activity by various humectants on germination of spores of *Bacillus* species with different germinants

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A B S T R A C T

The effect of water activity (a_w), as lowered by different dietary humectants, on the germination of *Bacillus subtilis, Bacillus megaterium* and *Bacillus cereus* spores with germinants that act by different mechanisms has been investigated and compared. Germination of spores of these species by all of the germinants investigated was inhibited as a_w decreased, with the general order of efficacy for these non-ionic humectants being sucrose > trehalose > glycerol. The effect of lowering a_w on germination by germinant receptor (GR)-dependent germinants was not appreciably altered by varying germinant concentrations, was generally not much more effective with spores lacking coats or an outer membrane, and was less pronounced with heat-activated spores. Analysis of the effect of a_w on spore germination via different mechanisms showed that GR-dependent germination was least sensitive to a_w, while germination via activation of spore cortex peptidoglycan hydrolysis or dipicolinic acid release was more sensitive. However, germination by high hydrostatic pressure was less sensitive to inhibition by low a_w, than was germination by other germinants. Examination of the GR-dependent germination of individual spores indicated that a_w acted most strongly in inhibiting the commitment step of germination, while exerting smaller effects on dipicolinic acid release or cortex peptidoglycan hydrolysis.

1. Introduction

Water activity (a_w) and pH are important parameters in food preservation, stabilization, and processing for preventing or limiting the growth of microorganisms, including molds, fungi and bacteria, as well as growth from bacterial spores, which can be infectious or have deleterious effects on food quality and safety (Fontana et al., 2008; Troller and Christian, 1978; Feeherry et al., 2003; Taub et al., 2003; Gulati et al., 2015). As a physical measure of the free water available to microorganisms and for chemical reactions in a food system, a_w is defined as the ratio of the water vapor pressure of a substance to the vapor pressure of pure water at the same temperature. Examples of a_w levels that control microorganisms in foods include: $a_w = 0.950$ controls *Pseudomonas*, *Shigella*, *Bacillus*, and *Clostridium perfringens* in highly perishable foods – canned and fresh fruits; $a_w = 0.910$ controls *Salmonella*, *Vibrio parahaemolyticus*, *Lactobacillus* and *Clostridium botulinum* in cured ham, or Cheddar, Swiss, Muenster, or Provolone cheeses; and $a_w = 0.870$ controls many yeasts in fermented sausage, dry cheeses, sponge cakes; and $a_w = 0.800$ controls most molds in fruits juice concentrates, chocolate and maple syrups, country style ham, and high-sugar cakes (adapted from Beuchat, 1981). According to international and national standards, foods with $a_w \leq 0.85$ and pH ≤ 4.6 are rendered non-potentially hazardous and do not require refrigeration to maintain safety from the rapid and progressive growth of infectious or toxigenic microorganisms. Foods with any component(s) having $a_w > 0.85$ and pH > 4.6 require a microbial challenge test to validate their safety for consumption (IFT/FDA, 2003). Food products with physical properties of {$a_w > 0.93$ and pH > 5.0} or {$a_w > 0.93$ and pH > 5.5}

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require challenge tests to ensure the products do not support the rapid and progressive growth specifically of the pathogenic endospore-formers *Bacillus cereus* or *Clostridium perfringens*, respectively.

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Low-water activity foods ($a_w < 0.6$) can occur naturally or be dried deliberately, and include cereals, chocolate, cocoa powder, dried fruits and vegetables, egg powder, fermented dry sausage, flour, meal and grits, herbs, spices and condiments, honey, hydrolyzed vegetable protein powder, meat powders, dried meat, milk powder, pasta, peanut butter, peanuts and tree nuts, powdered infant formula, grains, and seeds (Beuchat et al., 2013). Recent recalls and foodborne illnesses associated with low a_w foods (*Salmonella* in spices, dry nuts, chocolate, and peanut butter; *Cronobacter* in powdered infant formula; *Clostridium botulinum* in honey; and *Bacillus cereus* in rice cereal) have increased public concern for the safety of these foods, such that viable pathogenic microorganisms may not grow but may survive and persist for extended periods (Syamaladevi et al., 2016). These target pathogens may require additional processing and validation steps with low a_w foods, to ensure food safety, with particular consideration of the influence of low a_w on the thermal resistance of these and other microorganisms (Syamaladevi et al., 2016). With spores, for instance, lowering a_w from 0.9 to 0.2 is known to increase the thermal resistance of *Geobacillus stearothermophilus* and *C. botulinum* spores at temperature T = 110 °C (Murrell and Scott, 1966). Similarly, lowering a_w in the region of 1.0–0.9 with NaCl or sucrose increases the resistance of *B. amyloliquefaciens* spores to inactivation by heat (T = 105 and 115 °C) or to high pressure (HP) processing treatments that combine HP (P = 600 MPa (MPa)) with elevated heat (T = 105 and 115 °C), presumptively by retarding dipicolinic acid (DPA) release through interactions of the humectant molecules with the spore inner membrane (Sevenich et al., 2015).

Bacillus cereus is a gram-positive, facultative anaerobic rod-shaped endospore-forming bacterium found ubiquitously in soil and in many raw and processed foods, such as rice, vegetables, milk and dairy products, and spices. Food poisoning with *B. cereus* has been associated with rice, meats, sauces, desserts, rice, cereal grains and related products (pasta, focaccia); fats, oils and salad dressings; and milk and milk products (Beuchat et al., 2013). *B. cereus* infections occur as two types of gastrointestinal disorders: the emetic syndrome, which is characterized by vomiting and caused by ingestion of heat-stable toxin, which is usually pre-formed in starchy foods (cakes, pasta, cooked rice). The diarrhoeal syndrome is caused by a diarrheagenic toxin that can be formed in the food or in the small intestine. There are concerns of *B. cereus* contamination in pasteurized, refrigerated foods that may contain viable spores that can germinate and outgrow during storage, even at low temperatures (de Vries et al., 2004; Guinebretiere et al., 2003; Choma et al., 2000; Carlin et al., 2000). *B cereus* spores can also survive in dry foods such as rice cereal and in dry food processing environments for long periods of time and can germinate and grow in reconstituted products that are not properly processed or stored. Wet processing of dry foods (cereals) can also introduce conditions for growth and production of heat-stable toxins. While infection with *B. cereus* usually produces mild symptoms, a *B. cereus*-associated food poisoning outbreak from the consumption of pasta salad demonstrates the potential severity of the emetic syndrome and importance of determining factors for controlling *B. cereus* in foods for public health (Dierick et al., 2005).

The nonthermal technology of HP at high temperatures has also been used at P ≤ 600 MPa, and temperatures of ≤60 °C for 30 min in the study of the germination and inactivation of *B. cereus* spores (Van Opstal et al., 2004; Wei et al., 2009; Ju et al., 2008). If germination and outgrowth are not adequately controlled during food storage, HP treatments will have to eliminate *B. cereus* spores from foods, to ensure safety. The HP inactivation kinetics of *B. cereus* have also been studied under HP – high temperature conditions (H Luu-Thi et al., 2014; Daryaei et al., 2013). An important aspect to consider is that spores of *Bacillus cereus* are known to become more resistant to germination and inactivation by HP as a_w decreases (Al-Holy et al., 2007). Specifically, lowering a_w from 0.99 to 0.92 with sucrose inhibits germination of *B. cereus* spores by HP (250 MPa, 25 °C, 15 min) for spores made at temperatures of 37, 30, or 20 °C (there was no inactivation at these HPP conditions). Similarly, lowering a_w to 0.92 reduces germination of *B. cereus* spores by 3–5 logs and prevents 1–3 logs of inactivation that occurred at a_w ~0.99 with HP conditions of 690 MPa, 40 °C for 2 min (Raso et al., 1998). Together, these factors show the importance of determining mechanisms of spore resistance and germination, particularly as they relate to processing foods with low a_w.

Spore germination in *Bacillus* species is normally initiated by a variety of nutrient germinants; these include specific amino acids, sugars and purine nucleosides, presumably molecules that indicate the environment is favorable for growth of a particular organism (Setlow, 2013). The different nutrient germinants trigger spore germination by activating one or more germinant receptors (GRs) located in spores' inner membrane (IM) (Griffiths et al., 2011; Hudson et al., 2001; Paidhungat and Setlow, 2001). In *Bacillus subtilis*, spore germination is triggered by L-alanine or L-valine activating the GerA GR, or a mixture of L-asparagine, D-glucose, D-fructose, and KCl (AGFK) simultaneously activating both the GerB and GerK GRs (Setlow, 2013). Normally, L-asparagine alone does not trigger *B. subtilis* spore germination. However, a mutant form of GerB, termed GerB*, can be activated by L-asparagine alone (Atluri et al., 2006; Paidhungat and Setlow, 1999). *Bacillus megaterium* and *Bacillus cereus* spores also have multiple IM GRs, and their GR-dependent germinants include D-glucose and KBr for *B. megaterium* spores, and L-alanine and inosine for *B. cereus* spores