

Sporicidal Activity of Novel Formulations Containing Lipophilic Epigallocatechin-3-Gallate and Natural Ingredients

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ABSTRACT

Bacterial spores are resistant to harsh environments and to currently used hand hygiene products. Infections caused by spore-forming bacteria, which often occur in healthcare settings, long-term care facilities, food and beverage industries, are associated with high morbidity and mortality in the United States. The current recommendation from the US Center for Disease Control and Prevention (CDC) to prevent the spread of bacterial spores is to wash hands with soap and water. However, it is known that soap and water do not inactivate bacterial spores, but rather remove them from the skin into the water drainage system. Although there is a trend towards a reduction of healthcare associated infections (HAI) in the United States, spore-forming bacteria, such as *Clostridium difficile* (*C difficile*) and *Bacillus cereus* (*B. cereus*), still pose significant risks to the population. Thus, hand hygiene methods with high sporicidal activity, yet without toxicity, are needed to better protect the general population from infections caused by spore-forming bacteria. We reported previously that derivatives of green tea polyphenols, especially lipid-soluble green tea polyphenols such as EGCG-acyl esters, exhibit potential as sporicidal agents without toxicity to humans or the environment. We hypothesize that alcohol (ethanol) formulated with lipid-soluble EGCG and other plant-derived ingredients would achieve high sporicidal efficacy and become novel hand/surface disinfectants without toxicity. The objective of the current study was to determine if alcohol/EGCG-Palmitate (EGCG-P) formulations containing plant-derived ingredients are able to inactivate *B. cereus* spores effectively as a basis for future hand hygiene purposes. Methods used included suspension testing of different formulations against purified *B. cereus* spores and quantification of spore germination. The results demonstrated that several formulations containing only plant-derived ingredients were capable of reducing spore germination by greater than 10,000 fold (log 4 reduction) after 60-second exposure. Additional proof-of-concept studies are warranted to explore the suitability of these formulations for future hand hygiene products against spore-forming bacteria.

Keywords

EGCG, Lipophilic EGCG, EGCG-Palmitate, Bacterial spores, Natural sporicidal product.

Introduction

Some bacteria, such as various *Bacilli* and *Clostridia* species, are able to undergo a sporulation process under prolonged and

unfavorable conditions to form dormant spores [1-4]. Spores have features which enable them to become resistant to various harsh environmental conditions, including heat, chemical solvents, and UV radiation, potentially surviving for hundreds of years or even longer [1,2,5-7]. These features consist of having layers of hard shells, low water content, high core mineralization, high calcium dipicolinate content, inactivated enzymes, low levels of energy

compounds (ATP and NADH), and diminished active metabolism [1,4,7,8]. Bacterial spores are commonly found in soil samples, hot springs, and the gastrointestinal tract of metazoans [3,6,9]. When the environment is favored for growth, the spores then undergo the process of germination, which enables them to become vegetative cells again [1,7,8,10].

Since spores are resistant to harsh environmental conditions and survive for many years, they have become a threat to the food industry as they can cause food spoilage and food poisoning [7,11]. Common spore-forming bacteria that can cause food poisoning are *Clostridium botulinum*, *Clostridium perfringens*, and *Bacillus cereus*. *Clostridium botulinum* produces a neurotoxin, and is primarily found in smoked blood sausages, improper canning, and honey [12,13]. *Clostridium perfringens* is commonly found in the meat industry (meat and equipment), hospitals (medical devices), restaurants (furniture and utensils), and homes for the elderly. According to the Centers for Disease Control and Prevention (CDC), *C. perfringens* is one of the most common types of foodborne illness and causes nearly one million cases of food poisoning each year. *Bacillus cereus* is commonly found in the dairy industry, rice, fresh fruits and vegetables, spices, dried foods, water, plant material, and soil, and is much more difficult to control than *C. perfringens* [11,14,15]. In addition, an opportunistic pathogen, *Clostridium difficile* (*C. difficile*), causes *Clostridium difficile* infection (CDI) in the healthcare system. It damages the intestinal cells and causes inflammation in the gut of patients following antibiotic treatment. Increasing concern regarding CDI reflects an increased morbidity, mortality, severity and rates of recurrent [16]. According to CDC, almost 500,000 *C. difficile* infections are reported each year in the United States, and CDI is responsible for 15,000 deaths (CDC, Healthcare-associated infections). In American hospitals alone, 1 in 25 patients acquires at least one infection from hospital care (healthcare-associated infections, HAI). From 2011 to 2014, CDI only decreased 8% while other HAI decreased significantly more [17]. It was estimated that HAI account for 1.7 million infections and 99,000 associated deaths each year in the United States, contributing to one-third of unexpected in-hospital deaths [18].

Green tea, prepared from the plant, *Camellia sinensis*, is the second most consumed beverage in the world next to water. It is mostly consumed in Asian countries such as China, Japan, and Southeast Asia, due in part to its medicinal benefits [19-23]. Previous studies have shown that green tea extract has anti-inflammatory, antioxidant, anti-cancerous, anti-Alzheimer, anti-Parkinsonism, antifibrotic, anti-collagen-induced arthritis, antibacterial, antiviral, antifungal, antiangiogenic, antihypertensive, and hypoglycemic properties [19,21,22,24-41]. Previous studies have reported that green tea polyphenols are able to prevent the growth and development as well as reduce the heat resistance of *B. stearothermophilus* and *C. thermoaceticum* [42], and to inhibit spore germination of *C. botulinum* and *B. cereus* by damaging the outer shell structure of spores [43].

However, the water-soluble green tea polyphenols, such as EGCG,

are relatively unstable and difficult to formulate into disinfectants [44]. Therefore, several modifications, such as lipophilic tea polyphenol (LTP), epigallocatechin-3-gallate-sterate (EGCG-S) [45], and different EGCG fatty acid monoester derivatives (e.g. palmitoyl or lauroyl) [46] have been tested in different laboratories. We reported recently that both LTP and EGCG-S (1% and 5%) were found to inhibit 98-100% of spore germination in three *Bacillus* spp.: *B. cereus*, *B. megaterium*, and *B. subtilis* [47]. We hypothesize that alcohol (ethanol) formulated with lipid-soluble EGCG and other plant-derived ingredients would achieve more potent sporicidal efficacy and become the basis for novel hand/surface disinfectants without toxicity.

In this study, EGCG-Palmitate (EGCG-P) was used alone and with different concentrations of ethanol, as well as in different formulations, to examine their inhibitory effects on the spores of *Bacillus cereus*. The reason to choose EGCG-P was because it is a major component of tea polyphenol palmitate, which has been approved to be a food additive by the FDA of China, and classified as generally recognized as safe (GRAS) by the US FDA (GRAS notice 772, Palmitoylated green tea catechins). The purpose of this study was to explore the potential to develop novel sporicidal products in order to reduce bacterial spore-induced HAI and to aid in the prevention of spore-forming bacteria contamination in the food and beverage industries.

Materials and Methods

Names of plant-derived natural ingredients and concentrations are confidential information due to current pending patent applications (US and CPT patents pending).

EGCG-P was purchased from Camellix, LLC (Evans, GA), and dissolved in 100% ethanol (Fisher Scientific, Hampton, NH) as a 10% stock prior to formulation. Natural carrier (99.7% USP Kosher) was purchased from ChemWorld.com. Ultrez 20 was supplied by Voyageur Soap and Candle Company (Surrey, BC, Canada). Triethanolamine (TEA) was provided by Carolina Biological Supply Company, and naturally occurring acid (100% anhydrous fine granules) was purchased from HaleFresh.com. Benzalkonium chloride was supplied by Nature's Innovation, Buford, GA.

Formulations

1. Hand gel (hand sanitizer): ethanol, EGCG-P, natural carrier, Ultrez 20 (polymer), TEA (polymerizer), and water.
2. Hand rub/spray: ethanol, EGCG-P, naturally occurring acid and water.
3. Hand rub/spray: ethanol, EGCG-P, natural carrier, naturally occurring acid and water.
4. Hand rub/spray: ethanol, EGCG-P, 0.1% benzalkonium chloride, and water.
5. Hand rub/spray: ethanol, EGCG-P, 0.1% benzalkonium chloride, naturally occurring acid and water.

All formulations were either with neutral pH (Hand gel) or between pH 3 and 4.

Controls included 70%, 78%, and 85% v/v ethanol. These concentrations of alcohol were also tested with EGCG-P.

Bacterial Cultures

Bacillus cereus (*B. cereus*) (Carolina Biological Supply Co., Item# 154870A) was grown aseptically on nutrient agar or in nutrient broth. All stock cultures were maintained at 4°C. Prior to every experiment the microorganism was grown from a stock culture overnight at 37°C in an incubator shaker at 250 rpm. Throughout the study, the microorganism was maintained and tested routinely for purity prior to carrying out each experiment.

Spore Enrichment and Purification

B. cereus was incubated on modified nutrient agar plates (supplemented with 0.06 g of MgSO₄ and 0.25 g of KH₂PO₄ per liter) at 37°C for 10 days to enhance spore formation [48]. After 10 days, Schaeffer Fulton differential staining was conducted to observe the spores and vegetative cells [49]. The spores were then purified by centrifugation at room temperature for 10 min at 10,000 rpm twice. The supernatant was discarded and the spores were suspended in sterile deionized water and vortexed to create a homogenous suspension. The suspension was heated for 20 min at 75°C to eliminate any remaining vegetative cells and obtain pure spores. Purified spores from *B. cereus* were mixed for 1 min (60 sec) with the formulations #1, 2, 3, 4 or 5. After treatment, serial 10 X dilutions were made immediately, plated onto nutrient agar plates, and subsequently incubated at 37°C for 24 h. After incubation, the colony forming unit (CFU) value was counted, the % of inhibition and log₁₀ reduction were calculated. Non-treated spore samples (i.e., suspended in media for 60 sec) were used as a negative (treatment) control. The 80% Ethanol was used as positive control. Three independent experiments were carried out and the mean and standard deviation of the results were calculated. The log₁₀ (fold) reduction was calculated with the following equation: Log reduction = Log₁₀ (CFU control/ CFU treated).

Statistical Analysis

All assays were performed three times. The statistical analyses were carried out in GraphPad Prism 5.0 (GraphPad Software Inc., California). The log₁₀ reduction results were analyzed using a repeated measure two-way and one-way analysis of variance (ANOVA) for Ethanol and EGCG-P/alcohol combinations and different formulations, respectively.

Results

Effect of EGCG-P, different concentrations of ethanol and EGCG-P/alcohol combinations on the spore germination

Spore suspensions were treated with media (control) or different concentrations of ethanol (70%, 78% and 85%) respectively for 60 seconds. The combination of 0.2% EGCG-P with different concentrations of ethanol was also used to treat the spore suspension for 60 seconds. Results are shown in Figure 1. The different concentrations of ethanol alone inhibited spore germination of *B. cereus*; the percentage inhibition in for all three concentrations was above 99%. The average log₁₀ reduction for 70%, 78% and 85% ethanol was 2.43, 2.58 and 2.45, respectively. The average

log₁₀ reduction of EGCG-P+70% ethanol, EGCG-P+78% ethanol and EGCG-P+85% ethanol was 2.45, 2.94 and 2.77, respectively. Although the combination of EGCG-P with different concentrations of ethanol gave higher mean values than alcohol alone at that concentration, the differences were not statistically significance (p>0.05). These results suggested that this combination was not ideal as a sporicidal formulation, which aims for more than log₁₀ 4 reduction. The combination with the highest log₁₀ reduction was EGCG-P with 78% Ethanol. Therefore, this was used as a base for novel formulations with addition of other agents.

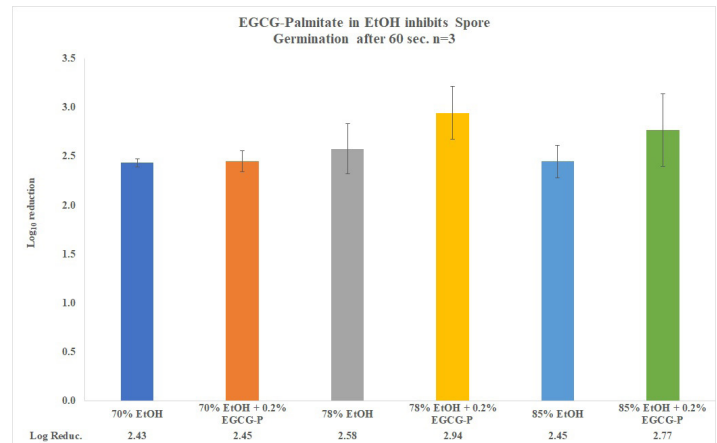


Figure 1: Log₁₀ reduction of ethanol and EGCG-P/ethanol inhibition of spore re-germination after 60 seconds. Mean are shown with standard deviation (n=3).

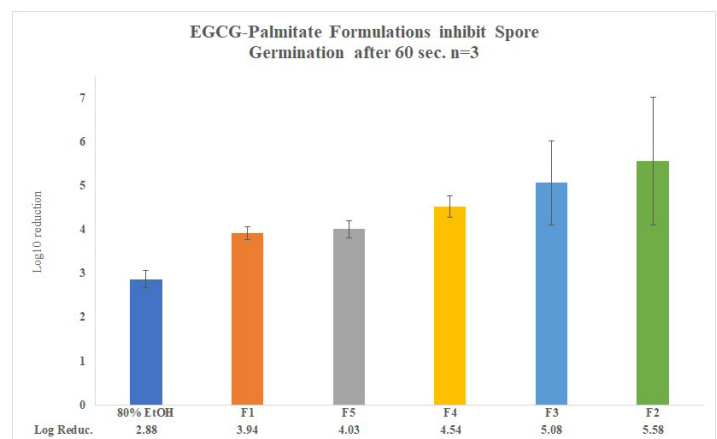


Figure 2: Log₁₀ reduction of spore re-germination by 5 EGCG-P formulations and a positive control (80% Ethanol) after 60 seconds. Mean are shown with standard deviation (n=3).

Effect of different novel formulations on spore germination

Five different novel EGCG-P formulations were used in this study to determine and evaluate the sporicidal activities of these formulations. As shown in Figure 2, the novel formulations were able to reduce spore germination by an average of >4 log₁₀, with two formulations (F2 and F3) reducing spore germination by an average of >5 log₁₀ (99.999%), after a 60-sec incubation with spores of *B. cereus*. All formulations showed significantly (p=0.0093; 0.0008; 0.0001; 0.0128; and 0.0052 for F1-F5) higher log₁₀ reduction when compared with the positive control (80% ethanol). As described above, F2 and F3 only contain food grade

plant-derived ingredients that are commonly found in popular beverages. These formulations exhibited extremely powerful sporicidal activity against spores of *B. cereus*, and demonstrated that our new formulations improved the sporicidal activity by about 1000-fold compared to our previously published data [47], even without considering the shortened time from 5 min to 60 sec.

Discussion

Currently, there are no effective sporicidal hand hygiene products on the market. Hand wash with soap and water is the only recommended method to prevent the spread of *C. difficile* either in healthcare settings or at home [50]. This is because commonly used hand sanitizers, hand rubs or scrubs, either containing alcohol or bactericidal agents, are not able to eradicate *C. difficile* or other bacterial spores, which are resistant to alcohol and other bactericidal agents. Standardized *in vivo* tests based on American Society for Testing and Materials (ASTM) protocols showed that various hand hygiene products (4% chlorhexidine gluconate hand wash, 0.3% triclosan antimicrobial hand wash, regular liquid hand wash or body wash, heavy-duty hand cleaner for printer's ink, with tap water as control) only gave less than 1 log₁₀ reduction of *C. difficile* germination, except for the heavy-duty hand cleaner, which gave 1.21 log₁₀ reduction [51]. These levels of reduction in *C. difficile* germination are not considered to be sporicidal (>2 log₁₀ reduction *in vivo* and >4 log₁₀ reduction *in vitro*, <5 min exposure). Even commercially available "sporicidal" wipes for hospital cleaning purposes failed to reach a 4 log₁₀ reduction of *C. difficile* spore germination, highlighting the lack of methods to control bacterial spore associated infections [52].

Efforts have been focused on improving the efficacy of alcohol in order to increase the sporicidal activity, which include manipulation of the physical properties of alcohol itself by alteration of pH with acids or bases [53]. Indeed, a 5-min exposure to 70% ethanol with extremely high (>11, by NaOH) or low (<1, by HCl) pH at room temperature resulted in reduction of *C. difficile* germination by >2.5 log₁₀ (<4 log₁₀), but not *B. subtilis* or *B. thuringiensis* germination [53]. There was no significant difference among acids used for acidification of ethanol (hydrochloric acid, sulfuric acid, citric acid and lactic acid). Importantly, the sporicidal effect (>4 log₁₀ reduction, <5 min, *in vitro*) only became apparent when the temperature was increased to 80°C. This temperature is 2 degrees higher than the boiling point of ethanol. Another attempt to increase the efficacy of ethanol involved combination with peracetic acid (PPA), a sporicidal oxidizing agent, and alcohol/ethanol acidified with HCl. At pH 2, 70% acidified ethanol and PPA was able to reduce *C. difficile* spore germination by 2 log₁₀ and *B. subtilis* spore germination by less than 0.2 log₁₀ [54]. At pH 1.5 and PPA concentration increased to 450 ppm, reduction of *C. difficile* spore germination reached almost 3 log₁₀, but no further reduction for *B. subtilis* was obtained, indicating *B. subtilis* is significantly more resistant to these agents than *C. difficile*. According to the US Environmental Protection Agency (USEPA), the Acute Exposure Guideline Level (AeGL) for peracetic acid exposure to humans reaches 3 (AeGL-3) when exposing 18 ppm PPA for 10 min (<https://www.epa.gov/aegl/peracetic-acid-results>

aegl-program). AeGL-3 represents a level of exposure that could cause life-threatening health effects or death [55]. Thus, developing sporicidal hand hygiene products without harmful ingredients is in urgent need.

In the current study, the goal was to use formulations of plant-derived agents to inactivate *B. cereus* spores by 4 log₁₀ in 60 sec. We initially tested 70%, 78% and 85% ethanol with distilled water alone or in combination with EGCG-P (from a stock of 10% EGCG-P with 100% ethanol) at room temperature. As shown in Figure 1, none of the formulations achieved a 3-log₁₀ reduction. In addition, there was no significant difference among the formulations (p>0.05); there was an only trend for the addition of EGCG-P to enhance the efficacy in 78% and 85% ethanol. Therefore, we next tested formulations containing additional plant-derived ingredients either in a hand gel formulation (F1) or in ethanol formulations. As shown in Figure 2, F1 exhibits a significantly higher efficacy in comparison to 85% v/v ethanol alone. It reduced *B. cereus* spore germination by 3.94 log₁₀, close to the desired 4 log₁₀ reduction. F2 and F3 were formulated with 100% plant-derived ingredients, which gave the highest average sporicidal activity of 5.58 and 5.08 log reductions after 60 sec exposure. There was no statistical difference between the two formulations. Interestingly, when the formulations included a potent bactericidal agent benzalkonium chloride (F4, F5), the sporicidal activity was decreased to 4.54 and 4.03 log₁₀ respectively, indicating the plant-derived ingredients in EGCG-P ethanol formulations are sufficient to inactivate *B. cereus* spores. To the best of our knowledge, this is the first observation that formulations with 100% plant-derived, nontoxic ingredients are able to reduce bacterial spore germination within 60 sec.

Here we report, for the first time, novel formulations of alcohol and EGCG-P with nontoxic plant-derived ingredients effectively inactivated *B. cereus* spore germination within 60 sec. The efficacy ranged from log₁₀ 4 to > log₁₀ 5 reduction of spore germination. To further investigate the sporicidal properties of alcohol/EGCG-P formulations, other species of pathogenic Bacillus and *C. difficile* need to be tested to validate the proof-of-concept regarding novel approaches to infection control against spore-forming bacteria.

In conclusion, enhancement of sporicidal activity on formulations containing alcohol and EGCG-P can be achieved with naturally occurring plant-derived ingredients. Since these nontoxic formulations have been tested for their virucidal and bactericidal activities [56,57], they could be used to develop various sporicidal hand/skin hygiene products with comprehensive germicidal activities (bactericidal and virucidal), pending future research and development efforts.

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