Enhanced Sporicidal Activity of Alcohol and Epigallocatechin-Palmitate-Based Hand Hygiene Formulations Comprised of Plant-Derived Compounds

Tinchun Chu1*, Lee H. Lee2#, Ayuni Yussof1, Sabrina Lopez1, Gabriela Herrera2, Priscilla Luna2, Mahfuza Uddin2, Laying Wu3, John A. Murzaku4, Douglas Dickinson5, Stephen Hsu5,6#

1Department of Biological Sciences, Seton Hall University, South Orange, NJ, USA
2Department of Biology, Montclair State University, Montclair, NJ, USA
3Microscopy and Microanalysis Research Laboratory, Montclair State University, Montclair, NJ, USA
4Department of Mathematics and Computer Science, Seton Hall University, South Orange, NJ, USA
5Camellix Research Laboratory, Augusta, GA, USA
6Department of Oral Biology & Diagnostic Sciences, Augusta University, Augusta, GA, USA

Email: *Tin-Chun.Chu@shu.edu

Abstract

Pathogenic spore-forming bacteria pose high risks to healthcare settings, as well as in the food and beverage industries. We reported recently that novel alcohol-based formulations containing plant-derived compounds, including epigallocatechin-3-gallate-palmitate (EGCG-P), a green tea polyphenol ester, provide > 99.99% inactivation of bacterial spores within 60 sec. Based on recently published data from our group and others, we hypothesize that a combination of EGCG-P and alcohol formulated with other plant-derived ingredients would achieve high sporicidal efficacy against a wide spectrum of bacterial spores and can provide novel hand hygiene methods against bacterial spores without toxicity. The objectives of the current study were to optimize two novel formulations with combinations of glycerol, citric acid, and EGCG-P to increase sporicidal activity and explore the rapid inactivation mechanisms and suitability for sporicidal products with broad-spectrum activities against aerobic and anaerobic bacterial spores. Methods included suspension testing of two formulations against spores from Bacillus cereus and Clostridium sporogenes, quantification of spore germination, and scanning electron microscopy. The results demonstrated that these novel formulations were able to reduce spore germination by >99.99% after 30 sec exposure in suspension tests, and rapidly caused physical damage to the spores. Additional studies are...
warranted to determine the suitability of the novel formulations for future hand hygiene use.

**Keywords**

Alcohol, Hand Hygiene, Sporicidal, Bacterial Spores, Plant-Derived Compounds

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**1. Introduction**

The current guidelines from United States Center for Disease Control and Prevention (CDC) and World Health Organization (WHO) for a hand hygiene method to prevent bacterial spore infection (and also norovirus infection) are still limited to hand wash with soap and water [1] [2]. This is based on evidence that the available hand hygiene products do not have sporicidal or virucidal activity (>4 log reduction of infectivity within 60 sec in a standard suspension test, or >2 log reduction of infectivity within 5 min in a human hand test: WHO guidelines attached) [1].

As of today, there is no established sporicidal test standard in the world for hand hygiene products, due to the lack of a reported nontoxic method with >4 log reduction (99.99% reduction) of spore germination under a 2 min-exposure. The European Union (EU) only has hand hygiene product standard/pass criteria for fungal spores, which requires >4 log reduction under 60 sec (EN 13624, EN 1650) [3] [4]. For surface and environmental cleaning sporicidal claims, EN 14347 and EN 13704 are European standards using *Bacillus subtilis* (*B. subtilis*) and *Clostridium sporogenes* (*C. sporogenes*) (but do not address *C. difficile*) for 60 min exposure time with pass criteria of >4 log reduction [5] [6] [7]. In the US, the Environmental Protection Agency (EPA) is the regulatory body for sporicidal disinfectant tests and claims using a protocol with SOP Number: MB-15-03, based on Association of Analytical Communities (AOAC) method 966.04, to test *B. subtilis*, or American Society for Testing and Materials (ASTM) method E2197-11 to test *B. subtilis* and *C. sporogenes* on a hard surface. The pass criteria are >4 log reduction in up to a 60 min-exposure [5]. Collectively, criteria for a sporicidal hand hygiene product might be expected to be a >4 log reduction in 30 - 60 sec against relevant bacterial spore species.

We previously tested the potential sporicidal activities of green tea polyphenols and their acyl esters. These plant-derived compounds in aqueous solutions exhibited sporicidal activities and demonstrated a capability to cause physical alteration of the spore coat after 2 h exposure [8]. However, the exposure time of 2 h used in that study is beyond the required exposure timeframe for hand hygiene use (30 - 120 sec). Since alcohol is a plant-derived bactericidal agent with low adverse effect on human skin [9], we next explored different alcohol-based formulations containing EGCG-P, together with other plant-derived compounds for delivery. The rationale for the formulations was based on the strong dena-
turing capability of alcohol combined with the physical destruction of the spore coat by EGCG-P. The combination of ingredients in these nontoxic formulations (F1, F2 and F3 do not contain benzalkonium chloride, a quaternary ammonium compounds (QAC)) reached the sporidical range with 60 sec exposure [10].

The purpose of the current study was to determine whether combinations of plant-derived compounds (i.e. a natural formulation) could provide greater than 5 log reduction of spore germination in alcohol-based formulations containing EGCG-P, and the potential fast-acting mechanism of these formulations against both aerobic and anaerobic spores.

2. Materials and Methods

2.1. Chemicals, Reagents and Cultures

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. Glycerol (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 citric acid (100% anhydrous fine granules) was purchased from HaleFresh.com. B. cereus and C. sporogenes were obtained from Carolina Biological Supply Co., Item#154870A, and Item#154995A, respectively.

2.2. Formulations

To further increase the efficacy of F1 and F2 described previously [10], adjustments to the formulations were made for the current study. The current F1 formulation (cF1 hereafter) is in a hand sanitizer gel form (pH > 6.0) that contains 78% ethanol, 0.2% epigallocatechin-3-gallate-palmitates (EGCG-P), glycerol, water, gelling agent, and triethanolamine (polymerization catalyst). The current F2 formulation (cF2 hereafter) is in hand rub form that contains 80% ethanol, 0.2% EGCG-P, citric acid and water (pH > 3.0).

2.3. Bacterial Spore Enrichment and Purification

For B. cereus, the methods were described previously [10]. For C. sporogenes, the cells were grown in reinforced clostridial medium (RCM) (BD#218081) and supplemented with 5% yeast extract and 2% agar. After inoculating 100 μL of C. sporogenes onto the plate, parafilmed, and placed it in a vacuum-sealed bag. The bag was filled with argon gas to remove any remaining oxygen. After incubating at 37˚C for 10 days, the C. sporogenes spores were harvested with the same procedure as reported previously [8].

2.4. Suspension and Neutralization Assay

Purified endospores from B. cereus were mixed for 30 sec with cF1 or cF2 at a 1:9 ratio between spores and the alcohol-based formulations, prior to neutralization of the reaction by immediate 10× dilution in phosphate buffer saline (PBS).
The serial 10× dilutions of the neutralized mixes were plated out in triplicate in 100 mm Petri dishes with nutrient agar, and incubated at 37^o^C for 24 h. The CFU of spore germination was counted, and the % of inhibition and log_{10} reduction calculated. Untreated spore samples (i.e. suspended in PBS for 30 sec) were used as control. Three independent experiments were carried out and the mean and standard deviation of the results calculated. The log_{10} (fold) reduction was calculated with the following equation: Log reduction = log_{10} (CFU control/CFU treated).

To determine if a 10× dilution of cF1 and cF2 by PBS completely neutralized the sporicidal activity of the formulations, neutralization tests were performed to validate the test conditions by 30 sec exposure of the spores with 10× diluted cF1 and cF2, followed by serial 10× dilution, plating, incubation and CFU calculation as described in suspension test.

### 2.5. Scanning Electron Microscopy (SEM)

Purified spores of B. cereus and C. sporogenes were exposed to cF1 or cF2 for 60 sec or 5 min (B. cereus only) prior to neutralization by dilution in PBS, as described under suspension tests. Untreated spores were mixed with PBS for 60 sec as control. Once the treatment was completed, 100 µL of the samples were dispensed and vacuum filtered using 0.2 µm polycarbonate membrane (EMD Millipore Isopore #GTTP01300) with a Nalgene filter unit (Fisher Scientific #09-740-23A). Samples were rinsed with PBS (pH 7.2) or 0.1 mol·l⁻¹ sodium cacodylate buffer [Na(CH₃₂)₂AsO₂·3H₂O] three times for 5 min each; fixed with 2.5% glutaraldehyde in 0.1 mol·l⁻¹ cacodylate buffer for 30 min at room temperature. The samples underwent a dehydration series and were immersed in ethanol, providing additional fixation, followed by drying with liquid CO₂ at 1072 psi and 31˚C in Denton Critical Point Dryer. Samples were mounted on a stub and coated with a thin layer of copper metal film using Denton IV Sputter Coater. Images were captured with a Hitachi S-3400N Scanning Electron Microscope.

### 3. Results

A pilot study was conducted to improve the antimicrobial activity of F1 (hand sanitizer formulation) and F2 (hand rub formulation). Glycerol was added as an emollient to reduce the irritant effect of alcohol to the skin, rather than as an antimicrobial agent. It was found that when glycerol was added to F1 within a certain concentration range, the antimicrobial activity was significantly improved (data not shown, patent pending). Accordingly, we incorporated glycerol at an appropriate concentration in F1 to make cF1. For F2 improvement, it was found the antimicrobial activity increased significantly with the addition of citric acid within a certain concentration range. This was not due to the lowered pH because other pH reducing acids were not able to increase the antimicrobial activity of F2 (data not shown, patent pending). In fact, alcohol-based formulations containing different acids, with pH < 1, failed to reduce bacterial spore germina-
tion by >2.5 log after 5 min exposure [11]. In addition, this low concentration of citric acid did not lead to a pH drop to <3.3. Thus, it is possible the combination of citric acid and EGCG-P results in a synergistic effect in the alcohol-based formulation now referred to as cF2.

3.1. Suspension Tests

To determine if the sporicidal activity of cF1 and cF2 was enhanced with the combination of alcohol, EGCG-P and other plant-derived compounds, purified spores of B. cereus were exposed to cF1 and cF2 for 30 sec before the reactions were stopped by neutralization. Untreated spores (control) were incubated with PBS for 30 sec. The log reduction of CFUs was evaluated by analysis of variance (ANOVA). As shown in Figure 1, both cF1 and cF2 rapidly inactivated the spore germination capacity (p = 0.004). A highly significant difference between groups was found (p < 0.005). cF1 treatment resulted in 5.4 ± 0.12 log reduction of germination from B. cereus spores, while cF2 caused 5.84 ± 0.39 log reduction of B. cereus spore germination (the average log number for the control is 11.58). One sample t-test comparison showed that neutralized cF1 (cF1-N) and cF2 (cF2-N) failed to inactivate the spores, with only 0.12 ± 0.32 and 0.17 ± 0.35 log reduction respectively (p < 0.001). These results demonstrate that cF1 and cF2 rapidly inactivated B. cereus spores in the suspension tests, and the efficacy of the alcohol-based formulations were significantly improved by combination of plant-derived compounds, with both cF1 and cF2 capable of reducing the germination of B. cereus spores by >5 log10 (>99.999%).

3.2. SEM Validation Tests

Figure 2 shows two types of bacterial spores (Bacillus cereus and Clostridium sporogenes) treated with cF1 and cF2 for 60 sec in comparison with untreated control. In comparison to the untreated control, cF1-treated spores of B. cereus (upper row) exhibited an altered morphology with a reduction in size and porous spore coats. Additionally, there was a film-like appearance between the spores. The cF2-treated spores show a complete destruction of spore structures. Similarly, morphology changes were apparent after treatments of spores of C. sporogenes (lower row). Untreated control spores had a uniform spherical morphology. The cF1-treated spores were misshapen and clustered, and the surface was damaged while cF2-treated spores were shriveled and exhibited collapsed spore coats. These structural alterations suggested that both cF1 and cF2 were able to rapidly alter the spore morphology, damage the coat of the spores and lead to inactivation of these bacterial spores by collapsing the spore coats and by allowing ethanol to penetrate the outer coat of the spores.

To further understand the destructive capacity of the alcohol-based formulations, spores of B. cereus were treated with cF1 and cF2 for 5 min prior to SEM observation and imaging. Figure 3 shows the result comparing 60 sec and 5 min treatments of the spores. The cF1-treated spores demonstrated broken spore
coats with debris on the filter surface after 5 min suspension treatment, suggesting a complete fragmentation of the spores. The 5 min cF2-treated spores demonstrated additional morphological alterations compared to the 60 sec treatment, with reduced size and collapsed spore coats.

**Figure 1.** Log₁₀ reduction of spore re-germination by cF1 & cF2 after a 30 sec incubation. cF1-N and cF2-N refer to the formulations neutralized with PBS (1:9 v/v). Mean are shown with standard deviation (n = 3). Log₁₀ reduction by cF1 and F2, compared to untreated control, was statistically significant (p < 0.005).

**Figure 2.** Morphological alterations of spores treated with cF1 and cF2. Left column: untreated spores of *B. cereus* (upper) and *C. sporogenes* (lower); Center column: spores of *B. cereus* (upper) and *C. sporogenes* (lower) treated with cF1 in suspension assay for 60 sec; Right column: spores of *B. cereus* (upper) and *C. sporogenes* (lower) treated with cF2 in suspension assay for 60 sec.

**Figure 3.** Morphological alterations of spores treated with cF1 and cF2 for 1 min and 5 min.
4. Discussion

Unlike surface disinfectant and device disinfectant products with virucidal or sporicidal activity, hand hygiene products are not allowed to contain toxic chemicals, since they are not suitable for human use. Another difference between hand hygiene products and surface/device disinfectants is that hand hygiene products must be able to rapidly inactivate the pathogenic organisms (within 30 - 120 sec vs. 60 min or longer). The Food and Drug Administration (FDA) regulates hand hygiene products by issuing monographs for approved active ingredients. Some alcohols such as ethanol and isopropanol have been recognized as bactericidal agents that can be used in hand hygiene products to reduce bacterial infections. The current FDA monograph for over-the-counter hand hygiene products allows only ethanol, isopropanol, and benzalkonium chloride as the active ingredients [12]. However, alcohol is known to be relatively ineffective for sporicidal purposes. According to current regulations covering surgical hand hygiene products (mostly alcohol-based products), they must be bactericidal, fungicidal (against yeasts), and virucidal against enveloped viruses, but they are not required to have sporicidal, mycobactericidal, or virucidal activity against non-enveloped viruses [9]. Thus, nosocomial infections (NIs), or healthcare associated infections (HAIs) caused by bacterial spores or non-enveloped viruses are not well-controlled with currently used methods. In fact, HAIs remain one of the major causes of death in most countries [13].

During the 2020 coronavirus pandemic, hand hygiene was essential to help protect the population from infection by the virus. In fact, Coronaviridae, an enveloped virus family, is sensitive to most hand hygiene products containing alcohol. Unfortunately, alcohol-based hand hygiene products are not effective against non-enveloped viruses such as norovirus, or against bacterial spores [14] [15]. Therefore, there is an urgent need for alcohol-based hand hygiene products with broad-spectrum activities against non-enveloped viruses and bacterial spores.

Alcohols, especially ethanol and isopropanol, are able to rapidly denature most biomolecules in microorganisms. However, pathogenic bacterial spores are not sensitive to these alcohols, or several commonly used ingredients in hand hygiene products. As a matter of fact, ethanol, isopropanol, and n-propanol do not possess significant sporicidal activity; nor do other agents such as Clorisan, chlorohexidine, and plain soap [9]. Other agents possessing bactericidal but not sporicidal activity include organic acids/esters, phenols, quaternary ammonium compounds (QACs), biguanides and organomercurials [16].

Hand hygiene products typically contain multiple ingredients to provide a combination of antimicrobial activity with cosmetic functions such as emollients, and fluid properties to facilitate use, such as viscous gels. There is an increased public desire for products with a “natural” connotation. In a previously published study, we tested the sporicidal activity of alcohol-based formulations comprised of plant-derived ingredients, including EGCG-P, either in a hand sanitizer formulation (F1) or in liquid hand rub formulations (F2), using B. cereus
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as the test organism. We found that F1 exhibited a significantly higher sporicidal efficacy in comparison to 80% v/v ethanol alone [10], reducing B. cereus spore germination by 3.94 log₁₀, close to the desired 4 log₁₀ reduction. F2 with 100% plant-based ingredients gave the highest average sporicidal activity, with a 5.58 log reduction after a 60 sec exposure. To the best of our knowledge, this was the first observation that a formulation (F2) with 100% plant-derived ingredients was able to reduce bacterial spore germination by more than 5 log after 60 sec-exposure [10].

The current study was designed to determine whether improved plant-derived formulations of cF1 and cF2 (for hand sanitizer gel and hand rub use, respectively) have higher in vitro efficacies with shorter exposure time, and if 60 sec exposure results in physical damage to the spore coats of two bacterial spore species. Results from the suspension tests demonstrated that both cF1 and cF2 possessed strong sporicidal activity, with >5 log reduction of spore germination at a 30 sec exposure (Figure 1). These results indicated that when certain plant-derived compounds are formulated with alcohol (ethanol), the alcohol-based formulations became sporicidal, instead of sporostatic. This finding provides important evidence to support the possibility of developing novel alcohol-based sporicidal products for hand hygiene use. However, it is not known what mechanisms and the role glycerol or citric acid play lead to the significant increase in sporicidal activity found in cF1 and cF2, which warrant further exploration. Another important observation was the damage caused by the formulations to spore coats of both aerobic and anaerobic bacterial spores, as shown in Figure 2. This result demonstrated the rapid inactivation of the spores was associated with physical damage of spore coats, which could lead to permeability of alcohol into the core of the spores causing denature of intrinsic biomolecules. This rapid structural alteration of the spore coats has not been reported previously for nontoxic hand hygiene or disinfectant formulations. When exposure time was increased to 5 min, damage to the spore structure became more apparent, as shown in Figure 3, which further validated the potential mechanism for the sporicidal activity.

In order to develop sporicidal hand hygiene products, additional work must be performed. To test the potential broad-spectrum of sporicidal activity, Bacillus subtilis (B. subtilis) and Clostridium difficile (C. difficile) need to be used. If these in vitro tests produced satisfactory results, clinical efficacy tests will be required to determine the in vivo efficacy using ASTM E-1174 method (for claims in the United States and Health Canada) according to efficacy criteria of the FDA’s Tentative Final Monograph (TFM) [17] [18]. This standard requires a 2-log₁₀ reduction of the indicator organism on each hand within 5 min after the first use, and a 3-log₁₀ reduction of the indicator organism on each hand within 5 min after the tenth use. For claims in the European Union, similar clinical tests are needed using EN 1499 [19] or EN 1500 [20] methods. Again, as noted above, there are no current criteria specifically regulating test standards and efficacy for sporicidal hand hygiene products [21].

In conclusion, a strong and rapid-action sporicidal activity of the alcohol-based
formulations containing plant-derived ingredients was achieved. The mechanism of action of the formulations could depend on the plant-derived components to initially alter the structure of spore coats, and allow alcohol to penetrate into the matrix of the spore, leading to a rapid and irreversible inactivation. Since similar nontoxic formulations have been tested for their virucidal and bactericidal activities [15] [22], these alcohol-based formulations could be used to develop various sporicidal hand/skin hygiene products with comprehensive germicidal activities (bactericidal, sporicidal, fungicidal, and virucidal), pending future research and development efforts.

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Conflicts of Interest
The authors declare no conflict of interest.

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