

DEVELOPMENT OF GERMINANT MIXTURES AND INACTIVATION OF ADHERED CLOSTRIDIUM SPOROGENES SPORES ON FOOD CONTACT SURFACES

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Abstract

Adhered spores are one of the major causes of surface-derived contamination in food industry. In this study, the feasibility of a germination-induced inactivation strategy on adhered *Clostridium sporogenes* spores was assessed. Mixtures of L-alanine (Ala), L-cysteine (Cys), and/or L-serine (Ser) were more effective to induce spore germination than an individual amino acid. The germinant mixture containing 50 mM Ala+25 mM Cys+50 mM Ser in 50 mM NaHCO₃+50 mM L-lactate+25 mM Na₂HPO₄ at pH 6.0 can promote maximum germination extent for various strains of *C. sporogenes* spores. Spores adhered onto stainless steel (SS) and plastic cutting board (PB) coupons were incubated with this germinant for 30 min at 30°C prior to inactivation with 200 ppm NaOCl, 25 ppm iodophor, 200 ppm Quaternary Ammonium Compounds (QACs), and electrolyzed oxidizing water (EOW). The incorporation of a germination-inducing step before sanitizing treatments enhanced an antimicrobial efficacy of NaOCl and EOW under both clean and dirty conditions on SS coupons. Upon implementing a germination step, EOW showed the strongest sporicidal activity among all sanitizers tested with 3.78 and 3.16 log reductions on dirty SS and clean PB coupons, respectively. Collectively, these results suggest an incorporation of germination step with the selected amino acid mixture could sensitize adhered *C. sporogenes* spores on SS and PB surfaces to activities of sanitizing agents, and demonstrate the potent activity of EOW against adhered *C. sporogenes* spores.

Keywords: *Clostridium sporogenes*, food contact surface, germination, electrolyzed oxidizing water, germinant mixture capacity, adjustment factors

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Introduction

Resistance of bacterial endospores to various stresses encountered in food manufacturing facilities causes difficulties to eliminate spores from food processing environments (Hornstra *et al.*, 2007; André *et al.*, 2012). Spores are able to survive for extended period of time and strongly attach to food contact surfaces, partly due to their high hydrophobicity and the presence of appendages and exosporium of some spore-forming species; however, information regarding the attachment of spores of *Clostridium* spp. on food contact surfaces is lacking (Parker *et al.*, 2001; Faille *et al.*, 2002; Klavenes *et al.*, 2002; Faille *et al.*, 2007; Joshi *et al.*, 2012; Alzubeidi *et al.*, 2018). Once attached to surfaces, it is difficult to remove or inactivate spores and this could act as a continuous source of cross-contamination to food products affecting quality, shelf-life, or even compromising safety of consumers (Blatchley *et al.*, 2005; Hornstra *et al.*, 2007; Udombijitkul *et al.*, 2013). Generally, a procedure to inactivate spores adhered to food contact surfaces is costly and requires elevated concentrations and temperature of sanitizers that might potentially cause detrimental effects to the environment and equipment (Russell, 1990; Udombijitkul *et al.*, 2013; Omotade *et al.*, 2014). In order to overcome these burdens, an alternative strategy that can effectively eliminate spores on food contact surfaces is imperative.

Spore germination occurs rapidly when spores sense the presence of the specific compound, termed germinant, via their germinant receptors (GRs) (Paredes-Sabja *et al.*, 2011; Setlow, 2014). It is well established that resistance properties of spores are compromised upon germination. (Akhtar *et al.*, 2009; Omotade *et al.*, 2014). Thus, inducing spore germination could terminate the extreme inert condition of spores rendering them more susceptible to various stressors and present an improved measure to inactivate or reduce the number of contaminated spores (Nerandzic and Donskey, 2010; Løvdal *et al.*, 2011; Nerandzic and Donskey, 2016). Since heterogeneity in spore germination within population exists as

well as a diversity in germinants required to trigger germination of different strains within the same species is well perceived (Paredes-Sabja *et al.*, 2008; van der Voort *et al.*, 2010; Wang *et al.*, 2011; Udombijitkul *et al.*, 2014), the critical factors determining success of the germination-induced inactivation measure are the germination capability of spores within population and the extent to which the developed germinant stimulates spore germination (Udombijitkul *et al.*, 2013). Taking advantages of the current knowledge and insight of spore germination mechanisms, development of an effective and practical germinant is feasible. This highlights a significance of our attempt to intensively characterize a simple germinant mixture for maximizing germination rate and extent by employing *Clostridium sporogenes* as a model food-related spore-forming bacterium.

C. sporogenes, a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium, is able to survive in under- or minimally-processed food products (Chaturvedi *et al.*, 2015). *C. sporogenes* is one of the important food spoilage microorganisms (McClure, 2006; Olguín-Araneda *et al.*, 2015). Owing to its high genetic and physiological similarities, non-toxicogenicity, and its spore resistance to heat which exceeds that of *Clostridium botulinum* spores, *C. sporogenes* PA 3679 is a widely used surrogate microorganism for proteolytic *C. botulinum* for practical reasons. Furthermore, both *C. botulinum* and *C. sporogenes* possess an outermost exosporium on spore surface; nevertheless, its role for adhesion to inanimate surfaces has not been elucidated (Bradbury *et al.*, 2012; Brown *et al.*, 2012; Diao *et al.*, 2014; Janganan *et al.*, 2016). Previous studies indicated that spores of *C. botulinum* and *C. sporogenes* shared many common amino acid germinants and co-germinants (Broussolle *et al.*, 2002; Brunt *et al.*, 2014). Hence, there is a potential to develop a common germinant that can trigger spore germination in many species and implement it in the food contact surface sanitizing step by using various strains of *C. sporogenes* as a model microorganism.

The objectives of this study were to formulate the common germinant mixture that can maximize germination responses of various strains of *C. sporogenes* spores and to evaluate the efficacy of implementing germination step before inactivation with commonly used sanitizers against spores of *C. sporogenes* attached to SS and PB coupons. Additionally, this study was also undertaken to examine the effectiveness of EOW as a sanitizer against adhered *C. sporogenes* spores.

Materials and Methods

Bacterial Strains and Preparation of Purified Spores

C. sporogenes strains used in this study consisted of PA 3679, ATCC 3584 that were provided by Prof. Dr. Mahfuzur R. Sarker (Oregon State University, Corvallis, OR, USA), and TISTR 1452 and TISTR 1481 that were purchased from Thailand Institute of Scientific and Technology Research (Pathum Thani, Thailand). The actively growing cultures were prepared in Fluid Thioglycollate Medium (FTG) (Difco, BD Diagnostic Systems, Sparks, MD, USA) followed by overnight incubation at 37°C.

C. sporogenes spores were prepared as described previously with some modifications (Paredes-Sabja *et al.*, 2007). The actively growing culture was transferred to Duncan Strong (DS) sporulation medium (Duncan and Strong, 1968), and incubated for 5 days at 37°C. Spores were purified by repeated washing with cold sterile water until obtaining >99% of purified spores, free of cell debris, sporulating, and germinating cells as examined by phase contrast microscopy (Leica, Heerbrugg, Switzerland). Purified spores were suspended in sterile distilled water and adjusted to an optical density at 600 nm (OD_{600}) of ~6.0 and stored at -20°C until used.

Preparation of Diluted Meat Juice

Diluted meat juice was prepared and used to suspend the purified spores in some experiments to simulate a low hygienic condition and present the worst-case scenario

that might encounter in food processing plants (Souza *et al.*, 2014). Ground beef (100 g) were manually cut into small pieces, and then mixed with 900 mL of distilled water. Beef slurry was ground with a sterile food processor until it became homogeneous and sterilized at 121°C for 15 min to eliminate all indigenous microorganisms. After cooling to room temperature, a clear supernatant was carefully removed and stored in a sterile microtube at -20°C until used.

Development of Germinant Mixtures and Germination Assay

L-alanine (Ala), L-cysteine (Cys), and L-serine (Ser) (Merck, Darmstadt, Germany) solutions were prepared with 25 mM Na_2HPO_4 buffer (pH 7.0) (Merck). To examine the effect of pH, each tested amino acid was prepared at 100 mM and adjusted to various pHs ranging from 4.5-10.0. The effect of amino acid concentrations was determined at 1-100 mM by preparing each tested amino acid with 25 mM Na_2HPO_4 and adjusting to pH 6.0. Subsequently, spore germination assay was also conducted with mixed germinants containing various selected amino acids and/or L-lactate (Sigma-Aldrich, Co., St. Louis, MO, USA) and $NaHCO_3$ (Merck) as co-germinants. The formulas of 6 mixed germinants were shown in Table 1. All germinants were filter-sterilized (0.45 μ m, Minisart[®] syringe filter, Satorious, Goettingen, Germany). For the germination assay, 100 μ L of standardized spore suspension (OD_{600} ~ 6.0) were heat activated at 80°C for 10 min and mixed with pre-warmed germinant at 30°C (Brunt *et al.*, 2014). Heat activation of spores is a typical procedure to induce spore germination and it is quite common that spores are exposed to high temperature under food manufacturing conditions (Andersson *et al.*, 1995; Udompijitkul *et al.*, 2013; Setlow, 2014). Spore germination was routinely monitored every 10 min by measuring the change in OD_{600} , which decreases by approximately 40% upon complete germination as confirmed by the percent of phase dark spores under phase contrast microscopy.

Table 1. Composition of the mixed amino acid germinants

Germinant ^a	Composition
Formula 1	50 mM L-alanine+25 mM L-cysteine in 25 mM Na ₂ HPO ₄
Formula 2	50 mM L-alanine+25 mM L-cysteine in 25 mM Na ₂ HPO ₄ +50 mM NaHCO ₃ +50 mM L-lactate
Formula 3	50 mM L-alanine+25 mM L-cysteine+50 mM L-serine in 25 mM Na ₂ HPO ₄
Formula 4	50 mM L-alanin +25 mM L-cysteine+50 mM L-serine in 25 mM Na ₂ HPO ₄ +50 mM NaHCO ₃ +50 mM L-lactate
Formula 5	100 mM L-alanin +100 mM L-cysteine in 25 mM Na ₂ HPO ₄ +50 mM NaHCO ₃ +50 mM L-lactate
Formula 6	100 mM L-alanin +100 mM L-cysteine+100 mM L-serine in 25 mM Na ₂ HPO ₄ +50 mM NaHCO ₃ +50 mM L-lactate

^a Each germinant mixture was prepared with an indicated buffer and adjusted to pH 6.0.

Preparation of Model Food Contact Surfaces

SS coupons (Grade 304, 2B finish) purchased from Chockchai All Metal Service Co., Ltd. (Samut Prakan, Thailand) and cut into 5×5 cm² pieces were first rinsed with tap water, and then treated with an alkaline detergent, 5% (v/v) Extran[®] MA 01 (Merck), for 2 h, rinsed with water, dried, and wrapped individually with aluminum foil before sterilizing at 121°C for 15 min. Sterile SS coupons were stored at room temperature until used. The polypropylene PB (New Star Foodservice, Chino, CA, USA) were cut into of 5×5 cm² pieces and washed with 5% (v/v) Extran[®] MA 01 for 2 h, rinsed with water, and then dipped in hot distilled water at 90°C for 20 min followed by dipping in 70% ethanol for 5 min prior to drying inside a class II biosafety cabinet for 30 min according to the previously published protocol with some modifications (Monnin *et al.*, 2012).

Sanitizer Preparation

NaOCl solution was prepared by diluting an appropriate amount of commercial

NaOCl solution (Haite Bleach, Kao Industrial Thailand Co., Ltd., Bangkok, Thailand) containing 6% (w/w) of NaOCl with sterile water to obtain a concentration of 200 ppm free chlorine. Iodophor (Agrodyne, Agromed Co., Ltd., Bangkok, Thailand) containing 2.6% (w/v) of available iodine was prepared at 25 ppm by diluting iodophor with sterile water. QACs were prepared by diluting an appropriate amount of QACs containing 80% Benzalkonium chloride (Chemipan Crop. Co., Ltd., Bangkok, Thailand) with sterile water to obtain the desired concentration of 200 ppm. All of these sanitizing agents were prepared and used within 1 h. EOW was prepared from 10% (w/v) NaCl solution by using the Hoshizaki ROX-10WA-E, Hoshizaki water electrolyzer (Hoshizaki Electric Co., Ltd., Toyoake, Aichi, Japan). EOW was collected from the anode side prior to experiment, stored in a sterile 1-L bottle, and used within 1 h after production. EOW used in this study had the following properties: pH 2.97, oxidation reduction potential 1,166 ± 16 mV, and free chlorine 100 ppm.

Spore Inactivation on Model Food Contact Surfaces after Germination Induction

The 3-strain cocktail of *C. sporogenes* spores consisting of PA 3679, TISTR 1452, and ATCC 3584 was prepared by combining an equal number of purified spores (~10⁷ CFU/ml) and suspended in sterile distilled water and sterile diluted beef juice to represent tested surfaces under “clean” and “dirty” conditions, respectively. The decontamination experiments were conducted on both clean and dirty surfaces of SS and PB coupons. The cocktail spores were heat activated at 80°C for 10 min (Brunt *et al.*, 2014). Then, an aliquot of 100 µL of heat-activated spore suspension was inoculated onto each SS or PB coupon and spread with a sterile bent glass rod. The contaminated surfaces were dried in the class II biosafety cabinet for 1 h to promote spore attachment. For triggering spore germination, the contaminated surfaces were aseptically transferred to sterile petri dishes containing 30 mL of the control buffer or Formula 4 to cover an entire surface for 30 min at 30°C. The

treated surfaces were transferred to a sterile beaker containing 200 mL of tested sanitizers for 10 min followed by immersing in neutralizing buffer (Difco) for 1 min. The treated surfaces were aseptically transferred into a sterile petri dish before determining the number of surviving cells.

In each experiment, several SS or PB coupons were included to determine the number of bacteria in each step as follows: 1) Count A represented the number of heat-activated *C. sporogenes* spores that was contaminated on a tested coupon, dried for 1 h, and immediately determined the total viable cells. 2) Count B was the number of heat-activated and dried *C. sporogenes* spores on a tested coupon that was induced to germinate with the corresponding germinant for 30 min at 30°C. The number of total viable cells were determined. 3) Count C referred to the number of heat-activated and dried *C. sporogenes* spores on a tested coupon (the same coupon as Count B) that were induced to germinate with the corresponding germinant for 30 min at 30°C. The number of non-germinated (dormant) spores were determined after heat treatment at 80°C for 20 min, and 4) Count D denoted the number of total viable cells remaining on a tested coupon after germination induction and inactivation with the particular sanitizing agent.

Microbial Analysis of Food Contact Surfaces

The entire surface was swabbed with two sterile cotton swabs by employing the recommended directional swabbing technique (Bell *et al.*, 2005). These swabs were transferred into 10 mL of 25 mM Na₂HPO₄ buffer (pH 7.5) and mixed vigorously with a vortex mixer for 1 min. A 1-mL aliquot was subjected to heat treatment at 80°C for 20 min to enumerate the number of non-germinated spores (dormant spores) and the other portion without heat treatment to determine total cell counts. All plating was performed on Brain Heart Infusion Agar (Difco) and incubated anaerobically at 37°C for 48 h. In every trial of food contact surface decontamination experiment, SS or PB coupons without spore

inoculation were included as a negative control to ensure an absence of indigenous microorganisms.

Statistical Analysis

All experiments were performed in triplicate with 3 independent spore preparations. Data were analyzed by analysis of variance (ANOVA) using SPSS statistical software package (IBM Corp., Armonk, NY, USA). Multiple comparisons among mean values were performed with Duncan's Multiple Range Test at the significant level of 0.05.

Results and Discussion

Effects of pH and Concentration of Amino Acids on Inducing Germination of *C. sporogenes* Spores

Three amino acids, Ala, Cys, and Ser, were selected based on previous studies demonstrating that each of these amino acids was able to initiate germination of *C. botulinum* ATCC 3502, *C. sporogenes* ATCC 11437, and ATCC 15579 spores (Ishimori *et al.*, 2012; Brunt *et al.*, 2014). To identify the optimum pH and concentration of Ala, Cys, and Ser to induce germination, *C. sporogenes* PA 3679 and TISTR 1452 were selected as representative strains owing to the widely used of PA 3679 as the surrogate of proteolytic *C. botulinum* (Bradbury *et al.*, 2012; Brown *et al.*, 2012) and the high sporulation efficiency of these strains. The negligible extent of germination was observed when *C. sporogenes* spores were incubated with 25 mM Na₂HPO₄ buffer (pH 7.0) as a control at 30°C, reflecting less than 10% of OD₆₀₀ reduction. This observation was also confirmed by phase contrast microscopy in which ~ 99% of spores remained phase bright indicating no significant germination as spores lose refractility upon germination and become phase dark. Similar correlation between OD₆₀₀ decrease and percent of phase dark spores during germination was also reported for *C. botulinum* and *C. sporogenes* spores in the previous study (Brunt *et al.*, 2014). In this

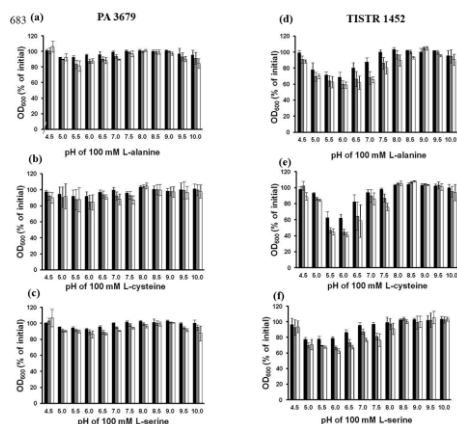


Figure 1. Effect of pH on germination of *C. sporogenes* PA 3679 (a-c) and TISTR 1452 (d-f) spores by Ala, Cys, and Ser. Heat activated *C. sporogenes* spores were incubated with 100 mM of each tested amino acid adjusted to various pHs at 30°C for 10 min (■), 30 min (▒), and 60 min (□)

work, significant germination was considered only for spores with an OD₆₀₀ reduction over 15% from the initial value.

The highest germination extent was observed for Ala, Cys, and Ser upon adjusting pHs to 5.5 and 6.0 for both tested strains (Figure 1(a-f)) within 60 min. For Ala-induced germination, the highest germination extent of TISTR 1452 was observed at pH 6.0 (Figure 1(d)) and PA 3679 at pH 5.5 (Figure 1(a)). Similarly, pH 6.0 maximized germination of both tested strains with Cys and Ser (Figures 1(b-c) and 1(e-f)). Although there was no significant difference in germination induction for tested amino acids adjusted to pH 5.5 and 6.0 ($p > 0.05$), pH 6.0 provided the highest germination extent for most of the tested amino acids for both strains. Thus, all further experiments were conducted with germinants adjusted to pH 6.0.

For Ala-induced germination, a significant germination was observed with a concentration of 10 mM for TISTR 1452 and 50 mM for PA 3679 spores after 60 min of incubation (Figures 2(a) and 2(d)). Only 1 mM and 25 mM of Cys

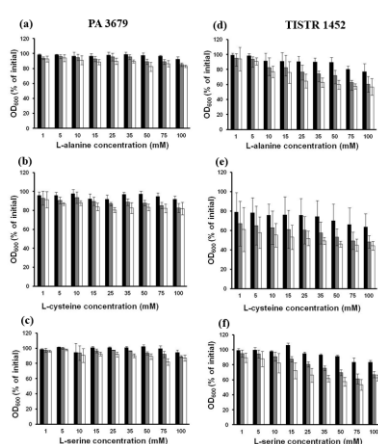


Figure 2. Effect of concentration on germination of *C. sporogenes* PA 3679 (a-c) and TISTR 1452 (d-f) spores by Ala, Cys, and Ser. Heat activated *C. sporogenes* spores were incubated with each tested amino acid adjusted to pH 6.0 at 30°C for 10 min (■), 30 min (▒), and 60 min (□)

were sufficient to stimulate germination response for TISTR 1452 and PA 3679, respectively (Figures 2(b) and 2(e)). Ser at 10 mM was able to induce germination of TISTR 1452. Overall, spores of PA 3679 had a much lower germination extent as reflected by smaller optical density reduction during 60 min of incubation to each amino acid regardless of tested concentrations compared with TISTR 1452 at the same time period. A diversity in spore germination response to the same germinant among strains of proteolytic *C. botulinum*, *Clostridium perfringens*, and *Bacillus megaterium* was also previously reported (Foerster and Foster, 1966; Alberto *et al.*, 2003; Paredes-Sabja *et al.*, 2009; Udompijitkul *et al.*, 2014; Banawas and Sarker, 2018). Owing to this phenomenon, the germination characteristic of one strain might not reflect germination response of other strains or mixed population of various strains. With this respect, PA 3679 could be an example of spores with an inert response to the single amino acid tested and highlight a significance of developing a common

germinant capable of initiating germination of various strains to indicate the actual environment and broaden a practical application to mixed-strain populations. Although *C. sporogenes* spore germination extent seemed to enhance in a concentration dependent manner, particularly for TISTR1452, no significant increase of germination extent was achieved with increasing concentrations of Ala, Cys, and Ser over 50 mM, 25 mM, and 50 mM, respectively ($p>0.05$).

Developing the Germinant Mixture to Trigger Germination of *C. sporogenes* Spores

There is no attempt to intensively formulate the germinant mixture for *C. sporogenes* spores available so far. The germinant mixtures, Formula 1, 2, 3, and 4, were capable of inducing higher extent of spore germination as compared to an individual amino acid germinant (Figures 3(a-b)), especially germination of PA 3679 spores was substantially improved. This study indicates that the combinations of Ala, Cys, and/or Ser at 50, 25, and/or 50 mM, respectively, were superior germinants compared with an individual amino acid tested. However, we found that a combination of 100 mM of each tested amino acid triggered a lower germination rate and response for both strains (Figures 3(a-b)). Among tested mixtures, Formula 4 was the most effective germinant to induce germination of both tested strains with approximately 40% reduction in OD₆₀₀, indicating that most spores within a population germinated within the time period of 30-60 min. To exclude the possibility that the germination-inducing capacity of Formula 4 was specific to limited strains, two additional strains of *C. sporogenes*, ATCC 3584 and TISTR 1481, were included in the germination assay. Figure 4 shows that both strains were able to germinate in the presence of Formula 4 although at different extents. Collectively, these results indicate that Formula 4 was an effective germinant for a wide range of *C. sporogenes* isolates. Besides monitoring the reduction of OD₆₀₀, spore germination with Formula 4 was also confirmed by examining phase bright and phase dark spores under

phase contrast microscope. The majority of spores of 4 tested *C. sporogenes* with approximately 86-88% became phase dark after 60 min post-incubation. Thus, Formula 4 was selected for inducing spore to germinate before inactivating with sanitizers on model food contact surfaces.

The better germination response of *C. sporogenes* in germinant mixtures at moderate concentrations of mixed amino acids observed in this study implies that these amino acid germinants might act synergistically to initiate spore germination. It was suggested that the synergistic effect of nutrient germinants involves an increase in the number of activated GRs and enhance interactions among different GR proteins (Yi *et al.*, 2011; Setlow, 2014).

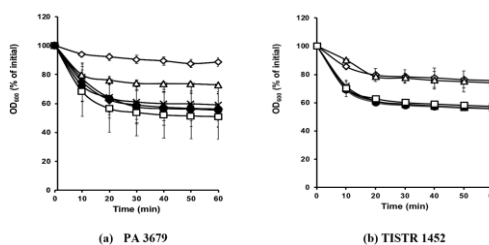


Figure 3. Germination of *C. sporogenes* PA 3679 (a) and TISTR 1452 (b) with various germinant mixture formulas. Heat activated spores were induced to germinate with Formula 1 (◆), Formula 2 (●), Formula 3 (×), Formula 4 (□), Formula 5 (△), and Formula 6 (◇)

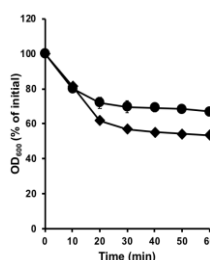


Figure 4. Germination of strains of *C. sporogenes* spores with Formula 4 germinant mixture. Heat activated spores of ATCC 3584 (●) and TISTR 1481 (◆) were incubated with Formula 4 at 30°C for 60 min

Table 2. Inactivation of *C. sporogenes* spores on clean SS coupons with various sanitizing agents for 10 min at room temperature

Sanitizer/ concentration	Germinant	Initial	Germination (total cells)	Germination (dormant spores)	After treatment	Log reduction after sanitizer treatment ^f
		Count A ^b	Count B ^c	Count C ^d	Count D ^e	
NaOCl (200 ppm)	Control ^a	5.09±0.24	5.17±0.26	4.35±0.38	4.58±0.52	0.51
	Formula 4		4.85±0.32	3.37±0.19	3.40±0.38	1.69
Iodophor (25 ppm)	Control	5.13±0.12	5.21±0.07	4.75±0.16	4.86±0.19	0.27
	Formula 4		4.97±0.07	3.58±0.06	3.90±0.20	1.23
QACs (200 ppm)	Control	5.11±0.20	5.16±0.17	4.88±0.30	5.19±0.19	-0.08
	Formula 4		4.66±0.22	3.50±0.32	3.67±0.16	1.44
EOW	Control	5.05±0.39	4.89±0.13	4.50±0.09	3.72±0.19	1.33
	Formula 4		3.92±0.07	2.93±0.20	2.44±0.46	2.61

^a Control represents 25 mM Na₂HPO₄ buffer (pH 7.5)

^{b, c, d, e} Count A, Count B, Count C, and Count D values are mean ± SD in log CFU/cm² as described in Materials and Methods.

^f Log reduction values are the reduction of total cells in log CFU/cm² following a treatment with the indicated sanitizer and calculated as the value of Count A - Count D.

As spores germinate in response to separate threshold concentrations of germinants that activate one or multiple GRs, increasing concentrations over a particular level might not further enhance the rate and extent of germination. Studies on *Bacillus subtilis* spores proved that the synergistic effect of nutrient germinants on germination rate is only observed at low concentrations and the synergy is lost at higher concentrations (Yi *et al.*, 2011).

Inactivation of *C. sporogenes* Spores on Model Food Contact Surfaces after Germination Induction

It was established that germinated spores are more sensitive to various stressors than their dormant form (Hornstra *et al.*, 2007; Akhtar *et al.*, 2009; Nerandzic and Donskey, 2010; Udombijitkul *et al.*, 2013; Omotade *et al.*, 2014; Nerandzic and Donskey, 2016), we tested our working hypothesis that inducing *C. sporogenes* spore germination with Formula 4 prior to a surface decontamination step could enhance the sporicidal activities of commonly-used sanitizers against adhered spores. Two model foods contact surfaces, SS and PB, were selected based on the widely used in food industry and domestic kitchens (Holah and Thorpe, 1990; Liu *et al.*, 2006). Since the germination extent of *C. sporogenes* spores of

all tested strains with Formula 4 was relatively constant within 30 min of germination (Figures 3(a-b) and (4)), we decided to induce spore germination with Formula 4 for 30 min before inactivating with sanitizers. Results from Tables 2, 3, 4, and 5 clearly show that Formula 4 was able to induce germination of *C. sporogenes* spores adhered onto SS and PB surfaces within 30 min as demonstrated by the number of non-germinated (dormant) spores (Count C) that was consistently lower for spores incubated with Formula 4 compared with control buffer. These results also suggest that *C. sporogenes* spores maintained their germination capability after 1-h drying under aerobic condition. Thus, there is a potential to implement germination-induction step to adhered spores before inactivating with sanitizers.

For clean SS coupons, EOW showed the greatest sporicidal activity against dormant spores (control) with the log reduction of 1.33 log CFU/cm², while NaOCl, iodophor, and QACs had limited efficacy to inactivate dormant *C. sporogenes* spores with negligible changes in Count D compared with initial counts (Count A). Spores germinated with Formula 4 showed a higher susceptibility to all tested sanitizers compared with their respective control spores ($p < 0.05$) with log reductions of 1.69, 1.23, 1.44, and 2.61 for NaOCl, iodophor, QACs, and EOW,

Table 3. Inactivation of *C. sporogenes* spores on dirty SS coupons with various sanitizing agents for 10 min at room temperature

Sanitizer/ concentration	Germinant	Initial	Germination (total cells)	Germination (dormant spores)	After treatment	Log reduction after sanitizer treatment ^f
		Count A ^b	Count B ^c	Count C ^d	Count D ^e	
NaOCl (200 ppm)	Control ^a	4.46±0.38	4.80±0.48	4.37±0.41	3.45±0.15	1.01
	Formula 4		5.03±0.88	2.76±0.10	1.87±0.46	2.59
Iodophor (25 ppm)	Control	4.19±0.22	4.23±0.32	4.27±0.91	3.80±0.45	0.39
	Formula 4		3.91±0.25	3.21±1.15	3.73±0.44	0.46
QACs (200 ppm)	Control	4.49±0.10	4.35±0.25	4.15±0.37	4.08±0.35	0.41
	Formula 4		3.98±0.08	2.58±0.63	3.45±0.17	1.04
EOW	Control	4.65±0.14	4.79±0.12	3.96±0.10	2.43±0.21	2.22
	Formula 4		4.71±0.05	2.72±0.22	0.87±0.41	3.78

^a Control represents 25 mM Na₂HPO₄ buffer (pH 7.5)^{b, c, d, e} Count A, Count B, Count C, and Count D values are mean ± SD in log CFU/cm² as described in Materials and Methods.^f Log reduction values are the reduction of total cells in log CFU/cm² following a treatment with the indicated sanitizer and calculated as the value of Count A - Count D.**Table 4. Inactivation of *C. sporogenes* spores on clean PB coupons with various sanitizing agents for 10 min at room temperature**

Sanitizer/ concentration	Germinant	Initial	Germination (total cells)	Germination (dormant spores)	After treatment	Log reduction after sanitizer treatment ^f
		Count A ^b	Count B ^c	Count C ^d	Count D ^e	
NaOCl (200 ppm)	Control ^a	5.00±0.16	4.86±0.27	4.88±0.09	3.64±0.25	1.36
	Formula 4		4.70±0.17	3.42±0.22	2.62±0.17	2.38
Iodophor (25 ppm)	Control	5.19±0.16	5.13±0.09	4.94±0.10	5.10±0.40	0.09
	Formula 4		4.66±0.26	3.47±0.23	3.92±0.41	1.27
QACs (200 ppm)	Control	4.92±0.32	5.03±0.46	4.77±0.58	4.93±0.42	- 0.01
	Formula 4		4.31±0.29	3.44±0.47	3.44±0.20	1.48
EOW	Control	5.31±0.08	5.21±0.06	5.04±0.10	2.67±0.10	2.64
	Formula 4		4.52±0.11	3.51±0.34	2.15±0.58	3.16

^a Control represents 25 mM Na₂HPO₄ buffer (pH 7.5)^{b, c, d, e} Count A, Count B, Count C, and Count D values are mean ± SD in log CFU/cm² as described in Materials and Methods.^f Log reduction values are the reduction of total cells in log CFU/cm² following a treatment with the indicated sanitizer and calculated as the value of Count A - Count D.

respectively (Table 2). QACs is a sporostatic agent and the resistance of dormant *C. sporogenes* spores upon adhered to SS surface to 200 ppm QACs activity is consistent with the previous report with spores of *C. perfringens* food poisoning isolates adhered to clean SS coupons, while germinated *C. perfringens* spores are significantly more susceptible to this sanitizer (Russell, 1990; Udombijitkul *et al.*, 2013). Although the iodine-based sanitizer possesses sporicidal activity against spores of *B. subtilis* and *Bacillus thuringiensis* as recently reported (Li *et al.*, 2017), we observed limited efficacy of 25 ppm iodophor against dormant spores of

C. sporogenes and this may highlight the diverse in the sensitivity of different spore-forming bacteria to iodine compounds. A significant reduction in populations of *C. sporogenes* spores on clean PB coupons (Table 4; Count D) treated with all tested sanitizers was only achieved for Formula 4-induced spores compared with initial counts (Count A) ($p < 0.05$). After implementing germination step with Formula 4, EOW was the most effective sanitizer with 3.16 log CFU/cm² reduction. An insignificant increase in cell counts after sanitizing treatment (Count D) as compared to the initial cell count (Count A) in some experiments might be attributed to

Table 5. Inactivation of *C. sporogenes* spores on dirty PB coupons with various sanitizing agents for 10 min at room temperature

Sanitizer/ concentration	Germinant	Initial	Germination (total cells)	Germination (dormant pores)	After treatment	Log reduction after sanitizer treatment ^f
		Count A ^b	Count B ^c	Count C ^d	Count D ^e	
NaOCl (200 ppm)	Control ^a	4.60 ± 0.11	4.40 ± 0.23	4.06 ± 0.38	3.30 ± 0.51	1.30
	Formula 4		4.12 ± 0.21	3.43 ± 0.35	2.71 ± 0.29	1.89
Iodophor (25 ppm)	Control	4.67 ± 0.07	4.50 ± 0.21	3.49 ± 0.31	4.37 ± 0.20	0.30
	Formula 4		4.27 ± 0.25	3.26 ± 0.39	3.43 ± 0.49	1.24
QACs (200 ppm)	Control	4.72 ± 0.56	4.43 ± 0.65	3.84 ± 0.97	4.37 ± 0.75	0.35
	Formula 4		4.18 ± 0.47	3.47 ± 0.05	3.31 ± 0.57	1.41
EOW	Control	4.30 ± 0.27	4.86 ± 0.07	3.93 ± 0.05	2.78 ± 0.28	1.52
	Formula 4		4.93 ± 0.26	2.86 ± 0.16	1.99 ± 0.50	2.31

^a Control represents 25 mM Na₂HPO₄ buffer (pH 7.5)

^{b, c, d, e} Count A, Count B, Count C, and Count D values are mean ± SD in log CFU/cm² as described in Materials and Methods.

^f Log reduction values are the reduction of total cells in log CFU/cm² following a treatment with the indicated sanitizer and calculated as the value of Count A - Count D.

a slight variation in the number of inoculum size of each coupon.

The beef juice contaminated surfaces were included in this work in order to mimic a food contact surface condition that might exist in real food processing environment. For dirty surfaces, EOW was able to significantly inactivate dormant spores (incubated with control buffer) by 1.52 log on dirty PB (Table 5) and 2.22 log on dirty SS surfaces (Table 3) ($p < 0.05$) compared with initial spores (Count A). For germination-induced spores, the number of log reductions by 3.78 after EOW treatment on dirty SS coupons was significantly higher than other tested sanitizers ($p < 0.05$), but the inactivation capacity on dirty PB coupons was comparable ($p > 0.05$) between NaOCl and EOW against germinated spores. It is important to note that EOW was able to retain its strong sporicidal activity, especially against germinated spores even in the presence of beef juice. These results contradict with the previous study with *Bacillus cereus* and *B. subtilis* spores that the effectiveness of EOW was dramatically decreased in the presence of organic matters on SS disks (Zhang *et al.*, 2016).

It is crucial to evaluate the efficacy of a surface decontamination measure in the presence of organic materials or food residues since this would be a potential scenario facing in food processing environments. The lower

initial counts (Count A) after 1-hour drying on dirty surfaces compared with clean surfaces was probably due to change in surface characteristics that occurred when the surfaces were modified in the presence of meat juice. Furthermore, the presence of beef juice on food contact surfaces might act as a nutrient germinant to induce germination of some spores in the population rendering them more susceptible to oxygen or desiccation during drying or further sensitizing spores to tested sanitizers. Indeed, we found that beef juice used in this study was able to induce significant spore germination of all tested *C. sporogenes* isolates (data not shown). These factors might, at least in part, account for the relatively strong sporicidal activity of tested sanitizers, particularly NaOCl and EOW despite the presence of organic materials as observed in this work.

It is notable that EOW was the most effective sanitizer observed in this study for both surfaces with a significant reduction achieved even with dormant spores or in the presence of organic materials. Although the precise mode of action of EOW against bacterial spores is not fully elucidated, treatments of *C. sporogenes* PA 3679 spores with electro-activated water having acidic pH and high oxidation reduction potential suggested that electro-activated water damages spore coat, cortex, and core (El Jaam

et al., 2016). In the suspension tests, EOW shows the sporicidal activity against *B. subtilis*, *B. cereus*, and *Clostridium difficile* (Zhang *et al.*, 2016; Tkhawkho *et al.*, 2017). Nevertheless, information regarding the inactivation efficacy of EOW against adhered spores on real food contact surfaces is still lacking. This information is very crucial since microorganisms were known to respond differently to sanitizers once attached to food contact materials and generally are less susceptible to the action of various commonly-used surface sanitizers (Kreske *et al.*, 2006; André *et al.*, 2012; Udombijitkul *et al.*, 2013). Thus, it is necessary to evaluate the antimicrobial efficacy of tested sanitizers against target microorganisms on real food contact surfaces to be able to better understand microbial response than the suspension test in laboratory medium. Owing to its simple preparation, environmental- and user- friendly, high efficacy, and cost effectiveness (Hricova *et al.*, 2008; Huang *et al.*, 2008), EOW should be considered as a promising sporicidal agent to combat with adhered spores on food contact surfaces.

Conclusions

In this study, we present the inactivation measure for spores of *C. sporogenes*, a closely-related bacterium to *C. botulinum*, that adhered to SS and PB coupons. The germinant mixture containing 50 mM Ala+25 mM Cys+50 mM Ser in 50 mM NaHCO₃+50 mM L-lactate+25 mM Na₂HPO₄ (pH 6.0) was able to efficiently stimulate spore germination of all tested strains of *C. sporogenes*. The implementation of a germination step sensitized adhered spores to the action of tested sanitizers and further enhance their efficacy in inactivating *C. sporogenes* spores on SS and PB coupons. For the first time, our study also showed a strong antimicrobial activity of EOW as a sanitizer against *C. sporogenes* spores adhered to food contact surfaces. The information gained from this work could serve as a prototype procedure for further optimizing the universal germinant mixture with simple

amino acids that can simultaneously induce germination of a variety of food-related spore-forming bacteria. This would provide an immense benefit of the germination-induced secondary decontamination strategy and aid in improving safety of consumer.

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