Summer 8-2021

Synthesizing Inactivation Efficacy of Treatments against *Bacillus cereus* through Systematic Review and Meta-Analysis and Evaluating Inactivation Efficacy of Commercial Cleaning Products against *B. cereus* Biofilms and Spores Using Standardized Methods

Minho Kim
University of Nebraska-Lincoln, mkim30@huskers.unl.edu

Synthesizing inactivation efficacy of treatments against *Bacillus cereus* through systematic review and meta-analysis and evaluating inactivation efficacy of commercial cleaning products against *B. cereus* biofilms and spores using standardized methods

By

MINHO KIM

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science & Technology

Under the supervision of Professors Jayne E. Stratton and Andréia Bianchini-Huebner

Lincoln, Nebraska

August 2021

Synthesizing inactivation efficacy of treatments against *Bacillus cereus* through systematic review and meta-analysis and evaluating inactivation efficacy of commercial cleaning products against *B. cereus* biofilms and spores using standardized methods

Minho Kim, M.S.

University of Nebraska, 2021

Advisor: Jayne Stratton

Bacillus cereus is ubiquitous and can be easily carried in food processing facilities. It is problematic because it can survive various treatments such as heat, radiation, and chemical cleaning by forming spores and biofilms. Some review papers have discussed inactivation efficacies of different treatments against *B. cereus*, but they are narrative without quantitative summaries. This study first aimed to find effective and food industry-applicable treatment candidates against *B. cereus* spores and biofilms by synthesizing and comparing the efficacy of treatments using systematic review and meta-analysis. After screening, 17 studies were included, but only nine studies were used for meta-analysis due to the absence of statistical data. Oxidizing agents presented the best efficacy with an average of 2.51 $\log_{10}$ inactivation. For inactivating *B. cereus* biofilms, Clean-In-Place (CIP) procedures with acid or alkaline detergents generally demonstrated good efficacy ranging from 0-6 $\log_{10}$ inactivation. However, not all treatments and conditions being tested were applicable in the food processing environment. Also, diverse methods were used for measuring inactivation efficacy which made a direct comparison of treatments unavailable. To deal with these issues, studies were conducted to evaluate

the sporicidal and anti-biofilm efficacy of commercially available cleaning products in food industry-applicable conditions with standardized methods. For the sporicidal efficacy, four commercially available cleaning products were tested. A sanitizer with an Environmental Protection Agency (EPA) biofilm claim, Synergex™, showed the best efficacy with over 5.33 $\log_{10}$ reduction in 5 min at 2% concentration. Less efficacy was shown at 1% concentration with a 0.5-2.8 $\log_{10}$ reduction. A sanitizer with an EPA sterilant claim, P3-oxonia™ at 2.5% concentration, showed about 1-$\log_{10}$ reduction in 10 minutes, and at 5% concentration showed 1 to 5 $\log_{10}$ reduction. An alkaline detergent Lift™ III and an acidic detergent HD PL-10™ showed <1 $\log_{10}$ inactivation. For the anti-biofilm efficacy, three commercial cleaning products were tested. Synergex™ showed over 5-$\log_{10}$ reductions in every condition tested. HD PL-10™ showed 0-2.1 $\log_{10}$ reduction with better efficacy when application time, concentration, and temperature increased. Lift™ III showed a 0.3-1.7 $\log_{10}$ reduction in tested conditions.

This thesis is dedicated to my beloved father,

Kim, Sungcheol

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude and warm appreciation to the following people who contributed much to helping me finish this valuable work.

Dr. Jayne Stratton and Dr. Andréia Bianchini-Huebner, my thesis advisors, for their guidance, immense knowledge, motivation, and the opportunity to work in the food micro lab.

Dr. Bing Wang and Curtis Weller, my committee members, for their insightful comments on developing the thesis.

Waraporn Mahlman, our lab manager, for being the nicest instructor with kindness and patience.

Dr. Bing Wang, Onay Dogan, Anna Rose Pilapil, and Constanza Avello for helping me in systematic review and meta-analysis part of my thesis.

Emily Robinson, for helping me in the statistical analysis.

Jesse Hines and ECOLAB for sponsoring a sanitizer and giving advice on experiments.

Walker Carson, Brooke Parrish, Constanza Avello, Cody Brown and Onay Dogan, fellow graduate students, for warmheartedness and friendship.

Kirk Martin, Chloe Bartek, and Ian Murphree, my roommates, for kindness and support.

Finally, I would like to thank my family for their continuous and unparalleled love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 1. SYNTHESIZING INACTIVATION EFFICACY OF TREATMENTS AGAINST *B. CEREUS* USING SYSTEMATIC REVIEW AND META-ANALYSIS

1. Abstract

Bacillus cereus has been widely found in food processing facilities, surviving various treatments such as heat, radiation, and chemical cleaning. Some review papers discuss inactivation efficacies of different interventions against *B. cereus*, but they are narrative rather than quantitative. A systematic literature review and meta-analysis were performed through three electronic databases (Web of Science Core Collection, SCOPUS, BIOSIS citation index). Seventeen studies met the inclusion criteria that were subjected to data extraction. Nine studies were available for the meta-analysis. For the meta-analysis, according to treatments' mechanism of action, treatments were divided into three subgroups: oxidizing agents, surface-active compounds, and others. For the inactivation efficacy against *B. cereus* spores, oxidizing agents presented the best efficacy with an average of 2.51 $\log_{10}$ inactivation. For inactivating *B. cereus* biofilms, Clean-In-Place (CIP) procedures with acid or alkaline detergents generally presented good efficacy with reported inactivation efficacy ranging from 0-6 log. Numerous papers excluded because of missing critical statistic data and high heterogeneity in measuring methods hindered the direct comparison of efficacies of different treatments. Thus, for future research, by adopting existing standardized methods and validation procedures, the food industry will be able to determine the most effective protocols to eliminate this resilient organism in their food processing facilities.

2. Introduction

Bacillus cereus is a gram-positive, motile, rod-shaped, aerobic or facultative anaerobic, endospore-forming bacterium that belongs to *Bacillus* genus. It is widespread

in nature and often isolated from soil and plant sources. It can easily spread from these habitats to foods carried by insects and animals (Stenfors Arnesen, Fagerlund, & Granum, 2008). *B. cereus* is problematic because it can cause two different types of food poisoning: The diarrhoeal type caused by complex enterotoxins and the emetic type caused by emetic toxins (cereulides) (Granum & Lund, 2006). In the United States, foodborne illness attributable to *B. cereus* contaminated foods were estimated as 63,400 cases per year (Scallan et al., 2011). However, the number of cases might be underestimated due to its mild symptoms (Messelhäusser et al., 2014). *B. cereus* can survive extreme conditions by forming endospores that are highly resistant to various treatments including heat, radiation and chemical treatments (Soni, Oey, Silcock, & Bremer, 2016). They can also form biofilms, which are defined as a community of bacteria surrounded by extracellular polymeric substances (EPS) and attached to a surface (Kwon, Hussain, & Oh, 2017). Both spores and vegetative cells can be embedded inside the biofilm and protected from cleaning regimes (Majed, Faille, Kallassy, & Gohar, 2016). These features allow *B. cereus* to survive regular processing or cleaning procedures and persist in the food processing environment as a continuous source of food contamination (Gopal et al., 2015; Kumari & Sarkar, 2016).

A systematic review is a review process that formulates a research question with methods to identify, select, and evaluate relevant research. A meta-analysis may follow the systematic review to quantitatively synthesize data extracted from the included studies (Moher, Liberati, Tetzlaff, & Altman, 2009). Systematic reviews and meta-analysis have been mainly used in human medicine field (Ahn & Kang, 2018). Although the application in food science and agriculture is still at early stage, systematic review

and meta-analysis have shown benefits in supporting the decision making of food safety risk management (Aiassa et al., 2015; Omer et al., 2018; Ortuzar et al., 2018; Zhang & Wang, 2018). In addition to qualitative characterization in a narrative review, a quantitative summary of findings from a systematic review can be achieved by meta-analysis allowing for quantitative evidence synthesis based on statistical algorithm (Garg, Hackam, & Tonelli, 2008).

There are a few review papers describing the efficacy of different treatments against *B. cereus* (Choi & Kim, 2020; Galié, García-Gutiérrez, Miguélez, Villar, & Lombó, 2018). However, they are narrative rather than quantitative. Additionally, not much knowledge is available on persistent *B. cereus* contamination or cleaning techniques for *B. cereus*. Therefore, to compare the efficacies of interventions against *B. cereus*, the aim of this research was to perform a systematic review and meta-analysis to synthesize the current research on inactivation efficacies of treatments against *B. cereus*.

3. Materials and Methods

3.1. Research Question, Search Strategy and Data Source

This review was designed to answer the following research question: "Which intervention can be effective in inactivating *B. cereus* spores and/or biofilms in food processing settings?". To collect relevant evidence to answer this question, a search strategy was developed integrating terms related to three main concepts, i.e., microbial organisms, interventions, and context related to food processing (Table 1). Search terms for each concept were finalized with the consultation of University of Nebraska-Lincoln librarian expertise in Food Science. Key terms for each concept were combined using the

Boolean operator "OR", and the concepts were combined using the Boolean operator "AND". The search syntax was verified by ensuring a full capture of a list of 20 relevant citations that were obtained before the systematic search based on a hand search and recommendations from the supervisory committee.

The last search was conducted on May 11, 2020, in three electronic bibliographic databases, including Web of Science Core Collection (via Web of Science, 1900 to date of search), Scopus (via the University of Nebraska-Lincoln Scopus interface, 1959 to date of search) and BIOSIS Citation Index (via Web of Science, 1926 to date of search) with no restrictions placed on the search beyond the inception dates of databases. Similarly, there was no restriction placed upon language to maximize the literature caption, although those published in English were selected during the screening process. Search results from multiple databases were uploaded to EndNoteX9 (Clarivate Analytics, Philadelphia, PA). Duplicated citations identified by EndnoteX9 deduplication function and hand search were removed.

Table 1. Search strategy

Concept	Search terms
Organism	"*Bacillus cereus*" or "*B. cereus*"
Treatment	"treat*" OR "disinfect*" OR "inactivat*" OR "biocid*" OR "anti spore" OR "decontaminat*" OR "lethality" OR "efficacy" OR "sporicid*" OR "deactivat*" OR "killing" OR "removal"
Commonly used cleaning methods, environments, and sanitizers	"CIP" OR "clean-in-place" OR "cleaning" OR "chemical agent" OR "disinfectant" OR "peroxide" OR "peracetic acid" OR "PAA" OR "chlorine" OR "hypochlorite" OR "cupric ascorbate" OR "surface" OR "stainless steel"

Note. "*" symbol retrieves zero or more characters.

3.2. Relevance Screening

Relevance screening was done on initially retrieved citations based on 1) title and abstract, and 2) full-text using the software EndNoteX9.

3.2.1. Title and abstract screening

Title and abstract screening were peer-reviewed to prevent the exclusion of relevant citations: An additional researcher did title and abstract screening using same inclusion and exclusion criteria and the result were combined after each step. Title screening focused on rapidly excluding citations irrelevant to our research question. Three inclusion criteria were used in this stage of screening: 1) Treatment should be applicable in the food industry; 2) Data should be reported in the log CFU unit; and 3) The target organism must be shown as *B. cereus* or its general definition such as *Bacillus* spp. or a sporeformer.

Relative *Bacillus* spp. and general terms for target organism such as sporeformers were kept for preventing missing relevant citations. Primary research article was the main data source; and for this reason, the other types such as the review and the book chapter were excluded. Non-English citations were excluded.

3.2.2. Full-text screening

After the preliminary screening based on title and abstract, included citations were downloaded for the full-text screening using University of Nebraska-Lincoln subscriptions and interlibrary loan services. Available files were attached in the Endnote library. In consistence to the aforementioned inclusion criteria, the eligibility of included citations were confirmed based on detailed information presented in the full

texts. Citations were excluded in this stage for the following reasons: no full texts available, no numerical data retrievable.

3.3. Data Extraction

Relevant data were manually extracted and organized in an Excel spreadsheet. The collected information from relevant citations includes author, year, country, sample size, cell form (vegetative, spore and biofilm), strain, surface, treatment, temperature, treatment time, concentration, and the efficacy of treatment—including unit and statistical descriptors of control and experimental groups such as mean and standard deviation (when available). For data analysis purpose, citations were divided into three subgroups according to the treatments' mode of action: 1) oxidizing agents including chlorine-based compounds, hydrogen peroxide, ozone and PAA, 2) surface-active compounds including quaternary ammonium compounds and acid anionic compounds, and 3) others including physical treatments and detergents.

3.4. Data Analysis

Due to the scarcity of necessary statistics reported, only a portion of citations relevant to our research questions were included for meta-analysis. To quantify the inactivation efficacy of each treatment, mean differences in $\log_{10}$ reduction were estimated. Heterogeneity measure (I^2) was also reported to explain differences under varying conditions of the same treatment and differences within each subgroup. Random effects meta-analysis models were built on trial-level data using inverse-variance weighting and restricted maximum likelihood method for variance estimation using the

'metafor' package in R version 4.0 (R Core Team 2020). Each trial shows different treatment conditions in the same study. Results were visualized in forest plots. Meta-regression was not applied due to the low number of citations.

4. Results and Discussion

4.1. Search Result and Study Selection

Figure 1 summarizes the systematic review process. A search from three databases on May 11, 2020, yielded 2,202 records. After de-duplication, 1,342 records remained for the relevance screening. Peer-review was done for the relevance screening and combined after each step of the two-stage screening. Of the 1,342 records, 1,008 studies were excluded for irrelevance based on title and abstract. Of the 334 studies assessed for eligibility based on full texts, ten studies were excluded as full texts not found using UNL's available tools and 307 studies were excluded by not passing the inclusion criteria. After the initial search, a total of 17 studies were included for the data extraction. However, due to the absence of statistical data such as means and standard deviations, eight studies were excluded and nine studies were used for the meta-analysis. An updated search was done on Jan 12, 2021, but no additional studies meeting the inclusion criteria was found.

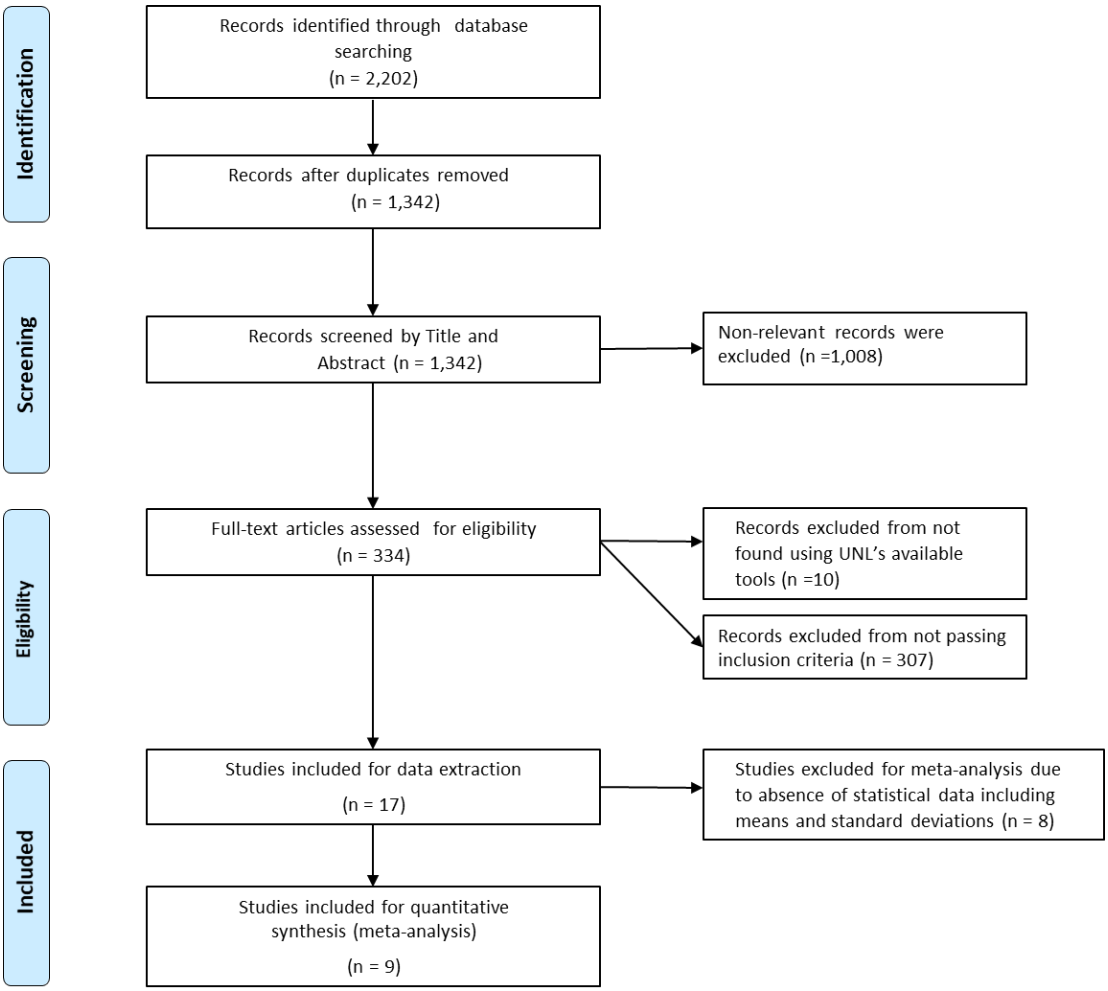


Figure 1. Flow diagram of systematic review

4.2. Characteristics of Relevant Studies and Extracted Data

Data were extracted from 17 included studies, which are summarized in Table 2. Among them, most studies evaluated the inactivation efficacy of various treatment methods against *B. cereus* biofilms (n=9), followed by spores (n=5) and vegetative cells (n=2). All studies followed a challenge testing design, consisting of artificial contamination of *B. cereus* in various experimental matrix. Different methods were being used to measure the efficacy and the results were reported with various units.

Challenged spores were prepared either by nutrient depletion caused by incubating over five days (S. S. Kim, Kim, Park, & Kang, 2020) or by growing in modified agar containing manganese and incubating over two days (Caballero Gómez, Grande, Pérez Pulido, Abriouel, & Gálvez, 2013; Giffel, Beumer, VanDam, Slaghuis, & Rombouts, 1995; Khadre & Yousef, 2001; Lv et al., 2019; Sagripanti et al., 2007). Quantifying spore purity is important to investigate their physiology, chemistry, and industrial applications but is often neglected (Harrold, Hertel, & Gorman-Lewis, 2011; Yang, Crow-Willard, & Ponce, 2009). Only half of included studies studying spores performed quantification and reported spore purity. For the spore purification, Giffel et al. (1995) used the heat shock treatment and Khadre and Yousef (2001) used a sonication method. A reported spore purity ranged from 90 to 99%.

For the biofilm preparation, static conditions were most used (five studies). The study by Deal, Klein, Lopolito, and Schwarz (2016) was the only one that used a dynamic condition where continuous flow existed. Biofilms built under continuous flow are known to be more resistant (Van Der Veen & Abee, 2011). This type of biofilm formed using the device such as the CDC biofilm reactor is also more realistic and repetitive than static biofilms (Paredes et al., 2012).

For vegetative cells and spores, the efficacy was measured in suspension except a study conducted by Sagripanti et al. (2007). The ASTM standard method E-2414-05 was used in this study with stainless steel and rubber surfaces. Diverse efficacies were observed from different treatments in the range of 0-6 $\log_{10}$ reduction in vegetative cells and spores. For the vegetative cells, 3-4 $\log_{10}$ reductions were achieved with 50 ℃ and 80 ℃ heat treatment. On the other hand, for spores, 250 ℃ superheated steam with UV-C

light could only achieve 1-3 $\log_{10}$ inactivation This shows the extreme heat resistance of spores compared to vegetative cells.

For measuring treatments' inactivation efficacy against biofilms, stainless steel was the most tested surface, followed by plastic surfaces. Stainless steel is widely used in the food industry as they have better cleanability and disinfectability than other surface materials (Boulané-Petermann, 1996). The reported efficacy ranged from 0-8 $\log_{10}$ inactivation as different biofilm preparation methods, surfaces, treatments, and units were used. Using a standardized method with a CDC biofilm reactor is a good option for antibiotic efficacy testing because biofilms can be built in a steady state and different surfaces can be tested simultaneously (Gomes et al., 2018). Using a standardized method will allow future researchers to better compare the inactivation efficacy against biofilms under similar conditions as diverse methods have been used.

Table 2. Summary of collected treatments' inactivation efficacy against *B. cereus*

Reference	Type of cell	Strain	Contact surface	Treatment	Efficacy	Measure outcome
Al-Qadiri et al. (2019)	Vegetative cell	ATCC 11778	Suspension	Neutral electrolyzed water	1-3 $\log_{10}$ inactivation	Log CFU/mL
Caballero Gómez et al. (2013)	Spore	Six strain cocktail (B47, B70, CRG5, ERG1, LWL1 and CECT 148T)	Suspension	Quarternary Ammonium Compounds (QACs), QACs+bacteriocin AS-48	0-6 $\log_{10}$ inactivation	Log CFU/mL
				Oxidizing agents (P3 oxonia, P3 topax), oxidizing agents+bacteriocin AS-48	0-6 $\log_{10}$ inactivation	
Cronin and Wilkinson (2008)	Vegetative cell	NCTC7464	Suspension	Heat (50 ℃, 80 ℃)	3-4 $\log_{10}$ inactivation	Log CFU/mL
				Sonication	<1 $\log_{10}$ inactivation	
				Preservative (Nacl, Potasium Sorbate)	1-2 $\log_{10}$ inactivation	
				Chemical treatment (pH, nisin, peroxide, alcohol)	0-6 $\log_{10}$ inactivation	
Deal et al. (2016)	Biofilm	ATCC 14759	Polycarbonate	Formulated alkaline detergent, sporicide (Hydrogen peroxide+peroxygen)	1-3 $\log_{10}$ inactivation	Log CFU/coupon
Fernandes, de Oliveira, Cheriegate, and de Abreu Filho (2018)	Biofilm	NA	Stainless steel in semi-finished gelatin	CIP regime using 2% Dicopan and 0.2% peracetic acid at RT	2-4 $\log_{10}$ inactivation	Log CFU/cm^2
Giffel et al. (1995)	Spore	Dairy factory isolated strains (A, B, C, D, E), ATCC 9139 and ATCC 12826	Suspension, stainless steel and rubber	Commercial sporicides (Puremel, Alfablink, PE Oxonia BK) at 50 ℃	0-3 $\log_{10}$ inactivation	Log CFU/mL
Huang, Lin, Ren, Song, and Guo (2019)	Biofilm	A1	Stainless steel	CIP regime using Benzalkonium Bromide	4-8 $\log_{10}$ inactivation	Log CFU/cm^2
Khadre and Yousef (2001)	Spore	OSU11	Suspension	Aqueos ozone	6-$\log_{10}$ inactivation	Log CFU/mL
				Hydrogen Peroxide	1-$\log_{10}$ inactivation	
H. Kim, Moon, Kim, and Ryu (2019)	Biofilm	ATCC 10876, ATCC 13061, BH09-3 (isolated from perilla leaf), and BH09-5(isolated from sprout)	Stainless steel, glass, polyethylene, polypropylene, wood (Smooth or scratched)	Oxidizing agents (Chlorine, Chlorine Dioxide)	1-3 $\log_{10}$ inactivation	Log CFU/10cm^2
				QAC	0-2 $\log_{10}$ inactivation	
				Alcohol	0-2 $\log_{10}$ inactivation	
S. S. Kim et al. (2020)	Spore	Three strain cocktail (ATCC 10876, 13061, 14579)	Stainless steel	Superheated steam (250 ℃) and UV-C	1-3 $\log_{10}$ inactivation	Log CFU/mL
Kreske, Ryu, Pettigrew, and Beuchat (2006)	Biofilm	Five strains (F4616A/90, F3812/84, F4810/72, O38-2, and C1)	Stainless steel	Oxidizing agent and detergent	0-3 $\log_{10}$ inactivation	Log CFU/coupon
Li, Liu, and Liu (2017)	Biofilm	CMCC 63303	Stainless steel	Acidic electrolyzed water	2-4 $\log_{10}$ inactivation	Log CFU/cm^2
Lv et al. (2019)	Spore	ATCC 14579	Suspension	Combined treatment with 80 ℃ heat, ultrasonication, and pressure	0-3 $\log_{10}$ inactivation	Log CFU/mL
J. S. Peng, W. C. Tsai, and C. C. Chou (2002)	Biofilm	Not defined	Stainless steel	CIP regime with detergents (Tak1000, Dilac, diver form) at 70 ℃ or RT	2-6 $\log_{10}$ inactivation	Log CFU/chip
Ryu and Beuchat (2005)	Biofilm	038-2	Stainless steel	Oxidizing agents (Chlorinated water and Chlorine Dioxide)	1-4 $\log_{10}$ inactivation	Log CFU/coupon
				Tsunami 200	<1 $\log_{10}$ inactivation	
Sagripanti et al. (2007)	Spore	ATCC 10702	Rubber and aluminum alloy	Commercial disinfectants (Decon green, Clorox, Sandia)	1-6 $\log_{10}$ inactivation	Log CFU/mL
Silva et al. (2018)	Biofilm	SAMN07414939	Stainless steel contaminated with milk	CIP regime with peracetic acid or Sodium Hypochlorite at 60 ℃	0-3 $\log_{10}$ inactivation	Log CFU/cm^2

4.3. Meta-analysis

For forest plots of outcomes, please refer to Appendix A: Forest plots.

Outcome 1: Inactivation of *B. cereus* spores by oxidizing agents

Caballero Gómez et al. (2013) was the only study used for meta-analysis in this forest plot, in which it reported results from 24 trials under various treatment conditions. Two commercial products were evaluated, i.e., P3 oxonia and P3 topax. P3 oxonia is an acidic commercial sanitizer containing hydrogen peroxide and peracetic acid, while P3 topax is an alkaline commercial sanitizer that has chlorine as an active compound. Three different temperatures, one-hour application time, and different concentrations were tested in suspension.

At 0.25% concentration, treatments with P3 oxonia showed about 6-$\log_{10}$ reduction in every tested temperature (22-60 ℃) with 1 h application time. In contrast, lower log reduction was observed with 0.025% concentration except 60 ℃. P3 oxonia at 0.025% showed higher efficacy as temperature increases. A similar efficacy as 0.25% was observed at 60 ℃ with over 6-$\log_{10}$ reductions. The manufacturer recommends using P3 oxonia at 0.2-2% and not to use at high temperature. The documented studies are well aligned with the recommendations showing good efficacy of P3 oxonia at recommended concentrations with over 6-$\log_{10}$ reduction under normal temperature ranges. It also explains lower inactivation efficacy observed with 0.025% concentration.

Treatments with P3 topax presented less than 1-$\log_{10}$ reduction on average and showed 0% heterogeneity which means there was no significant difference between different conditions. The manufacturer's recommended concentration for the P3 topax is 2-3%. The low concentrations tested (0.5% and 1%) could explain the low efficacy.

Different temperatures ranging from 22-60 ℃ were tested. However, using chlorinated sanitizer at a higher temperature requires caution as inhaling gaseous chlorine can be a safety hazard (Saroha, 2006). No significant difference was observed in adding bacteriocin AS-48 for both P3 oxonia and P3 topax.

Outcome 2: Inactivation of *B. cereus* spores by surface−active compounds

Surface-active compounds are known to be sporostatic; they cannot kill the spore but prevent the outgrowth of spores (Acosta-Gio, Herrero-Farias, & Mata-Portuguez, 2001; McDonnell & Russell, 1999). However, Caballero Gómez et al. (2013) reported the inactivation of *B. cereus* spores by several surface-active compounds.

Cetrimide presented slightly better efficacy at 60 ℃ with over 1-$\log_{10}$ reduction but showed no effect in the other temperatures and concentrations at 5% significance level. Hexadecylpyridinium chloride (HDP) and benzalkonium chloride showed 1-2 $\log_{10}$ inactivation at lower temperatures. Benzalkonium chloride had 6-$\log_{10}$ inactivation at 0.5% and 1% concentration at 60 ℃. HDP had 6-$\log_{10}$ reduction at 0.25% concentration. Future research is needed as few studies have been conducted on the efficacy of surface-active compounds in practical situation, and contradicting results were reported (Gerba, 2015). No noticeable difference was observed between treatments without AS-48 and treatments with AS-48.

Outcome 3: Inactivation of *B. cereus* spores by other treatments

Trichlosan, chlorohexidine, and polyhexamethylene guanidine (PHMG) were used in the study by Caballero Gómez et al. (2013). The heterogeneity of the four treatments was low as no significant difference between conditions was found.

This similar inactivation rate could be caused by killing vegetative cells mixed into spore suspension because no quantification of spores or spore purification was done in this study. Lv et al. (2019) studied physical treatments with heat, ultrasonication, and pressure. Each treatment separately did not present any meaningful inactivation. However, manothermosonication, a combined treatment of heat, ultrasonication, and pressure, showed an increasing inactivation rate as the application time increased.

Outcome 4: Inactivation of *B. cereus* biofilms by oxidizing agents

Acidic electrolyzed water, chlorine, chlorine dioxide, Clean-in-place (CIP) procedures with peracetic acid and sodium hypochlorite were tested against *B. cereus* biofilms from four studies. The random effects model for subgroups presented acidic electrolyzed water as the most effective treatment and chlorine more effective than chlorine dioxide generally. H. Kim et al. (2019) reported that sanitizers showed the least efficacy on a wood surface. However, no consistent result was shown between scratched surfaces and smooth surfaces.

Silva et al. (2018) showed that biofilms initiated from spores or pasteurized vegetative cells were more resistant to treatments than biofilms induced from vegetative cells. Direct comparison between different studies was not made, considering the diverse methods used for measuring efficacies and forming biofilms.

Outcome 5: Inactivation of *B. cereus* biofilms by surface−active compounds

A quaternary ammonium compound (QAC) presented an average of about 1-$\log_{10}$ reduction at 200 ppm in the study by H. Kim et al. (2019). A CIP procedure with benzalkonium bromide presented 4-7 $\log_{10}$ inactivation in the study by Huang et al.

(2019). Better efficacy was observed with higher concentrations. However, in the U.S., surface-active compounds are used around 200 ppm and the concentration used in the test was extremely high and can cause residual and toxicity concerns for actual use.

Outcome 6: Inactivation of *B. cereus* biofilm by other treatments

Seventy percent alcohol showed about 1-$\log_{10}$ inactivation and heterogeneity was moderate which supports there was no big difference under different conditions. CIP procedure using an alkaline detergent showed about a 3-$\log_{10}$ inactivation. Spek-Tak 1000 showed more than two times better efficacy at 70 ℃. For the CIP procedure using alkaline and acid cleaning, a similar condition was tested in the study by Huang et al. (2019), Silva et al. (2018), and J.-S. Peng, W.-C. Tsai, and C.-C. Chou (2002). However, considerable heterogeneity was observed, and this can be explained by static biofilms' lower repeatability and different methods used to evaluate the efficacy. In the study by J.-S. Peng et al. (2002), adding one more acid cleaning step showed slightly better efficacy at room temperature.

There are available standardized methods with higher reproducibility, including ASTM E3161-18 for preparing biofilm and ASTM E2871-19 for determining disinfectant efficacy against biofilm (Gomes et al., 2018). To achieve the efficacy claims against biofilm, these are the only approved methods by US Environmental Protection Agency (EPA) (Goeres et al., 2019). Still, there are not many types of research that tested biofilms with standardized methods. Therefore, using standardized methods is highly recommended for future research determining biocidal efficacy against biofilms.

5. Conclusions

For the inactivation efficacy against *B. cereus* spores, oxidizing agents showed the best efficacy with an average of 2.51 $\log_{10}$ inactivation. Oxidizing agents include halogen-based compounds, peracetic acid, ozone, and hydrogen peroxide and they are widely used in the food industry (Aryal & Muriana, 2019). Oxidizing agents are more applicable to the food industry than surface-compound agents as safety concerns exist for quaternary ammonium compounds (Melin et al., 2014). Other treatments included in the meta-analysis were not very effective except manothermosonication with an average efficacy of 0.63 $\log_{10}$ inactivation.

For inactivating *B. cereus* biofilms, direct comparison between different studies was not possible due to different methods and units used for testing. CIP procedures generally showed good efficacy ranging from 0-6 $\log_{10}$ inactivation. CIP procedures with acidic or alkaline detergents showed better or similar efficacy as disinfectants. One study reported that the detergent without enzymes could reduce not only Extracellular Polymeric Substances (EPS) mass but kill bacteria in the biofilm (Vickery, Pajkos, & Cossart, 2004). Another study found that the combination of detergent with a high-level disinfectant could achieve 3-5 $\log_{10}$ inactivation against *E. faecalis* and *P. aeruginosa* biofilm (Da Costa Luciano, Olson, Tipple, & Alfa, 2016). Applying detergents before the disinfection step plays an essential role for biofilm removal by reducing the amount of EPS protecting embedded cells (Vickery, Ngo, Zou, & Cossart, 2009).

There were several limitations to the study: 1) Caballero Gómez et al. (2013) showed that research testing of commercial cleaning products can be far removed from actual conditions that they are being used. For testing commercial sanitizers, adopting and simulating practical conditions will be more helpful for food processing facilities to

utilize them properly. 2) Almost half of the studies included were not available for meta-analysis due to missing statistical data including means and standard deviations. 3) Existing research measuring the inactivation efficacy of *B. cereus* spores and biofilms were done using diverse methods, making it hard to compare the efficacy of treatments. For future research, by using standardized methods, the food industry will be able to conduct more reliable validation studies when they encounter certain sporeforming organisms or biofilms in their facilities by comparing available sanitizer treatment options.

6. References

Acosta-Gio, E., Herrero-Farias, A., & Mata-Portuguez, V. H. (2001). Benzalkonium chloride is unacceptable to sterilize or disinfect medical or dental instruments. *Salud Publica De Mexico, 43*(6), 570-573. Retrieved from <Go to ISI>://WOS:000173356000008

Ahn, E., & Kang, H. (2018). Introduction to systematic review and meta-analysis. *Korean Journal of Anesthesiology, 71*(2), 103-112. doi:10.4097/kjae.2018.71.2.103

Aiassa, E., Higgins, J. P. T., Frampton, G. K., Greiner, M., Afonso, A., Amzal, B., . . . Verloo, D. (2015). Applicability and Feasibility of Systematic Review for Performing Evidence-Based Risk Assessment in Food and Feed Safety. *Critical Reviews in Food Science and Nutrition, 55*(7), 1026-1034. doi:10.1080/10408398.2013.769933

Al-Qadiri, H. M., Smith, S., Sielaff, A. C., Govindan, B. N., Ziyaina, M., Al-Alami, N., & Rasco, B. (2019). Bactericidal activity of neutral electrolyzed water against Bacillus cereus and Clostridium perfringens in cell suspensions and artificially inoculated onto the surface of selected fresh produce and polypropylene cutting boards. *Food Control, 96*, 212-218. doi:10.1016/j.foodcont.2018.09.019

Aryal, M., & Muriana, P. M. (2019). Efficacy of Commercial Sanitizers Used in Food Processing Facilities for Inactivation of Listeria monocytogenes, E. Coli O157:H7, and Salmonella Biofilms. *Foods, 8*(12), 639. doi:10.3390/foods8120639

Boulané-Petermann, L. (1996). Processes of bioadhesion on stainless steel surfaces and cleanability: A review with special reference to the food industry. *Biofouling, 10*(4), 275-300. doi:10.1080/08927019609386287

Caballero Gómez, N., Grande, M. J., Pérez Pulido, R., Abriouel, H., & Gálvez, A. (2013). Effect of enterocin AS-48 singly or in combination with biocides on planktonic and sessile B. cereus. *Food Control, 34*(2), 743-751. doi:10.1016/j.foodcont.2013.06.022

Choi, W., & Kim, S. S. (2020). Outbreaks, germination, and inactivation of bacillus cereus in food products: A review. *Journal of Food Protection, 83*(9), 1480-1487. doi:10.4315/0362-028X.JFP-19-429

Cronin, U. P., & Wilkinson, M. G. (2008). Physiological Response of Bacillus cereus Vegetative Cells to Simulated Food Processing Treatments. *Journal of Food Protection, 71*(11), 2168-2176. doi:10.4315/0362-028x-71.11.2168

Da Costa Luciano, C., Olson, N., Tipple, A. F. V., & Alfa, M. (2016). Evaluation of the ability of different detergents and disinfectants to remove and kill organisms in traditional biofilm. *American Journal of Infection Control, 44*(11), e243-e249. doi:10.1016/j.ajic.2016.03.040

Deal, A., Klein, D., Lopolito, P., & Schwarz, J. S. (2016). Cleaning and disinfection of Bacillus cereus biofilm. *PDA Journal of Pharmaceutical Science and Technology, 70*(3), 208-217. doi:10.5731/pdajpst.2014.005165

Fernandes, M. D. S., de Oliveira, A. M., Cheriegate, A. P. C., & de Abreu Filho, B. A. (2018). Bacteria isolated from the bovine gelatin production line: biofilm formation and use of different sanitation procedures to eliminate the biofilms. *Journal of Food Safety, 38*(5). doi:10.1111/jfs.12489

Galié, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J., & Lombó, F. (2018). Biofilms in the Food Industry: Health Aspects and Control Methods. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.00898

Garg, A. X., Hackam, D., & Tonelli, M. (2008). Systematic Review and Meta-analysis: When One Study Is Just not Enough. *Clinical Journal of the American Society of Nephrology, 3*(1), 253-260. doi:10.2215/cjn.01430307

Gerba, C. P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and Environmental Microbiology, 81*(2), 464-469. doi:10.1128/aem.02633-14

Giffel, M. C. T., Beumer, R. R., VanDam, W. F., Slaghuis, B. A., & Rombouts, F. M. (1995). Sporicidal effect of disinfectants on Bacillus cereus isolated from the milk processing environment. *International Biodeterioration & Biodegradation, 36*(3-4), 421-430. Retrieved from <Go to ISI>://WOS:A1995UQ56300018

https://sfamjournals.onlinelibrary.wiley.com/doi/pdfdirect/10.1111/1462-2920.14818?download=true

Goeres, D. M., Walker, D. K., Buckingham-Meyer, K., Lorenz, L., Summers, J., Fritz, B., . . . Parker, A. E. (2019). Development, standardization, and validation of a biofilm efficacy test: The single tube method. *Journal of Microbiological Methods, 165*, 105694. doi:10.1016/j.mimet.2019.105694

Gomes, I. B., Meireles, A., Gonçalves, A. L., Goeres, D. M., Sjollema, J., Simões, L. C., & Simões, M. (2018). Standardized reactors for the study of medical biofilms: a review of the principles and latest modifications. *Critical Reviews in Biotechnology, 38*(5), 657-670. doi:10.1080/07388551.2017.1380601

Gopal, N., Hill, C., Ross, P. R., Beresford, T. P., Fenelon, M. A., & Cotter, P. D. (2015). The Prevalence and Control of Bacillus and Related Spore-Forming Bacteria in the Dairy Industry. *Frontiers in Microbiology, 6*. doi:10.3389/fmicb.2015.01418

Granum, P. E., & Lund, T. (2006). Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Letters, 157*(2), 223-228. doi:10.1111/j.1574-6968.1997.tb12776.x

Harrold, Z. R., Hertel, M. R., & Gorman-Lewis, D. (2011). Optimizing Bacillus subtilis spore isolation and quantifying spore harvest purity. *Journal of Microbiological Methods, 87*(3), 325-329. doi:10.1016/j.mimet.2011.09.014

Huang, Z. Y., Lin, Y. Y., Ren, F. Z., Song, S. J., & Guo, H. Y. (2019). Benzalkonium bromide is effective in removing Bacillus cereus biofilm on stainless steel when combined with cleaning-in-place. *Food Control, 105*, 13-20. doi:10.1016/j.foodcont.2019.05.017

Khadre, M. A., & Yousef, A. E. (2001). Sporicidal action of ozone and hydrogen peroxide: a comparative study. *International Journal of Food Microbiology, 71*(2-3), 131-138. doi:10.1016/s0168-1605(01)00561-x

Kim, H., Moon, M. J., Kim, C. Y., & Ryu, K. (2019). Efficacy of chemical sanitizers against Bacillus cereus on food contact surfaces with scratch and biofilm. *Food Science and Biotechnology, 28*(2), 581-590. doi:10.1007/s10068-018-0482-2

Kim, S. S., Kim, S. H., Park, S. H., & Kang, D. H. (2020). Inactivation of bacillus cereus spores on stainless steel by combined superheated steam and UV-C irradiation treatment. *Journal of Food Protection, 83*(1), 13-16. doi:10.4315/0362-028X.JFP-19-133

Kreske, A. C., Ryu, J. H., Pettigrew, C. A., & Beuchat, L. R. (2006). Lethality of chlorine, chlorine dioxide, and a commercial produce sanitizer to Bacillus cereus and Pseudomonas in a liquid detergent, on stainless steel, and in biofilm. *Journal of Food Protection, 69*(11), 2621-2634. doi:10.4315/0362-028x-69.11.2621

Kumari, S., & Sarkar, P. K. (2016). Bacillus cereus hazard and control in industrial dairy processing environment. *Food Control, 69*, 20-29. doi:10.1016/j.foodcont.2016.04.012

Kwon, M., Hussain, M. S., & Oh, D. H. (2017). Biofilm formation of Bacillus cereus under food-processing-related conditions. *Food Science and Biotechnology, 26*(4), 1103-1111. doi:10.1007/s10068-017-0129-8

Li, N. W., Liu, G. L., & Liu, J. (2017). Inactivation of Bacillus cereus biofilms on stainless steel by acidic electrolyzed water. *Journal of Food Processing and Preservation, 41*(6). doi:10.1111/jfpp.13304

Lv, R., Zou, M., Chantapakul, T., Chen, W., Muhammad, A. I., Zhou, J., . . . Liu, D. (2019). Effect of ultrasonication and thermal and pressure treatments, individually and combined, on inactivation of Bacillus cereus spores. *Applied Microbiology and Biotechnology, 103*(5), 2329-2338. doi:10.1007/s00253-018-9559-3

Majed, R., Faille, C., Kallassy, M., & Gohar, M. (2016). Bacillus cereus Biofilms—Same, Only Different. *Frontiers in Microbiology, 7*. doi:10.3389/fmicb.2016.01054

McDonnell, G., & Russell, A. D. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews, 12*(1), 147-179. doi:10.1128/cmr.12.1.147

Melin, V. E., Potineni, H., Hunt, P., Griswold, J., Siems, B., Werre, S. R., & Hrubec, T. C. (2014). Exposure to common quaternary ammonium disinfectants decreases fertility in mice. *Reproductive Toxicology, 50*, 163-170. doi:10.1016/j.reprotox.2014.07.071

Messelhäusser, U., Frenzel, E., Blöchinger, C., Zucker, R., Kämpf, P., & Ehling-Schulz, M. (2014). Emetic Bacillus cereus are more volatile than thought: recent foodborne outbreaks and prevalence studies in Bavaria (2007-2013). *BioMed research international, 2014*, 465603-465603. doi:10.1155/2014/465603

Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *PLoS Medicine, 6*(7), e1000097. doi:10.1371/journal.pmed.1000097

Omer, M. K., Álvarez-Ordoñez, A., Prieto, M., Skjerve, E., Asehun, T., & Alvseike, O. A. (2018). A Systematic Review of Bacterial Foodborne Outbreaks Related to

Red Meat and Meat Products. *Foodborne Pathog Dis, 15*(10), 598-611. doi:10.1089/fpd.2017.2393

Ortuzar, J., Martinez, B., Bianchini, A., Stratton, J., Rupnow, J., & Wang, B. (2018). Quantifying changes in spore-forming bacteria contamination along the milk production chain from farm to packaged pasteurized milk using systematic review and meta-analysis. *Food Control, 86*, 319-331. doi:https://doi.org/10.1016/j.foodcont.2017.11.038

Paredes, J., Becerro, S., Arizti, F., Aguinaga, A., Del Pozo, J. L., & Arana, S. (2012). Real time monitoring of the impedance characteristics of Staphylococcal bacterial biofilm cultures with a modified CDC reactor system. *Biosensors and Bioelectronics, 38*(1), 226-232. doi:10.1016/j.bios.2012.05.027

Peng, J.-S., Tsai, W.-C., & Chou, C.-C. (2002). Inactivation and removal of Bacillus cereus by sanitizer and detergent. *International Journal of Food Microbiology, 77*(1-2), 11-18. doi:10.1016/s0168-1605(02)00060-0

Peng, J. S., Tsai, W. C., & Chou, C. C. (2002). Inactivation and removal of Bacillus cereus by sanitizer and detergent. *International Journal of Food Microbiology, 77*(1-2), 11-18. doi:10.1016/s0168-1605(02)00060-0

Ryu, J. H., & Beuchat, L. R. (2005). Biofilm formation and sporulation by Bacillus cereus on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *Journal of Food Protection, 68*(12), 2614-2622. doi:10.4315/0362-028x-68.12.2614

Sagripanti, J. L., Carrera, M., Insalaco, J., Ziemski, M., Rogers, J., & Zandomeni, R. (2007). Virulent spores of Bacillus anthracis and other Bacillus species deposited on solid surfaces have similar sensitivity to chemical decontaminants. *Journal of Applied Microbiology, 102*(1), 11-21. doi:10.1111/j.1365-2672.2006.03235.x

Saroha, A. K. (2006). Safe handling of chlorine. *Journal of Chemical Health & Safety, 13*(2), 5-11. doi:10.1021/acs.chas.8b13204

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., . . . Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases, 17*(1), 7-15. doi:10.3201/eid1701.p11101

Silva, H. O., Lima, J. A. S., Aguilar, C. E. G., Rossi, G. A. M., Mathias, L. A., & Vidal, A. M. C. (2018). Efficiency of Different Disinfectants on Bacillus cereus Sensu Stricto Biofilms on Stainless-Steel Surfaces in Contact With Milk. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.02934

Soni, A., Oey, I., Silcock, P., & Bremer, P. (2016). Bacillus Spores in the Food Industry: A Review on Resistance and Response to Novel Inactivation Technologies. *Comprehensive Reviews in Food Science and Food Safety, 15*(6), 1139-1148. doi:10.1111/1541-4337.12231

Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut:Bacillus cereusand its food poisoning toxins. *FEMS Microbiology Reviews, 32*(4), 579-606. doi:10.1111/j.1574-6976.2008.00112.x

Van Der Veen, S., & Abee, T. (2011). Generation of Variants in Listeria monocytogenes Continuous-Flow Biofilms Is Dependent on Radical-Induced DNA Damage and

RecA-Mediated Repair. *PLoS ONE, 6*(12), e28590. doi:10.1371/journal.pone.0028590

Vickery, K., Ngo, Q.-D., Zou, J., & Cossart, Y. E. (2009). The effect of multiple cycles of contamination, detergent washing, and disinfection on the development of biofilm in endoscope tubing. *American Journal of Infection Control, 37*(6), 470-475. doi:10.1016/j.ajic.2008.09.016

Vickery, K., Pajkos, A., & Cossart, Y. (2004). Removal of biofilm from endoscopes: evaluation of detergent efficiency☆. *American Journal of Infection Control, 32*(3), 170-176. doi:10.1016/j.ajic.2003.10.009

Yang, W. W., Crow-Willard, E. N., & Ponce, A. (2009). Production and characterization of pureClostridiumspore suspensions. *Journal of Applied Microbiology, 106*(1), 27-33. doi:10.1111/j.1365-2672.2008.03931.x

Zhang, Y., & Wang, B. (2018). Comparison of the efficacy of commercial antimicrobial interventions for reducing antibiotic resistant and susceptible beef-associated Salmonella and Escherichia coli strains. *Journal of Consumer Protection and Food Safety, 13*(1), 3-23. doi:10.1007/s00003-017-1141-x

CHAPTER 2. MEASURING INACTIVATION EFFICACY OF COMMERCIAL CLEANING PRODUCTS AGAINST *B. CEREUS* ENDOSPORES ON STAINLESS STEEL DISK CARRIERS

1. Abstract

Spores are a dormant and resilient form of bacteria with high hydrophobicity. They can survive extreme conditions including heat, radiation, and chemical treatments. *B. cereus* is ubiquitous. It can be carried into the food processing facility and survive by forming spores. *B. cereus* spores are more resistant than other spores with higher hydrophobicity and an additional layer called exosporium.

Previous efforts examined the inactivation efficacy of treatments against *B. cereus* spores. However, a quantitative comparison is impossible because various methods have been used. This study used a standardized carrier method for measuring the inactivation efficacy against the *B. cereus* spores on the stainless steel surface. Additionally, an optimized spore harvest method was used to reduce the error caused by lower spore purity. A three-way ANOVA was performed to analyze the impact of different treatments, application times, and concentrations.

Four commercially available cleaning products were tested using industry-applicable application times and concentration ranges. A sanitizer with a biofilm claim, Synergex™ (ECOLAB, St. Paul, MN) at 2% concentration, showed the best efficacy with greater than 5.33 $\log_{10}$ reduction in 5 min. On the other hand, Synergex™ at 1% concentration, showed a 0.5-2.8 $\log_{10}$ reduction. A sanitizer with a sterilant claim, P3-oxonia™ (ECOLAB, St. Paul, MN) at 2.5% concentration, showed about 1-$\log_{10}$ reduction in 10 min, and at 5% concentration showed a 1 to 5 $\log_{10}$ reduction as application time increased. An alkaline detergent, Lift™ III (ECOLAB, St. Paul, MN), and an acidic detergent, HD PL-10™ (ECOLAB, St. Paul, MN), exhibited a <1 $\log_{10}$ inactivation when using the manufacturer's recommended concentration ranges.

Sanitizers with peroxygens were effective against *B. cereus* spores, but maximum strength within the recommended concentration range was required to inactivate spores at room temperature. In the CIP cycle, adding a sanitizing step with an effective sporicide is recommended when it is known that spore contamination exists, as detergent alone was not enough for sanitizing and eliminating spores. Future studies may include temperature and physical removal as additional variables as they also perform an essential role in the inactivation of spores.

2. Introduction

Bacterial spores are metabolically dormant and extremely resistant to various stress factors (Peter Setlow, 2011). The spore coat and an additional layer, exosporium, that exist in several species such as *B. cereus* are known to play essential roles in protecting spores (Henriques & Moran Jr, 2007). Hydrophobic spore surfaces increase their ability to adhere to a processing surface (Joshi, Phillips, Williams, Alyousef, & Baillie, 2012). Attaching to the surface provides favorable conditions to microorganisms as nutrients become denser. This can lead to biofilm development, a stable and perpetuating bacterial community that is enclosed in an extracellular polymeric substance (EPS) matrix (Bower, McGuire, & Daeschel, 1996). These characteristics allow them to survive and persist in the food processing environment.

Understanding how to better remove *B. cereus* spores is important in preventing possible contamination of food products that can lead to spoilage and foodborne outbreaks. Additionally, *B. cereus* is an appropriate surrogate of a possible bioweapon, the human pathogen *Bacillus anthracis*, which causes anthrax (Montville, Dengrove, De Siano, Bonnet, & Schaffner, 2005). Aldehydes, chlorine releasing agents, iodine and

iodophors, peroxygens, ethylene oxide, ozone gas and β-Propiolactone are known as sporicidal agents (Russell, 1990). Some studies showed surfactants are effective against spores or increase sporicidal efficacy when combined with disinfectants by interacting with the cell membrane (W.-I. Cho, Cheigh, Hwang, & Chung, 2015; W. I. Cho & Chung, 2018; Ernst et al., 2006; Hamouda et al., 1999).

There were several efforts to evaluate the sporicidal efficacy of treatments against *B. cereus* (Caballero Gómez, Grande, Pérez Pulido, Abriouel, & Gálvez, 2013; Giffel, Beumer, VanDam, Slaghuis, & Rombouts, 1995; Khadre & Yousef, 2001; Lv et al., 2019; Sagripanti et al., 1997). However, limitations existed: 1) Conditions being tested were far removed from practical situations, 2) Different methods were being used to measure the efficacy and making it impossible to compare results from various studies, and 3) Spore quantification or purification was not done in every study, so low purity of spore suspension could cause errors. Thus, in this research, it was decided to use: 1) Conditions (Concentrations and application times) that can be applied inside the food processing environment, 2) An optimized spore harvesting method to minimize the error caused by sanitizers killing vegetative cells mixed into spore suspensions, and 3) An ASTM standardized method for evaluating sporicidal efficacy for comparing data from different studies. The aim was: 1) To examine if the product with an US Environmental Protection Agency (EPA) sterilant claim was effective against *B. cereus* spores; and 2) To compare the efficacy of four different commercial cleaning products (acidic detergent, alkaline detergent, and two disinfectants containing hydrogen peroxide and peracetic acid).

3. Materials and Methods

3.1. Bacterial Strain

A *Bacillus cereus* strain previously isolated from a mushroom powder ingredient used in beef broth formulations was used for the studies. The isolate was identified as *B. cereus* using 16S rRNA sequencing (Midi Labs, Inc., Newark, DE). The sequencing data was also analyzed by researchers at the University of Nebraska-Lincoln by comparing the sequence to information deposited in the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/BLAST.cgi). This tool confirmed the isolate as a strain closely related to *Bacillus cereus* ATCC 14579. Hemolysis was confirmed on blood agar (Hardy Diagnostics, Santa Maria, CA). The isolate was grown overnight in Tryptic Soy Broth (TSB) (Neogen Corp, Lansing, MI) and then stored in cryogenic vials with 20% sterile glycerol (Thermo Fisher Scientific, Waltham, MA) at -80 ℃.

3.2. Optimized Spore Harvest Method using Alcohol

This method was modified from Zhao, Krishna, Moudgil, and Koopman (2008). A *B. cereus* spore suspension was prepared by inoculating 100 µL of thawed *B. cereus* culture into 500 mL Erlenmeyer flasks containing 100 mL of Difico Columbia broth (Bioworld, Dublin, OH). A hemolytic *B. cereus* strain isolated from mushroom powder was used. The Difco Columbia broth was added with 1 mL of 10 mM $MnSO_4$ solution for improving sporulation efficacy and spore stability. The inoculated growth media covered with aluminum foil was incubated at 35 ± 2 ℃ in an orbital incubator shaker (Thermo Fisher Scientific, Waltham, MA, MaxQ 4000, Waltham, MA) at 250 rev/min for 3 days. The culture was transferred to two 50 mL centrifuge tubes (Thermo Fisher Scientific, Waltham, MA), 35 mL each. Two tubes

were centrifuged at 1,000 x *g* for 10 min at 4 ℃ using a Thermo Scientific Sorvall ST 16R centrifuge (Waltham, MA). The supernatant from the tubes was poured off and pellets were resuspended in 20 mL of sterile deionized water. A resuspended tube was vortexed for 20 s and washed by centrifuging at 1,000 x *g* for 10 min at 4 ℃. The supernatant from each tube was poured off and the pellet was resuspended into 20 mL of 1:1 sterile deionized water and ethanol (200 proof; 99.5%, Decon Labs, King of Prussia, PA). The centrifuge tube was capped and incubated at 22 ℃ for 12 h in an orbital shaker at 100 rev/min. Afterward, the suspension was centrifuged at 1,000 x *g* for 10 min at 4 ℃. The supernatant was poured off and the pellet was resuspended in 20 mL of sterile deionized water. This washing step was then repeated one more time.

3.3. Inactivation of B. cereus Spores on Stainless Steel Disk Carriers by Commercial Cleaning Products (ASTM E2197-17)

ASIS 314 type Stainless steel disks (Biosurface Technologies Corp, Bozeman, MT) were autoclaved and prepared for the study. Each stainless steel disk surface was inoculated with 10 µL of *B. cereus* spore suspension. Disks with inoculum were then dried for two hours inside a desiccator at room temperature. Disks containing dried inoculum were placed inside the bottom of a 30 mL sterile vial (Thermo Fisher Scientific, Waltham, MA). Disks were immersed in 50 µL of chemical agents added to the vials. Different contact times and concentrations of Synergex™, P3 oxonia™, Lift™ III, HD PL-10™ (ECOLAB, St Paul, MN) were tested. Controls received 50 µL of Phosphate Buffered Saline (PBS) (Remel, Lenexa, KS) for 10 min instead of the chemical agents. Ten mL of eluent with a neutralizer

was then added to the treated inoculum. Eluent used in this test was PBS with 0.1% (v/v) Tween-20 (VWR, Radnor, PA). Contents of the vial were vortexed for 30 s and serially diluted as required. For spread plating, 0.1 mL of appropriate dilutions were plated on Tryptic Soy Agar (TSA) with 3% agar (Neogen Corp, Lansing, MI). TSA plates were enumerated after incubating for 24 ± 2 h at 35 ± 2 ℃.

3.4. Statistical Analysis

Assays were carried out in triplicate. The PROC GLIMMIX procedure in SAS 9.4 (SAS Institute, Cary, NC) was used to analyze the log reduction as a Linear Model (LM) with a nested treatment structure among treatments, concentration, and application time. Residual and qq-plots were used to assess normality. Tukey's adjustment was used to determine pairwise significance at the α=0.05 level.

4. Results and Discussion

4.1. Efficacy of commercial cleaning Products against B. cereus spores

Four commercial cleaning products were evaluated for their efficacy of inactivating *B. cereus* spores on type 314-stainless steel carriers within the manufacturer's recommended concentration range and food industry-applicable time. Table 1 shows the results of a three-way ANOVA. According to the results, every factor showed distinguishable effects on the outcome. Figure 1 shows a visualized efficacy of each treatment and condition at 95% confidence limit.

Table 1. Three-way ANOVA results of sporicidal efficacy against *B. cereus*

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	3	32	162.41	<.0001
Treatment (Concentration)	4	32	87.18	<.0001
Application_Time	1	32	47.88	<.0001
Treatment*Application_Time	3	32	11.97	<.0001
Treatment*Application_Time (Concentration)	4	32	13.18	<.0001

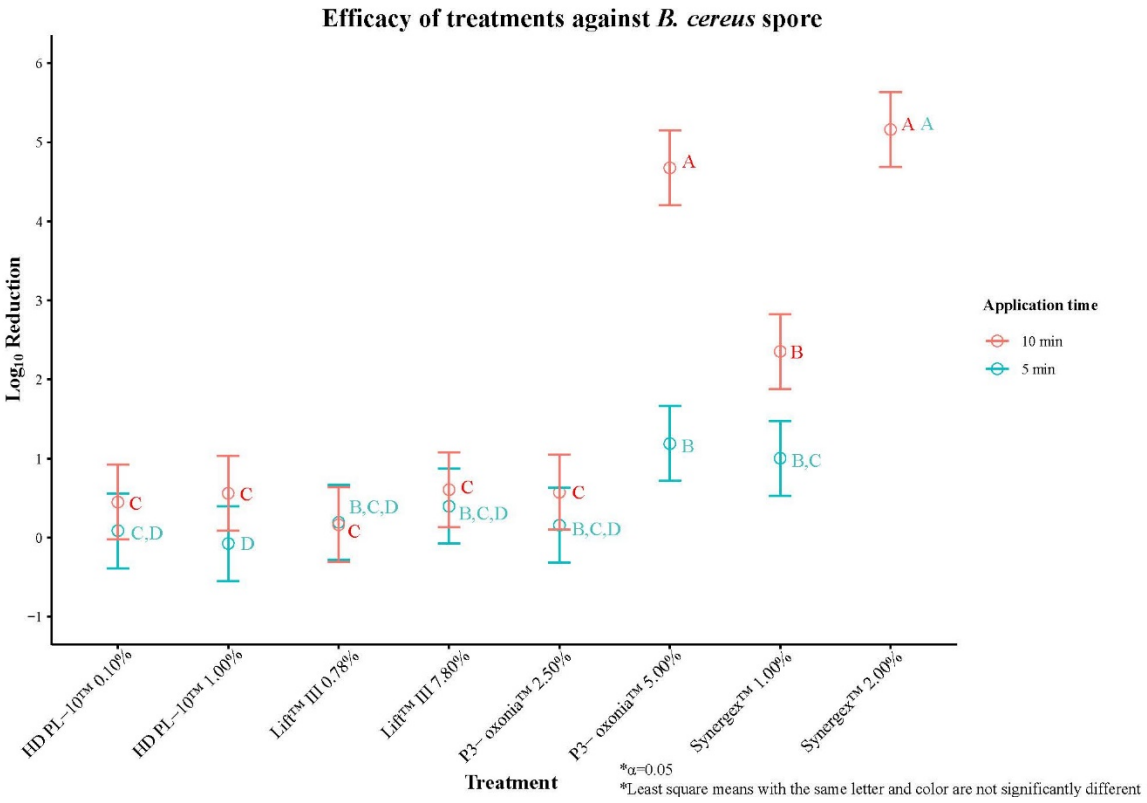


Figure 1. Visualized efficacy of treatments against *B. cereus* spore (Least square means at 95% confidence limit)

Among all treatments, Synergex™ at 2% concentration showed the best efficacy with greater than 5.33 $\log_{10}$ reduction (Detection limit: 5.33 $\log_{10}$ reduction) at both application times. On the other hand, Synergex™ at 1% concentration showed 0.5-2.8

$\log_{10}$ reduction. Table 2 shows that the efficacy increased as the application time increased. Table 3 supports that there was a significant difference in the efficacy between two different concentrations. Synergex™ is a sanitizer with an US EPA biofilm claim and consists of sulfuric acid, hydrogen peroxide, peroxyacetic acid, acetic acid, Secondary Alkane Sulphonates (SAS), octanoic acid and peroxyoctanoic acid as active ingredients. The result shows that it is effective as a sporicide as well. For inactivating biofilms, sporicidal effect can play an important role by killing embedded spores that sporulated when the biofilm developed and nutrients are depleted by generated gradients (Wilking, Angelini, Seminara, Brenner, & Weitz, 2011).

P3-oxonia™ is a sporicide containing hydrogen peroxide and peracetic acid. In the previous study, P3-oxonia™ showed over 6 $\log_{10}$ reductions at 0.25% concentration treated in the suspension for an hour against *B. cereus* spores (Caballero Gómez et al., 2013). However, when spores were treated on the stainless steel surface, a 2.5% concentration could only achieve around 1-$\log_{10}$ reduction in 10 minutes, and 5% concentration showed 1 to 5 $\log_{10}$ reduction as application time increased. Table 4 shows that the efficacy increases significantly as application time increases at a 5% concentration. Table 3 shows that there was a significant difference between the two different concentrations tested. The efficacy of sporicides is better on spores in suspension than on spores on surfaces, however, long exposure times are not realistic in cleaning regimes used by the food industry (Maillard, 2011). In food processing facilities, especially in the dairy industry, *B. cereus* tends to attach to the surface of pipelines where they can persist (Andersson, Ronner, & Granum, 1995). Therefore, testing the sporicidal efficacy of spores attached to a surface is more appropriate than in suspension. Using the

maximum strength within the manufacturer's recommended concentration range will be suitable for inactivating spores on the surface at room temperature. *B. cereus* spores are harder to control than other spores because of their hydrophobicity, resilience, and ubiquity (Andersson et al., 1995).

An alkaline detergent, Lift™ III, and an acidic detergent, HD PL-10™, both exhibited <1 $\log_{10}$ inactivation. Tukey's adjustment test result in the Figure 1 show no significant difference between different concentrations of HD PL-10™ and Lift™ III. A few studies discussed the sporicidal effect of surfactants by interacting with hydrophobic outer layers of spore (W.-I. Cho et al., 2015; W. I. Cho & Chung, 2018). However, lower efficacy was observed against *B. cereus* spores with commercial detergents. Spores are much more resistant to acid and base compared to vegetative cells (P. Setlow, 2014). The lower inactivation rate suggests that surfactants did not fully disrupt membrane properties, thus protecting spores from acid and base.

Table 2. Simple effect comparisons between different application times

Treatment	Application Time	Application Time	Estimated log reduction difference	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
HD PL-10™ 0.10%	10 min	5 min	0.3622	0.3285	32	1.10	0.2783	0.05	-0.3068	1.0313
HD PL-10™ 1.00%	10 min	5 min	0.6349	0.3285	32	1.93	0.0622	0.05	-0.03423	1.3039
Lift™ III 0.78%	10 min	5 min	-0.03073	0.3285	32	-0.09	0.9260	0.05	-0.6998	0.6384
Lift™ III 7.81%	10 min	5 min	0.2097	0.3285	32	0.64	0.5278	0.05	-0.4594	0.8787
Synergex™ 1.00%	10 min	5 min	1.3505	0.3285	32	4.11	0.0003	0.05	0.6814	2.0196
Synergex™ 2.00%	10 min	5 min	-366E-17	0.3285	32	-0.00	1.0000	0.05	-0.6691	0.6691
P3-oxonia™ 2.50%	10 min	5 min	0.4164	0.3285	32	1.27	0.2141	0.05	-0.2527	1.0855
P3-oxonia™ 5.00%	10 min	5 min	3.4863	0.3285	32	10.61	<.0001	0.05	2.8172	4.1553

Table 3. Simple effect comparisons between different concentrations

Treatment	Concentration	Concentration	Estimated log reduction difference	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
HD PL-10™ 10 min	0.1%	1.0%	-0.1106	0.3285	32	-0.34	0.7386	0.05	-0.7797	0.5585
HD PL-10™ 5 min	0.1%	1.0%	0.1620	0.3285	32	0.49	0.6252	0.05	-0.5071	0.8311
Lift™ III 10 min	0.78%	7.8%	-0.4438	0.3285	32	-1.35	0.1862	0.05	-1.1128	0.2253
Lift™ III 5 min	0.78%	7.8%	-0.2034	0.3285	32	-0.62	0.5402	0.05	-0.8725	0.4657
P3-oxonia™ 10 min	2.5%	5.0%	-4.1028	0.3285	32	-12.49	<.0001	0.05	-4.7719	-3.4337
P3-oxonia™ 5 min	2.5%	5.0%	-1.0329	0.3285	32	-3.14	0.0036	0.05	-1.7020	-0.3638
Synergex™ 10 min	1.0%	2.0%	-2.8054	0.3285	32	-8.54	<.0001	0.05	-3.4745	-2.1363
Synergex™ 5 min	1.0%	2.0%	-4.1559	0.3285	32	-12.65	<.0001	0.05	-4.8250	-3.4868

5. Conclusions

This research aimed to evaluate the efficacy of commercial cleaning products commonly used in the food industry against *B. cereus* spores using a standardized carrier method. Among four treatments, sanitizers containing peroxygens were effective at a higher concentration. Synergex™ showed the best sporicidal efficacy with a greater than 5.33 $\log_{10}$ reduction at 2% concentration, followed by P3-oxonia™ with a 4.68 $\log_{10}$ reduction at 5% concentration. Both sanitizers needed maximum recommended strength to achieve an excellent sporicidal efficacy at room temperature in 10 minutes. However, acidic and alkaline detergents showed only <1 $\log_{10}$ inactivation. No significant difference between different treatment conditions was observed.

These results indicate that using acidic and alkaline detergents solely for removing persistent *B. cereus* spores will not be enough. For the CIP cycle, a sanitizing step with a sporicide should be implemented after the cleaning step with a detergent when known spore contamination exists. Also, using maximum strength within the stated range

of the manufacturer and extended application time is recommended to remove spores effectively.

In previous research that measured the efficacy of treatments against bacterial spores, the methods commonly used could exaggerate the efficacy of treatments. This is due to the studies not using spore purification methods or testing sanitizer efficacy on spore suspensions. However, in this study, an optimized spore harvest method was used to reduce the error caused by vegetative cells mixed into the spore suspension. Also, a standardized carrier method was used to allow quantitative comparison of treatments from future studies and measure the efficacy against the surface contamination.

This study's limitation is that the current standardized method does not involve the physical removal of spores on the surface or the temperature effect. Both play an important role in the cleaning regimes of the food industry. Future studies with pilot-scale equipment may include physical removal and temperature as additional variables to complement these results.

Based on the conclusions, industry stakeholders will be able to decide the appropriate treatment and conditions for their daily cleaning regimes depending on the contamination level of the food processing environment and whether the removal of spores is a critical consideration.

6. References

Andersson, A., Ronner, U., & Granum, P. E. (1995). What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium

perfringens? *Int J Food Microbiol, 28*(2), 145-155. doi:10.1016/0168-1605(95)00053-4

Bower, C. K., McGuire, J., & Daeschel, M. A. (1996). The adhesion and detachment of bacteria and spores on food-contact surfaces. *Trends in Food Science & Technology, 7*(5), 152-157. doi:10.1016/0924-2244(96)81255-6

Caballero Gómez, N., Grande, M. J., Pérez Pulido, R., Abriouel, H., & Gálvez, A. (2013). Effect of enterocin AS-48 singly or in combination with biocides on planktonic and sessile B. cereus. *Food Control, 34*(2), 743-751. doi:10.1016/j.foodcont.2013.06.022

Cho, W.-I., Cheigh, C.-I., Hwang, H.-J., & Chung, M.-S. (2015). Sporicidal Activities of Various Surfactant Components against Bacillus subtilis Spores. *Journal of Food Protection, 78*(6), 1221-1225. doi:10.4315/0362-028x.jfp-14-401

Cho, W. I., & Chung, M. S. (2018). Sporicidal activities and mechanism of surfactant components against Clostridium sporogenes spores. *Journal of Food Science and Technology, 55*(11), 4675-4680. doi:10.1007/s13197-018-3384-7

Ernst, C., Schulenburg, J., Jakob, P., Dahms, S., Lopez, A. M., Nychas, G., . . . Klein, G. (2006). Efficacy of Amphoteric Surfactant– and Peracetic Acid–Based Disinfectants on Spores of Bacillus cereus In Vitro and on Food Premises of the German Armed Forces. *Journal of Food Protection, 69*(7), 1605-1610. doi:10.4315/0362-028x-69.7.1605

Giffel, M. C. T., Beumer, R. R., VanDam, W. F., Slaghuis, B. A., & Rombouts, F. M. (1995). Sporicidal effect of disinfectants on Bacillus cereus isolated from the milk

processing environment. *International Biodeterioration & Biodegradation, 36*(3-4), 421-430. Retrieved from <Go to ISI>://WOS:A1995UQ56300018

https://sfamjournals.onlinelibrary.wiley.com/doi/pdfdirect/10.1111/1462-2920.14818?download=true

Hamouda, T., Hayes, M. M., Cao, Z., Tonda, R., Johnson, K., Wright, D. C., . . . Baker Jr, J. R. (1999). A novel surfactant nanoemulsion with broad-spectrum sporicidal activity against Bacillus species. *Journal of Infectious Diseases, 180*(6), 1939-1949. doi:10.1086/315124

Henriques, A. O., & Moran Jr, C. P. (2007) Structure, assembly, and function of the spore surface layers. In*: Vol. 61. Annual Review of Microbiology* (pp. 555-588).

Joshi, L. T., Phillips, D. S., Williams, C. F., Alyousef, A., & Baillie, L. (2012). Contribution of Spores to the Ability of Clostridium difficile To Adhere to Surfaces. *Applied and Environmental Microbiology, 78*(21), 7671-7679. doi:10.1128/aem.01862-12

Khadre, M. A., & Yousef, A. E. (2001). Sporicidal action of ozone and hydrogen peroxide: a comparative study. *International Journal of Food Microbiology, 71*(2-3), 131-138. doi:10.1016/s0168-1605(01)00561-x

Lv, R., Zou, M., Chantapakul, T., Chen, W., Muhammad, A. I., Zhou, J., . . . Liu, D. (2019). Effect of ultrasonication and thermal and pressure treatments, individually and combined, on inactivation of Bacillus cereus spores. *Applied Microbiology and Biotechnology, 103*(5), 2329-2338. doi:10.1007/s00253-018-9559-3

Maillard, J. Y. (2011). Innate resistance to sporicides and potential failure to decontaminate. *J Hosp Infect, 77*(3), 204-209. doi:10.1016/j.jhin.2010.06.028

Montville, T. J., Dengrove, R., De Siano, T., Bonnet, M., & Schaffner, D. W. (2005). Thermal Resistance of Spores from Virulent Strains of Bacillus anthracis and Potential Surrogates. *Journal of Food Protection, 68*(11), 2362-2366. doi:10.4315/0362-028x-68.11.2362

Russell, A. D. (1990). Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews, 3*(2), 99-119. doi:10.1128/cmr.3.2.99

Sagripanti, J. L., Eklund, C. A., Trost, P. A., Jinneman, K. C., Abeyta Jr, C., Kaysner, C. A., & Hill, W. E. (1997). Comparative sensitivity of 13 species of pathogenic bacteria to seven chemical germicides. *American Journal of Infection Control, 25*(4), 335-339. doi:10.1016/S0196-6553(97)90026-2

Setlow, P. (2011). Resistance of Bacterial Spores. In *Bacterial Stress Responses, Second Edition*: American Society of Microbiology.

Setlow, P. (2014). Spore Resistance Properties. *Microbiol Spectr, 2*(5). doi:10.1128/microbiolspec.TBS-0003-2012

Wilking, J. N., Angelini, T. E., Seminara, A., Brenner, M. P., & Weitz, D. A. (2011). Biofilms as complex fluids. *MRS Bulletin, 36*(5), 385-391. doi:10.1557/mrs.2011.71

Zhao, J., Krishna, V., Moudgil, B., & Koopman, B. (2008). Evaluation of endospore purification methods applied to Bacillus cereus. *Separation and Purification Technology, 61*(3), 341-347. doi:10.1016/j.seppur.2007.11.002

CHAPTER 3. DETERMINING INACTIVATION EFFICACY OF COMMERCIAL CLEANING PRODUCTS AGAINST *B. CEREUS* BIOFILMS

1. Abstract

Biofilms are an assemblage of cells attached to the surface and enclosed in the Extracellular Polymeric Substances (EPS). EPS protects embedded cells by blocking penetrating chemicals. The food processing environment offers a favorable condition for biofilm formation with ample nutrients and other residues on the surface. Validation of cleaning agents against different organisms is essential because biofilm's architecture varies by organism. *B. cereus* has a strong tendency to form biofilms and is known to create a more resistant biofilm.

Previous research investigated the efficacy of different sanitizers on biofilms. Various methods have been used to create biofilms and measure this efficacy. However, different methods to create biofilms can result in dissimilar observed resistance. Therefore, quantitative comparison between different studies is not possible. This study employed standardized methods using a CDC biofilm reactor under continuous flow to create more realistic biofilms that could be replicated. An ASTM single tube method was used to measure the efficacy of treatments. A four-way ANOVA was performed to analyze the effect of different treatments, application times, concentrations, and temperatures.

Three commercial cleaning products were tested with food processing environment-applicable application times, concentrations, and temperatures. In summary, A disinfectant with a biofilm claim from EPA, Synergex™, showed greater than 5-$\log_{10}$ reductions in every condition tested. Synergex™ was effective not only against *S. aureus* and *P. aeruginosa* but also *B. cereus* biofilms. HD PL-10™ showed a 0-2.1 $\log_{10}$ reduction with better efficacy when application time, concentration, and temperature

increased. Lift™ III showed a 0.3-1.7 $\log_{10}$ reduction in the tested conditions. No significant difference was observed between different concentrations, and no consistent relationship was seen in different concentrations and contact times.

Inactivation efficacies displayed by acidic and alkaline detergents were not as dramatic as the peroxygen-based disinfectant, Synergex™. However, it is important to include detergents in the daily cleaning regime because they can help detach adherent food residues on the surface and reduce the viability of remaining bacteria. Adopting a cleaning regime consisting of a cleaning cycle with detergents and a sanitizing cycle with an effective disinfectant based on the contamination level will be the most effective strategy when biofilms are present in the food processing environment. Future studies may consider mechanical action as an additional variable because it plays an essential role by physically removing foreign matter with shear stress.

2. Introduction

A biofilm is an assemblage of cells attached to the surface and enclosed in the Extra Polymeric Substances (EPS). EPS consists of exopolysaccharides, extracellular DNA, proteins, and lipids (Flemming & Wingender, 2010; Kumar, Sharma, Parmar, Singh, & Singh, 2020). EPS protects embedded cells by restricting penetrating antibiotics (Lewis, 2001). A biofilm develops in multiple steps: Attaching, forming a microcolony, developing a three-dimensional biofilm with EPS, and then detaching (Watnick & Kolter, 2000). The structure of the biofilms can be influenced by hydrodynamic conditions such as shear stress and the type of organisms (Vanloosdrecht et al., 1995).

A *B. cereus* biofilm can be found in diverse environments including hospitals, paperboard production, and food and beverage industries. (Majed, Faille, Kallassy, & Gohar, 2016). The food production site is favorable for a *B. cereus* biofilm formation with ample nutrients and other organic components on the surface (Hussain & Oh, 2018). *B. cereus* is known to have a strong biofilm formation tendency and resilience (Ikram et al., 2019). The presence of biofilms containing *B. cereus* can harm food manufacturing by persisting and leading to spoilage, or worse, foodborne outbreaks.

Previous efforts were made to evaluate the antimicrobial efficacy of sanitizers by using several methods (Fernandes, de Oliveira, Cheriegate, & de Abreu Filho, 2018; Huang, Lin, Ren, Song, & Guo, 2019; Kim, Moon, Kim, & Ryu, 2019; Li, Liu, & Liu, 2017; Peng, Tsai, & Chou, 2002; Silva et al., 2018). Static biofilms have often been used to create biofilms for testing. However, a static biofilm is less realistic and repeatable than a biofilm built under continuous flow (Paredes et al., 2012). Furthermore, biofilms built under dynamic conditions are more resistant, making them more appropriate for measuring antibiotic efficacy (Van Der Veen & Abee, 2011).

This research evaluated the anti-biofilm efficacy of commercially available cleaning products against *B. cereus* using a CDC biofilm reactor and an ASTM E2871-19 single tube method. This study aimed to: 1) Measure the efficacy of sanitizer with a biofilm claim from Environmental Protection Agency (EPA) against *B. cereus* biofilm, 2) Compare the efficacy of acidic and alkaline detergents, 3) Find optimum conditions (application time, concentration, and temperature) that are practical for use in the food processing environment to deal with *B. cereus* biofilm.

3. Materials and Methods

3.1. Bacterial Strain

A *Bacillus cereus* strain previously isolated from a mushroom powder ingredient used in beef broth formulations was used for the studies. The isolate was identified as *B. cereus* using 16S rRNA sequencing (Midi Labs, Inc., Newark, DE). The sequencing data was also analyzed by researchers at the University of Nebraska-Lincoln by comparing the sequence to information deposited in the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/BLAST.cgi). This tool confirmed the isolate as a strain closely related to *Bacillus cereus* ATCC 14579. Hemolysis was confirmed on blood agar (Hardy Diagnostics, Santa Maria, CA). The isolate was grown overnight in Tryptic Soy Broth (TSB) (Neogen Corp, Lansing, MI) and then stored in cryogenic vials with 20% sterile glycerol (Thermo Fisher Scientific, Waltham, MA) at -80 ℃.

3.2. Biofilm Formation

B. cereus biofilms were prepared following ASTM international E3161-18 with modifications (ASTM, 2018). A CDC Biofilm reactor (Biosurface Technologies Corp, Bozeman, MT) was used to develop 48 h old biofilms on stainless steel surfaces with 24 h batch phase and 24 h continuous flow phase. CDC biofilm reactors and coupons were assembled and autoclaved prior to use. Coupons used for this test were 1 cm diameter AISI 314 type stainless steel coupons (Biosurface Technologies Corp, Bozeman, MT) Tryptic Soy Broth (TSB) (Neogen Corp, Lansing, MI) (3 g/L) 500 mL was aseptically transferred inside the vessel. One mL of *B. cereus* culture grown overnight in the TSB (3 g/L) with over 10^7 CFU/mL was then inoculated to the vessel. A

hemolytic *B. cereus* strain isolated from the mushroom powder was used. Batch phase without flow was kept for 24 h to reach the steady state population at 37 ± 2 ℃. For an additional 24 h, continuous flow was created by stirring at 130 rpm. TSB (1 g/L) from the 20 L carboy was pumped into the vessel at 10.8 mL/min speed for 24 hours at 37± 2 ℃ by a Masterflex L/S peristaltic tubing pump (Cole-Parmer Intrument Company, Chicago, IL). A carboy with TSB was placed inside the incubator at least two days prior to use. Another 20 L carboy was connected to the reactor for collecting waste.

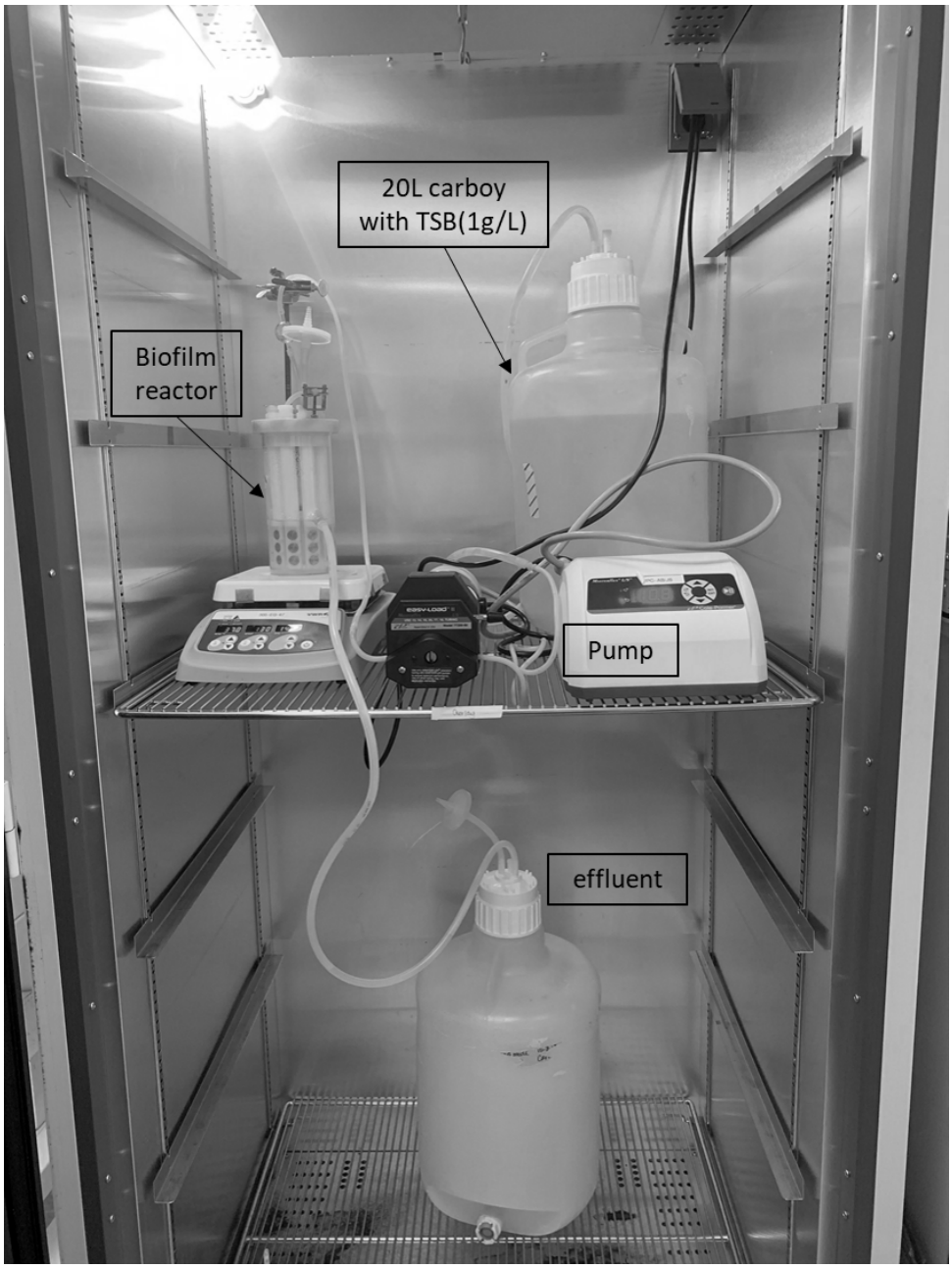


Figure 1. Setup of a CDC biofilm reactor in the continuous flow phase

3.3. Evaluating Efficacy of Commercial Cleaning Products against B. cereus Biofilms

The biocidal effect against *B. cereus* biofilm of commercial cleaning products was evaluated with an ASTM international E 2871-19 Determining

Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Method (ASTM, 2019). The rod containing the biofilm formed coupons were rinsed in sterile phosphate buffered dilution water (Remel, Lenexa, KS). Coupons were deposited into 50 mL conical tubes (Thermo Fisher Scientific, Waltham, MA). Each coupon was exposed to 4 mL of treatments for different contact times at two different temperatures (22 ± 2 ℃ or 60 ± 2 ℃). Thermo Scientific Precision 2870 water bath (Thermo Fisher Scientific, Waltham, MA) was used for heating cleaning products and keep the temperature at 60 ± 2 ℃ while treated. Three cleaning products including Synergex™, Lift™ III, and HD PL-10™ (ECOLAB, St. Paul, MN) were tested. For the control, coupons were treated with phosphate buffered dilution water for 10 min at room temperature. Two different manufacturer-recommended concentrations, two contact times, and two temperatures were tested. After appropriate exposure, 36 mL Dey-Engley neutralizing broth (Neogen Corp., Lansing, MI) was added to tubes. A combination of three vortexing for 30 ± 5 s and two sonications (55 ± 5 kHz for 30 ± 5 s) using Bransonic 52 (Branson Ultrasonics, Brookfield, CT) was used to remove the biofilm from the coupon. Sonication followed first two vortexing. The cell suspension was then enumerated with the standard plate count on Tryptic Soy Agar (TSA) with 3% agar (Neogen Corp., Lansing, MI) or membrane filtered with 0.45 μm filter (Thermo Fisher Scientific, Waltham, MA) and placed on TSA. TSA plates were recorded after incubating for 24 ± 2 h at 37 ± 2 ℃.

3.4. Statistical Analysis

Assays were carried out in triplicate and standard plate count was done in duplicate. The PROC GLIMMIX procedure in SAS 9.4 (SAS Institute, Cary, NC) was used to the log reduction as a Linear Model (LM) with a nested treatment structure among sanitizer, concentration, temperature, and application time. Residual and qq-plots were used to assess normality. Tukey's adjustment was used to determine pairwise significance at the $\alpha = 0.05$ level.

4. Results and Discussion

4.1. Efficacy of Commercial Cleaning Products against B. cereus Biofilms

Three commercial cleaning products' antimicrobial efficacy against *B. cereus* biofilm was measured using a CDC biofilm reactor and an ASTM E 2871-19 method. Two different temperatures, application times, and concentrations were tested for each cleaning product. Table 1 shows the results of four-way ANOVA in the efficacy of cleaning products. Figure 1 shows visualized estimated mean log reduction of cleaning products against *B. cereus* biofilms at 5 min contact time with 95% confidence limits, and Figure 2 visualizes estimated mean log reduction of cleaning products against *B. cereus* biofilms at 10 min contact time with 95% confidence limits.

Table 1. Four-way ANOVA results measuring efficacy of commercial cleaning products against *B. cereus* biofilms

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	2	48	1460.17	<.0001
Application time	1	48	0.18	0.6766
Temperature	1	48	4.94	0.0310
Treatment (Concentration)	3	48	21.22	<.0001
Treatment*Application time	2	48	0.38	0.6882
Treatment*Temperature	2	48	26.35	<.0001
Application time*Temperature	1	48	28.66	<.0001
Treatment*Application time (Concentration)	3	48	1.69	0.1815
Treatment*Temperature (Concentration)	3	48	0.02	0.9973
Treatment*Application time*Temperature	2	48	10.99	0.0001
Treatment*Application time*Temperature (Concentration)	3	48	2.16	0.1044

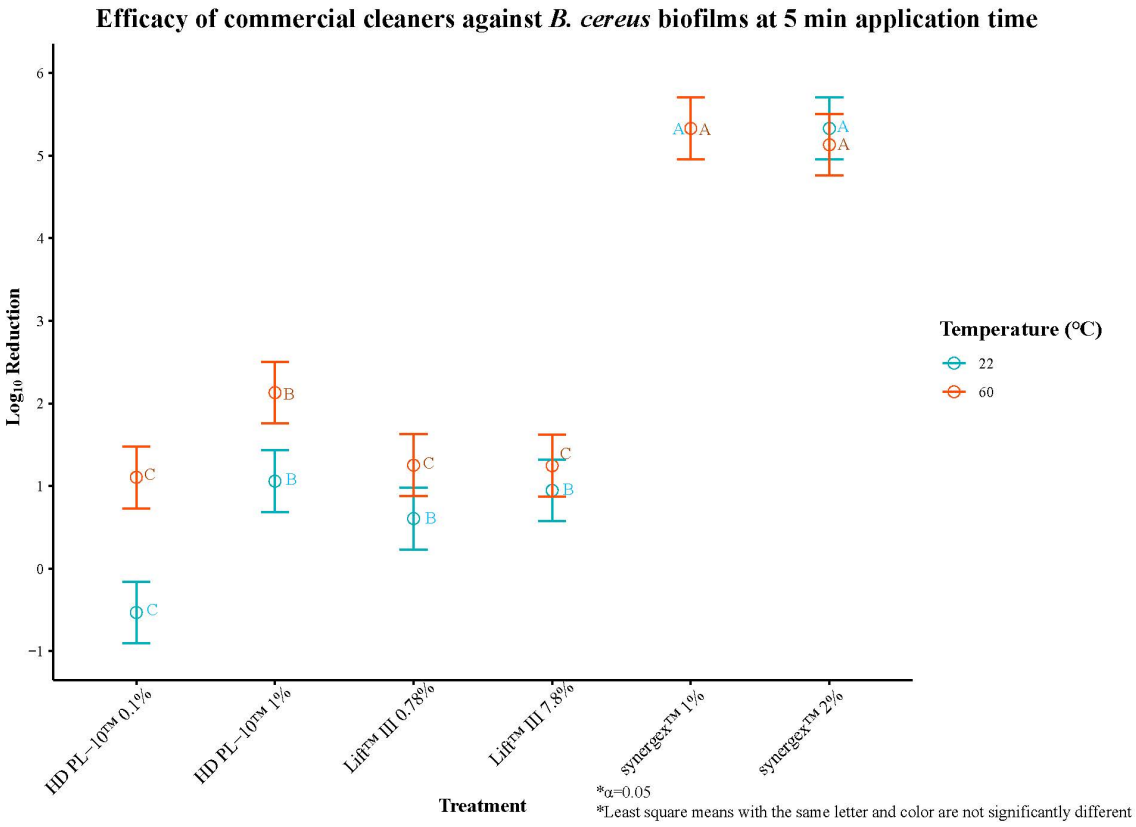


Figure 2. Visualized efficacy of commercial cleaning products against *B. cereus* biofilms at 5 min application time with Tukey's comparison test result (95% confidence limits)

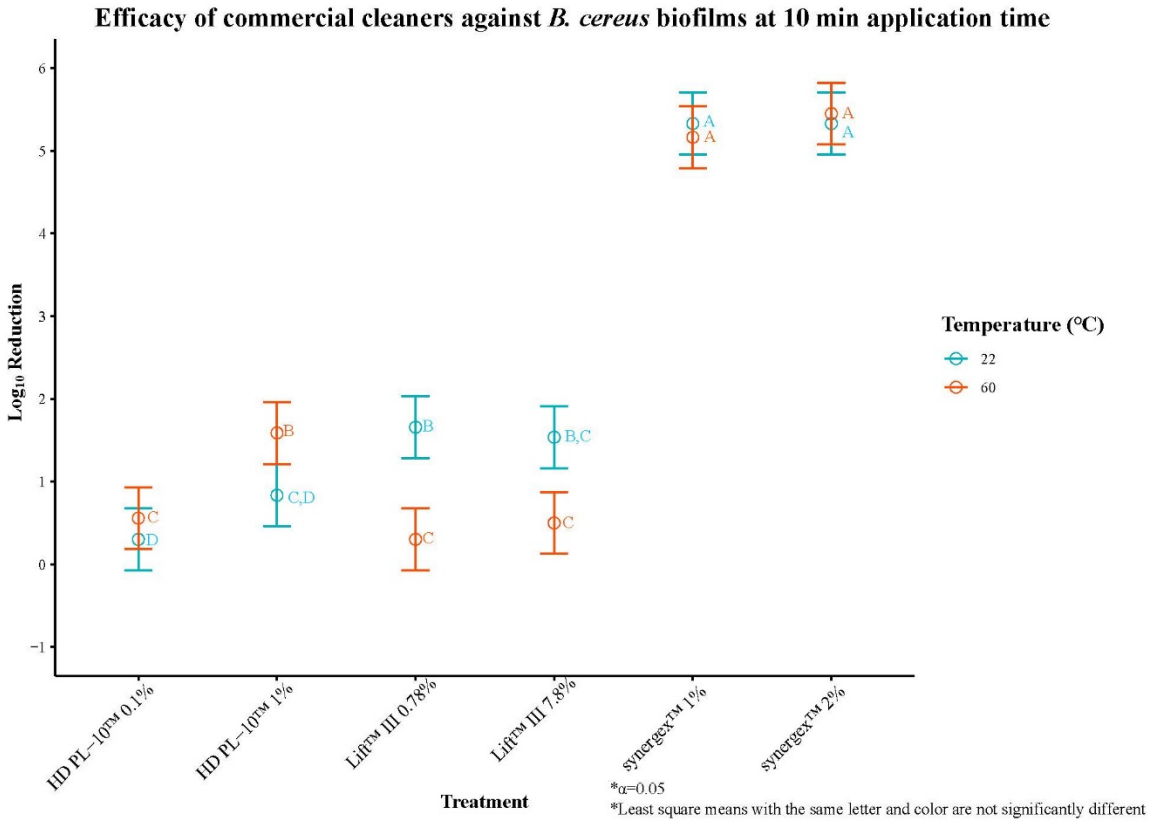


Figure 3. Visualized efficacy of commercial cleaning products against *B. cereus* biofilms at 10 min application time with Tukey's comparison test result (95% confidence limits)

Not every factor was significant in Table 1, but the simple effect comparison was performed in Table 2, Table 3, and Table 4 to see if there is any confounding effect of each factor while other factors are fixed. Figure 2 and Figure 3 shows that a commercial disinfectant with a biofilm claim, Synergex™, demonstrated the best efficacy among cleaning products tested. Synergex™ achieved over 5-$\log_{10}$ reductions in every condition tested. Synergex™ turned out to be an effective treatment against *B. cereus* biofilms as well as *S. aureus* and *P. aeruginosa* biofilms. The product was effective in cool and hot temperatures, with shorter application times, and at lower concentrations tested. Synergex™ consists of sulfuric acid, hydrogen peroxide, peroxyacetic acid, acetic acid,

Secondary Alkane Sulphonates (SAS), octanoic acid, and peroxyoctanoic acid. Several studies reported hydrogen peroxide and peracetic acids' effect against different biofilms, but the result varied by species (Lee, Cappato, Corassin, Cruz, & Oliveira, 2016; Lineback et al., 2018; Sindi et al., 2019). Therefore, it is important to validate the chemical agents against the specific organism.

At 22 ℃, an acidic detergent HD PL-10™ showed no efficacy with 0.1% concentration at 5 min application time, and the efficacy increased as an application time increase to 10 min. About 1-$\log_{10}$ reduction was observed with 1% concentration, but there was no significant difference between the two different application times according to Table 3. Table 4 supports that there was a significant difference between different concentrations in all application times and temperatures. At 60 ℃, HD PL-10™ generally showed better efficacy than at 22 ℃ with 1-2 $\log_{10}$ inactivation. This efficacy was as strong as chlorine-based oxidizing agents (Kim et al., 2019). Table 2 supports that there was a significant difference between two different temperatures. In summary, HD PL-10™ had better efficacy at higher concentrations, higher temperature, and longer application time in this test.

On the other hand, Figure 2 shows alkaline detergent Lift™ III showed no consistent relationship when tested at different temperatures. The alkaline detergent showed more than a 3-$\log_{10}$ higher reduction at 70 ℃ when compared to room temperature in previous research (Peng et al., 2002). In this study, however, no dramatic increase was observed at higher temperatures. The efficacy increased as application time increase in every condition, and Table 3 shows a significant difference between two different application times. Table 4 shows that there was no significant difference

between the two concentrations tested. Compared to HD PL-10™, Lift™ III showed better efficacy at 22 ℃ with longer contact time.

Even though the efficacy of detergents is not significant compared to disinfectant Synergex™, it is important to use acidic or alkaline detergents in the cleaning regime because they can reduce the viability of remaining bacteria (Gibson, Taylor, Hall, & Holah, 1999). Additionally, higher pH reduces the surface hydrophobicity and inhibits future biofilm development of survived bacteria (Nostro et al., 2012). CIP cycles with detergent also play an essential role in detaching attached spores, bacterial soil, and food residues (Faille et al., 2013; Moerman, Rizoulières, & Majoor, 2014).

Acidic and alkaline CIP cleaning agents showed greater than 6-$\log_{10}$ reductions against *E. coli* biofilm at 30 min application time with 1500 rpm revolution. In the same study, an acidic detergent was less effective against *S. aureus* biofilms when compared to *E. coli* biofilms, and alkaline cleaning generally showed better efficacy than acidic CIP cleaning agents (Furukawa, Akiyoshi, Komoriya, Ogihara, & Morinaga, 2010). Biofilms' resistance may vary depending on the methods used to form the biofilm and the resulting biofilm architecture (Mah & O'Toole, 2001). Still, this suggests the efficacy of detergents can be increased in practical situations where the water is flowing or pumped inside of pipes. Thus, future studies should consider physical removal as an additional variable with the pilot-lab scale test.

Table 2. Simple effect comparisons between different temperatures

Treatment	Temperature (℃)	Temperature (℃)	Estimated log reduction difference	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
HD PL-10™ 5min 0.10%	22	60	-1.6365	0.2634	48	-6.21	<.0001	0.05	-2.1660	-1.1069
HD PL-10™ 10min 0.10%	22	60	-0.2571	0.2634	48	-0.98	0.3339	0.05	-0.7866	0.2724
HD PL-10™ 10min 1.00%	22	60	-0.7533	0.2634	48	-2.86	0.0062	0.05	-1.2828	-0.2238
HD PL-10™ 5min 1.00%	22	60	-1.0726	0.2634	48	-4.07	0.0002	0.05	-1.6022	-0.5431
Synergex™ 5min 1.00%	22	60	-122E-17	0.2634	48	-0.00	1.0000	0.05	-0.5295	0.5295
Synergex™ 10min 1.00%	22	60	0.1658	0.2634	48	0.63	0.5320	0.05	-0.3637	0.6953
Synergex™ 5min 2.00%	22	60	0.1981	0.2634	48	0.75	0.4556	0.05	-0.3314	0.7276
Synergex™ 10min 2.00%	22	60	-0.1200	0.2634	48	-0.46	0.6507	0.05	-0.6495	0.4095
Lift™ III 5min 0.78%	22	60	-0.6461	0.2634	48	-2.45	0.0178	0.05	-1.1757	-0.1166
Lift™ III 10min 0.78%	22	60	1.3559	0.2634	48	5.15	<.0001	0.05	0.8264	1.8855
Lift™ III 5min 7.80%	22	60	-0.2985	0.2634	48	-1.13	0.2627	0.05	-0.8280	0.2311
Lift™ III 10min 7.80%	22	60	1.0372	0.2634	48	3.94	0.0003	0.05	0.5077	1.5667

Table 3. Simple effect comparisons between different application times

Treatment	Application time	Application time	Estimated log reduction difference	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
HD PL-10™ 22 ℃ 0.10%	10min	5min	0.8323	0.2634	48	3.16	0.0027	0.05	0.3028	1.3618
HD PL-10™ 60 ℃ 0.10%	10min	5min	-0.5470	0.2634	48	-2.08	0.0432	0.05	-1.0766	-0.01751
HD PL-10™ 22 ℃ 1.00%	10min	5min	-0.2237	0.2634	48	-0.85	0.4000	0.05	-0.7532	0.3059
HD PL-10™ 60 ℃ 1.00%	10min	5min	-0.5430	0.2634	48	-2.06	0.0447	0.05	-1.0725	-0.01349
Synergex™ 22 ℃ 1.00%	10min	5min	1.22E-15	0.2634	48	0.00	1.0000	0.05	-0.5295	0.5295
Synergex™ 60 ℃ 1.00%	10min	5min	-0.1658	0.2634	48	-0.63	0.5320	0.05	-0.6953	0.3637
Synergex™ 22 ℃ 2.00%	10min	5min	-555E-18	0.2634	48	-0.00	1.0000	0.05	-0.5295	0.5295
Synergex™ 60 ℃ 2.00%	10min	5min	0.3181	0.2634	48	1.21	0.2331	0.05	-0.2114	0.8476
Lift™ III 22 ℃ 0.78%	10min	5min	1.0520	0.2634	48	3.99	0.0002	0.05	0.5224	1.5815
Lift™ III 60 ℃ 0.78%	10min	5min	-0.9501	0.2634	48	-3.61	0.0007	0.05	-1.4796	-0.4206
Lift™ III 22 ℃ 7.80%	10min	5min	0.5900	0.2634	48	2.24	0.0297	0.05	0.06052	1.1196
Lift™ III 60 ℃ 7.80%	10min	5min	-0.7456	0.2634	48	-2.83	0.0068	0.05	-1.2751	-0.2161

Table 4. Simple effect comparisons between different concentrations

Treatment	Concentration	Concentration	Estimated log reduction difference	Standard Error	DF	t Value	Pr > |t|	Alpha	Lower	Upper
HD PL-10™ 5min 22 ℃	0.10%	1.0%	-1.5902	0.2634	48	-6.04	<.0001	0.05	-2.1198	-1.0607
HD PL-10™ 5min 60 ℃	0.10%	1.0%	-1.0264	0.2634	48	-3.90	0.0003	0.05	-1.5559	-0.4969
HD PL-10™ 10min 22 ℃	0.10%	1.0%	-0.5343	0.2634	48	-2.03	0.0481	0.05	-1.0638	-0.00474
HD PL-10™ 10min 60 ℃	0.10%	1.0%	-1.0304	0.2634	48	-3.91	0.0003	0.05	-1.5600	-0.5009
Synergex™ 5min 22 ℃	1.0%	2.0%	9.99E-16	0.2634	48	0.00	1.0000	0.05	-0.5295	0.5295
Synergex™ 5min 60 ℃	1.0%	2.0%	0.1981	0.2634	48	0.75	0.4556	0.05	-0.3314	0.7276
Synergex™ 10min 22 ℃	1.0%	2.0%	2.78E-15	0.2634	48	0.00	1.0000	0.05	-0.5295	0.5295
Synergex™ 10min 60 ℃	1.0%	2.0%	-0.2858	0.2634	48	-1.09	0.2833	0.05	-0.8153	0.2437
Lift™ III 5min 22 ℃	0.78%	7.8%	-0.3411	0.2634	48	-1.30	0.2014	0.05	-0.8707	0.1884
Lift™ III 5min 60 ℃	0.78%	7.8%	0.006536	0.2634	48	0.02	0.9803	0.05	-0.5230	0.5361
Lift™ III 10min 22 ℃	0.78%	7.8%	0.1208	0.2634	48	0.46	0.6486	0.05	-0.4088	0.6503
Lift™ III 10min 60 ℃	0.78%	7.8%	-0.1979	0.2634	48	-0.75	0.4560	0.05	-0.7275	0.3316

5. Conclusions

An acidic disinfectant with a biofilm claim, Synergex™, showed the best efficacy among treatments tested with over 5-$\log_{10}$ reductions in every condition. Peroxygens-based disinfectant with surfactant was significantly effective against *B. cereus* biofilms as well as *S. aureus* and P. *aeruginosa* biofilm. An acidic detergent HD PL-10™ showed no efficacy at 0.1% concentration. However, it showed better efficacy using a longer application time and higher temperature with about 1-$\log_{10}$ inactivation. At 1% concentration, HD PL-10™ showed better efficacy than at 0.1% concentration. Inactivation efficacy increased with 60 ℃ showing 1-2 $\log_{10}$ reduction, but longer application time did not improve efficacy. Alkaline detergent Lift™ III showed no significant difference between the two different concentrations. Lift™ III resulted in

about 1-$\log_{10}$ reduction at 5 min and had slightly better efficacy at 10 min at room temperature. No consistent relationship was shown from different temperatures.

When comparing acidic and alkaline detergents, the alkaline detergent showed slightly better efficacy using a longer application time at room temperature. However, at 60 °C, HD-PL 10™ showed better efficacy at the maximum recommended strength with greater than 2-$\log_{10}$ reductions. This efficacy is equivalent to the efficacy shown in another study using chlorine- based oxidizing agents. It is not as strong as the disinfectant Synergex™. Nevertheless, cleaning with detergents can help prevent the bacteria's outgrowth and persisting by reducing the remaining bacteria's viability.

Static biofilms and various methods were used in previous studies measuring the efficacy of biocides against biofilms. This study used standardized methods to create biofilms and measure the efficacy with better repeatability and real-world compatibility. Using standardized methods can also help future studies compare the efficacy of treatments directly. However, a limitation exists in this method as well. Mechanical action plays an important role by detaching adherent foreign matters with shear stress used in cleaning. Nevertheless, this effect is disregarded in the method used.

A cleaning regime consisting of cleaning with detergents followed by a rinsing cycle, plus an additional sanitizing step is the best option to prevent biofilm development in the food processing facility. This research will help the food processing environment adopt optimum treatments and conditions based on the contamination type (e.g., spores) and level. By using proper amount of disinfectants, industry stakeholders will be able to reduce the cost and the risk of environmental hazards caused by VOCs (Volatile organic compounds) (Wolkoff et al., 1998).

6. References

ASTM. (2018). Standard Practice for Preparing a Pseudomonas aeruginosa or Staphylococcus aureus Biofilm using the CDC Biofilm Reactor. In *ASTM E3161-18*. West Conshohocken, PA: ASTM International.

ASTM. (2019). Standard Test Method for Determining Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Method. In *ASTM E2871-19*. West Conshohocken, PA: ASTM International.

Faille, C., Bénézech, T., Blel, W., Ronse, A., Ronse, G., Clarisse, M., & Slomianny, C. (2013). Role of mechanical vs. chemical action in the removal of adherent Bacillus spores during CIP procedures. *Food Microbiology, 33*(2), 149-157. doi:10.1016/j.fm.2012.09.010

Fernandes, M. D. S., de Oliveira, A. M., Cheriegate, A. P. C., & de Abreu Filho, B. A. (2018). Bacteria isolated from the bovine gelatin production line: biofilm formation and use of different sanitation procedures to eliminate the biofilms. *Journal of Food Safety, 38*(5). doi:10.1111/jfs.12489

Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology, 8*(9), 623-633. doi:10.1038/nrmicro2415

Furukawa, S., Akiyoshi, Y., Komoriya, M., Ogihara, H., & Morinaga, Y. (2010). Removing Staphylococcus aureus and Escherichia coli biofilms on stainless steel by cleaning-in-place (CIP) cleaning agents. *Food Control, 21*(5), 669-672. doi:https://doi.org/10.1016/j.foodcont.2009.10.005

Gibson, H., Taylor, J. H., Hall, K. E., & Holah, J. T. (1999). Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms.

Journal of Applied Microbiology, 87(1), 41-48. doi:10.1046/j.1365-2672.1999.00790.x

Huang, Z. Y., Lin, Y. Y., Ren, F. Z., Song, S. J., & Guo, H. Y. (2019). Benzalkonium bromide is effective in removing Bacillus cereus biofilm on stainless steel when combined with cleaning-in-place. *Food Control, 105*, 13-20. doi:10.1016/j.foodcont.2019.05.017

Hussain, M. S., & Oh, D.-H. (2018). Impact of the Isolation Source on the Biofilm Formation Characteristics of Bacillus cereus. *Journal of Microbiology and Biotechnology, 28*(1), 77-86. doi:10.4014/jmb.1707.07023

Ikram, S., Heikal, A., Finke, S., Hofgaard, A., Rehman, Y., Sabri, A. N., & Økstad, O. A. (2019). Bacillus cereus biofilm formation on central venous catheters of hospitalised cardiac patients. *Biofouling, 35*(2), 204-216. doi:10.1080/08927014.2019.1586889

Kim, H., Moon, M. J., Kim, C. Y., & Ryu, K. (2019). Efficacy of chemical sanitizers against Bacillus cereus on food contact surfaces with scratch and biofilm. *Food Science and Biotechnology, 28*(2), 581-590. doi:10.1007/s10068-018-0482-2

Kumar, J., Sharma, V. K., Parmar, S., Singh, P., & Singh, R. K. (2020). Biofilm: A microbial assemblage on the surface—A boon or bane? In (pp. 139-150): Elsevier.

Lee, S. H. I., Cappato, L. P., Corassin, C. H., Cruz, A. G., & Oliveira, C. A. F. (2016). Effect of peracetic acid on biofilms formed by Staphylococcus aureus and Listeria monocytogenes isolated from dairy plants. *Journal of Dairy Science, 99*(3), 2384-2390. doi:10.3168/jds.2015-10007

Lewis, K. (2001). Riddle of Biofilm Resistance. *Antimicrobial Agents and Chemotherapy, 45*(4), 999-1007. doi:10.1128/aac.45.4.999-1007.2001

Li, N. W., Liu, G. L., & Liu, J. (2017). Inactivation of Bacillus cereus biofilms on stainless steel by acidic electrolyzed water. *Journal of Food Processing and Preservation, 41*(6). doi:10.1111/jfpp.13304

Lineback, C. B., Nkemngong, C. A., Wu, S. T., Li, X., Teska, P. J., & Oliver, H. F. (2018). Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. *Antimicrobial Resistance & Infection Control, 7*(1). doi:10.1186/s13756-018-0447-5

Mah, T.-F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology, 9*(1), 34-39. doi:10.1016/s0966-842x(00)01913-2

Majed, R., Faille, C., Kallassy, M., & Gohar, M. (2016). Bacillus cereus Biofilms—Same, Only Different. *Frontiers in Microbiology, 7*. doi:10.3389/fmicb.2016.01054

Moerman, F., Rizoulières, P., & Majoor, F. A. (2014). 10 - Cleaning in place (CIP) in food processing. In H. L. M. Lelieveld, J. T. Holah, & D. Napper (Eds.), *Hygiene in Food Processing (Second Edition)* (pp. 305-383): Woodhead Publishing.

Nostro, A., Cellini, L., Di Giulio, M., D'Arrigo, M., Marino, A., Blanco, A. R., . . . Bisignano, G. (2012). Effect of alkaline pH on staphylococcal biofilm formation. *Apmis, 120*(9), 733-742. doi:10.1111/j.1600-0463.2012.02900.x

Paredes, J., Becerro, S., Arizti, F., Aguinaga, A., Del Pozo, J. L., & Arana, S. (2012). Real time monitoring of the impedance characteristics of Staphylococcal bacterial biofilm cultures with a modified CDC reactor system. *Biosensors and Bioelectronics, 38*(1), 226-232. doi:10.1016/j.bios.2012.05.027

Peng, J.-S., Tsai, W.-C., & Chou, C.-C. (2002). Inactivation and removal of Bacillus cereus by sanitizer and detergent. *International Journal of Food Microbiology, 77*(1-2), 11-18. doi:10.1016/s0168-1605(02)00060-0

Silva, H. O., Lima, J. A. S., Aguilar, C. E. G., Rossi, G. A. M., Mathias, L. A., & Vidal, A. M. C. (2018). Efficiency of Different Disinfectants on Bacillus cereus Sensu Stricto Biofilms on Stainless-Steel Surfaces in Contact With Milk. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.02934

Sindi, A., Chawn, M. V. B., Hernandez, M. E., Green, K., Islam, M. K., Locher, C., & Hammer, K. (2019). Anti-biofilm effects and characterisation of the hydrogen peroxide activity of a range of Western Australian honeys compared to Manuka and multifloral honeys. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-54217-8

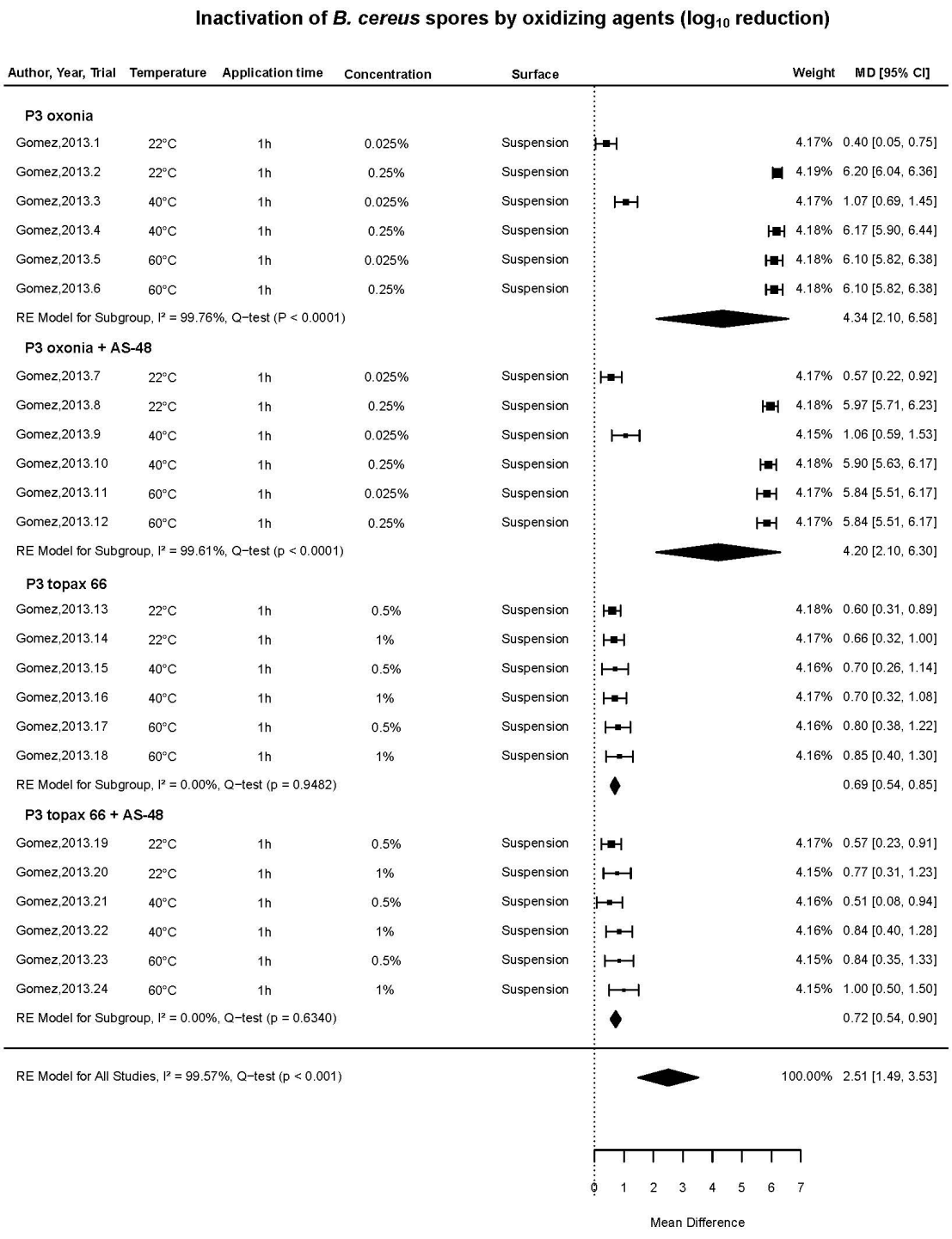
Van Der Veen, S., & Abee, T. (2011). Generation of Variants in Listeria monocytogenes Continuous-Flow Biofilms Is Dependent on Radical-Induced DNA Damage and RecA-Mediated Repair. *PLoS ONE, 6*(12), e28590. doi:10.1371/journal.pone.0028590

Vanloosdrecht, M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L., & Heijnen, J. (1995). Biofilm structures. *Water Science and Technology, 32*(8), 35-43. doi:10.1016/0273-1223(96)00005-4

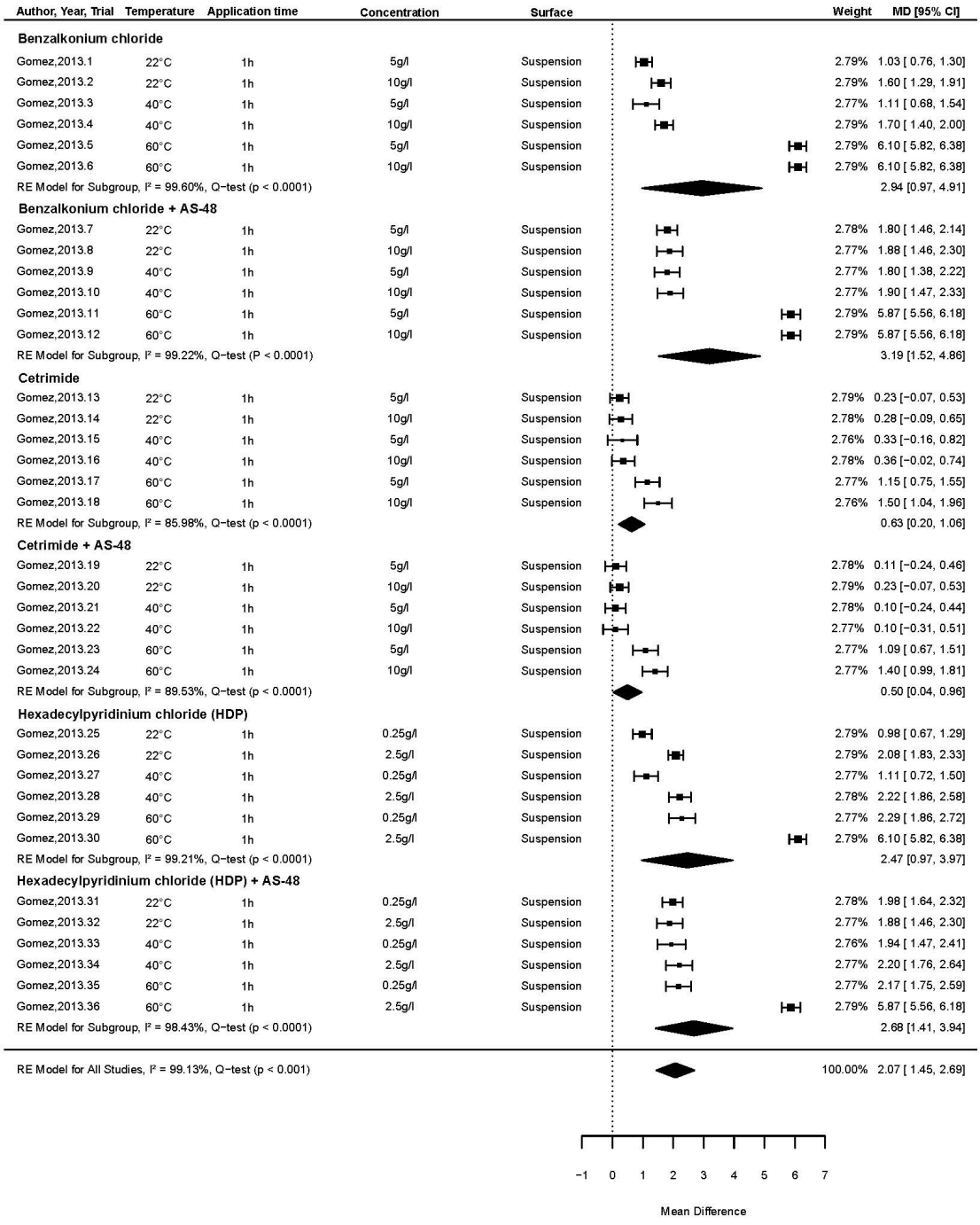
Watnick, P., & Kolter, R. (2000). Biofilm, City of Microbes. *Journal of Bacteriology, 182*(10), 2675-2679. doi:10.1128/jb.182.10.2675-2679.2000

Wolkoff, P., Schneider, T., Kildesø, J., Degerth, R., Jaroszewski, M., & Schunk, H. (1998). Risk in cleaning: chemical and physical exposure. *Science of The Total Environment, 215*(1), 135-156. doi:https://doi.org/10.1016/S0048-9697(98)00110-7

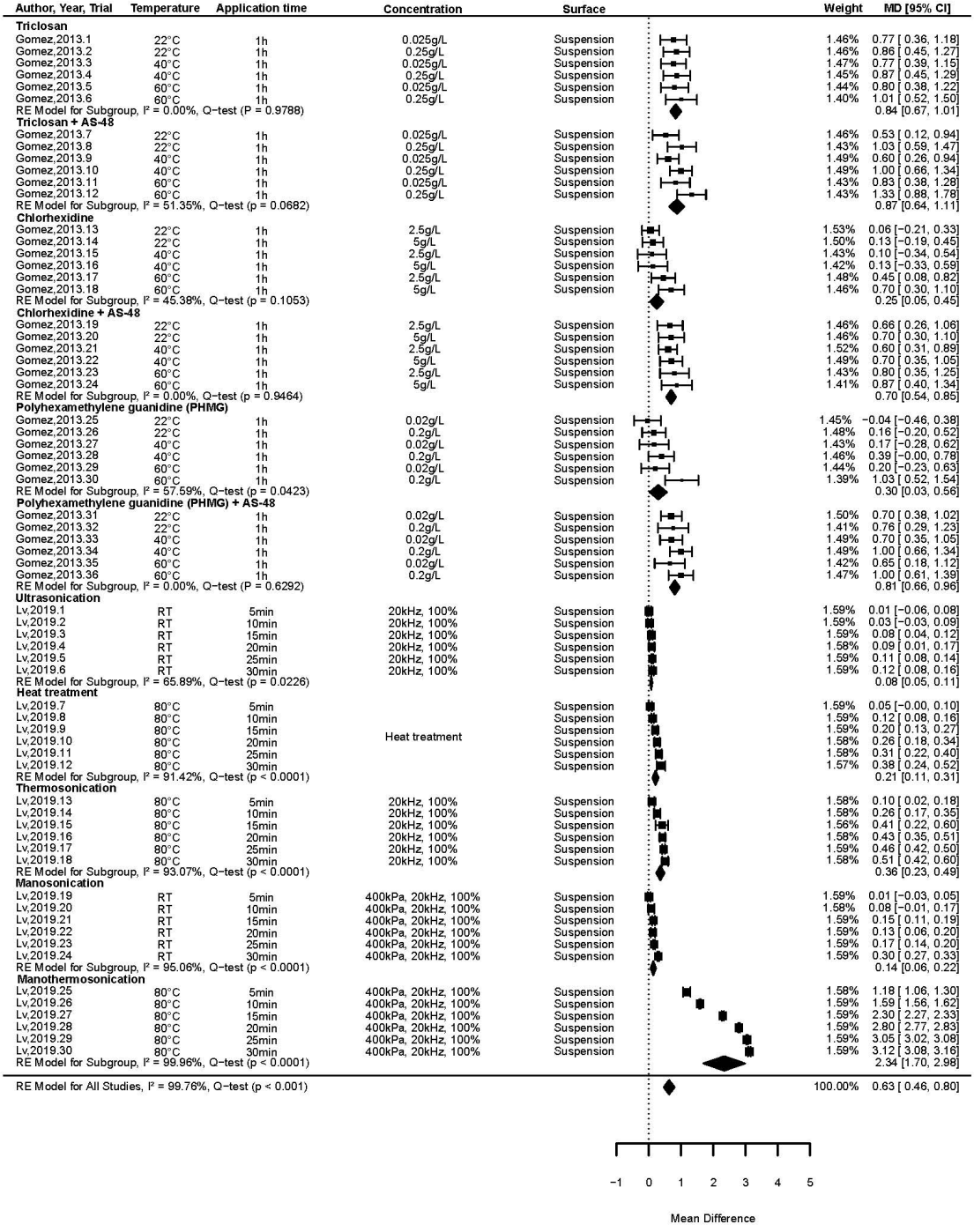
APPENDIX A: FOREST PLOTS



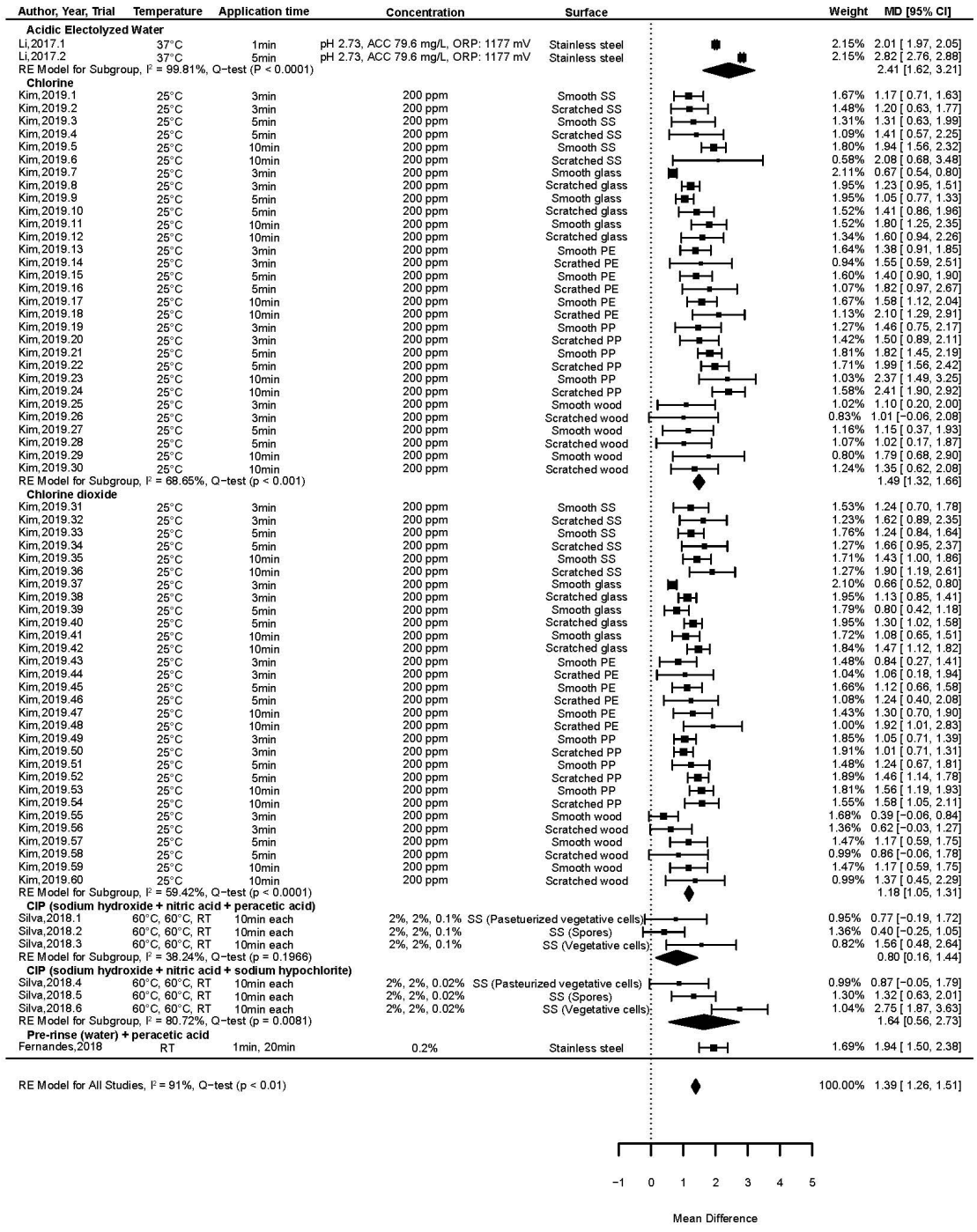
Inactivation of *B. cereus* spores by surface−active compounds (log_{10} reduction)

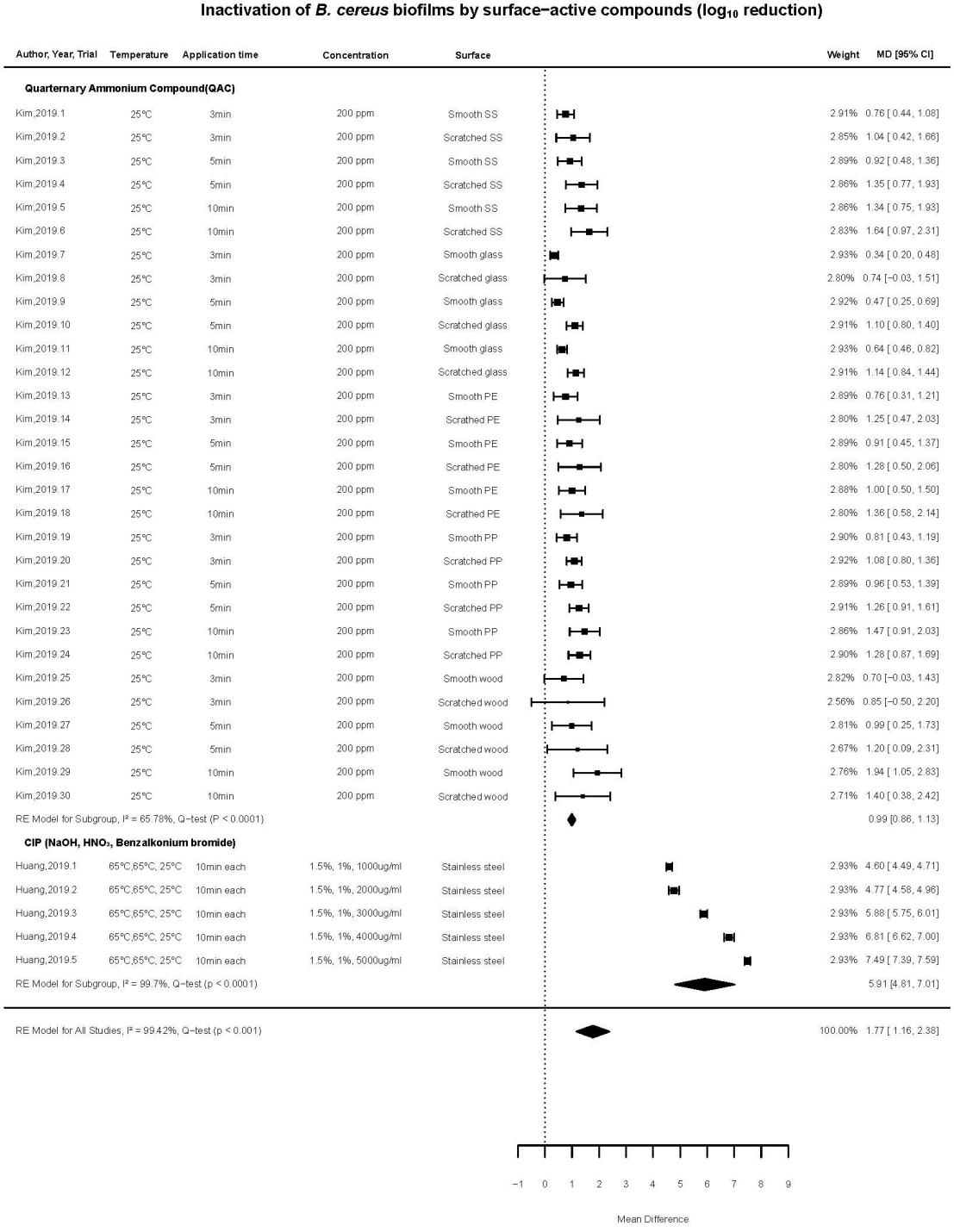


Inactivation of *B. cereus* spores by other treatments (log_{10} reduction)



Inactivation of *B. cereus* biofilms by oxidizing agents (log_{10} reduction)




Inactivation of B. cereus biofilms by surface−active compounds (log10 reduction)
Author, Year, Trial
Temperature
Application time
Concentration
Surface
Weight
MD [95% CI]
Quarternary Ammonium Compound(QAC)
Kim,2019.1 25℃ 3min 200 ppm Smooth SS 2.91% 0.76 [0.44, 1.08]
Kim,2019.2 25℃ 3min 200 ppm Scratched SS 2.85% 1.04 [0.42, 1.66]
Kim,2019.3 25℃ 5min 200 ppm Smooth SS 2.89% 0.92 [0.48, 1.36]
Kim,2019.4 25℃ 5min 200 ppm Scratched SS 2.86% 1.35 [0.77, 1.93]
Kim,2019.5 25℃ 10min 200 ppm Smooth SS 2.86% 1.34 [0.75, 1.93]
Kim,2019.6 25℃ 10min 200 ppm Scratched SS 2.83% 1.64 [0.97, 2.31]
Kim,2019.7 25℃ 3min 200 ppm Smooth glass 2.93% 0.34 [0.20, 0.48]
Kim,2019.8 25℃ 3min 200 ppm Scratched glass 2.80% 0.74 [−0.03, 1.51]
Kim,2019.9 25℃ 5min 200 ppm Smooth glass 2.92% 0.47 [0.25, 0.69]
Kim,2019.10 25℃ 5min 200 ppm Scratched glass 2.91% 1.10 [0.80, 1.40]
Kim,2019.11 25℃ 10min 200 ppm Smooth glass 2.93% 0.64 [0.46, 0.82]
Kim,2019.12 25℃ 10min 200 ppm Scratched glass 2.91% 1.14 [0.84, 1.44]
Kim,2019.13 25℃ 3min 200 ppm Smooth PE 2.89% 0.76 [0.31, 1.21]
Kim,2019.14 25℃ 3min 200 ppm Scrathed PE 2.80% 1.25 [0.47, 2.03]
Kim,2019.15 25℃ 5min 200 ppm Smooth PE 2.89% 0.91 [0.45, 1.37]
Kim,2019.16 25℃ 5min 200 ppm Scrathed PE 2.80% 1.28 [0.50, 2.06]
Kim,2019.17 25℃ 10min 200 ppm Smooth PE 2.88% 1.00 [0.50, 1.50]
Kim,2019.18 25℃ 10min 200 ppm Scrathed PE 2.80% 1.36 [0.58, 2.14]
Kim,2019.19 25℃ 3min 200 ppm Smooth PP 2.90% 0.81 [0.43, 1.19]
Kim,2019.20 25℃ 3min 200 ppm Scratched PP 2.92% 1.08 [0.80, 1.36]
Kim,2019.21 25℃ 5min 200 ppm Smooth PP 2.89% 0.96 [0.53, 1.39]
Kim,2019.22 25℃ 5min 200 ppm Scratched PP 2.91% 1.26 [0.91, 1.61]
Kim,2019.23 25℃ 10min 200 ppm Smooth PP 2.86% 1.47 [0.91, 2.03]
Kim,2019.24 25℃ 10min 200 ppm Scratched PP 2.90% 1.28 [0.87, 1.69]
Kim,2019.25 25℃ 3min 200 ppm Smooth wood 2.82% 0.70 [−0.03, 1.43]
Kim,2019.26 25℃ 3min 200 ppm Scratched wood 2.56% 0.85 [−0.50, 2.20]
Kim,2019.27 25℃ 5min 200 ppm Smooth wood 2.81% 0.99 [0.25, 1.73]
Kim,2019.28 25℃ 5min 200 ppm Scratched wood 2.67% 1.20 [0.09, 2.31]
Kim,2019.29 25℃ 10min 200 ppm Smooth wood 2.76% 1.94 [1.05, 2.83]
Kim,2019.30 25℃ 10min 200 ppm Scratched wood 2.71% 1.40 [0.38, 2.42]
RE Model for Subgroup, I² = 65.78%, Q−test (P < 0.0001) 0.99 [0.86, 1.13]
CIP (NaOH, HNO3, Benzalkonium bromide)
Huang,2019.1 65℃,65℃, 25℃ 10min each 1.5%, 1%, 1000ug/ml Stainless steel 2.93% 4.60 [4.49, 4.71]
Huang,2019.2 65℃,65℃, 25℃ 10min each 1.5%, 1%, 2000ug/ml Stainless steel 2.93% 4.77 [4.58, 4.96]
Huang,2019.3 65℃,65℃, 25℃ 10min each 1.5%, 1%, 3000ug/ml Stainless steel 2.93% 5.88 [5.75, 6.01]
Huang,2019.4 65℃,65℃, 25℃ 10min each 1.5%, 1%, 4000ug/ml Stainless steel 2.93% 6.81 [6.62, 7.00]
Huang,2019.5 65℃,65℃, 25℃ 10min each 1.5%, 1%, 5000ug/ml Stainless steel 2.93% 7.49 [7.39, 7.59]
RE Model for Subgroup, I² = 99.7%, Q−test (p < 0.0001) 5.91 [4.81, 7.01]
RE Model for All Studies, I² = 99.42%, Q−test (p < 0.001) 100.00% 1.77 [1.16, 2.38]
−1 0 1 2 3 4 5 6 7 8 9
Mean Difference

Inactivation of *B. cereus* biofilm by other treatments (log_{10} reduction)