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Commercial Household Disinfectant Inactivation of Bacillus cereus Spores on Fruit and Vegetable Surfaces

Helen Kerr
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To the Graduate Council:

I am submitting herewith a thesis written by Helen Kerr entitled "Commercial Household Disinfectant Inactivation of Bacillus cereus Spores on Fruit and Vegetable Surfaces." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

P. Michael Davidson, Major Professor

We have read this thesis and recommend its acceptance:

David Golden, John R. Mount

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Commercial Household Disinfectant Inactivation of *Bacillus cereus* Spores on Fruit and Vegetable Surfaces

A Thesis Presented
for the Master of Science Degree
The University of Tennessee, Knoxville

Helen Kerr
August 2009

DEDICATION

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I would like to thank my major professor, P. Michael Davidson, for giving me the opportunity to complete my M.S. degree. He has always challenged me to go beyond what was expected and truly get the most out of my education.

The Food Science Department as a whole has my appreciation. All of the faculty, staff, and students are of the highest caliber and make this department one of the best on campus. Everyone makes this an enjoyable environment for both working and learning. Last, but certainly not least, I would like to thank my fiancé, Mathew Stanke, and my family for their support. As new challenges have arisen, all were there to help work through them. I know that I would not have been able to get as far without their support.

ABSTRACT

Studies were conducted to test the efficacy of several common household products containing antimicrobial compounds for inactivating spores of *Bacillus cereus*, as a surrogate for *B. anthracis,* on fresh fruit or vegetables. Additionally, the effect of storage time on hypochlorite activity of household products was determined. *Bacillus cereus* ATCC 33018 and ATCC 49064 were used in a cocktail for all tests. Household disinfectant and/or cleaning products with potential for sanitization were purchased in a retail market and were selected based upon efficacy against *B. cereus* in previous tests in milk. The active components were NaOCl, HCl or H_2O_2. For produce, cantaloupe melons and spinach were obtained from a local retail market and rinsed with sterile deionized (DI) water. Cantaloupe rinds were removed, trimmed to remove the mesocarp, cut into 25 cm^2 sections and placed in sterile Petri dishes. Sections of melon and spinach leaves were spot inoculated with 0.1 ml of *B. cereus* and allowed to dry for 30 min at 25°C. Sanitizing products were sprayed onto the surface of the produce and the produce was allowed to stand for various times. Produce was then placed in neutralizer buffer to arrest the activity of sanitizing compounds and survivors were enumerated on non-selective media. To determine the effect of product age on activity, three commercial NaOCl-containing products that were past, at and 6 mo from the expiration dates were evaluated. Spinach and cantaloupe were tested as described previously. For the cantaloupe melon rind the control population mean was 7.15 $\pm$ 0.07 log CFU/cm^2. The log reduction was > 5.15 for undiluted NaOCl (Clorox®, 6.00% NaOCl) and inactivation took 120 min though the greatest reduction was observed in the first 10 min. For products containing 1.84%-2.40% NaOCl, log reductions were

2.75 to 3.40 over 180 min. For spinach, the control population mean was 7.37 ± 0.01 log CFU/leaf. A > 5.84 log reduction in *B. cereus* spores was found for both undiluted NaOCl (Clorox®) and HCl (The Works Drain Opener, 20.00% HCl). However, the former reduced the viable spore population to the lowest detection level in 10 to 60 min while the latter took approximately 3 h. A 4.23-4.60 log reduction occurred with the 1.84%-2.40% NaOCl-containing products after 180 min. Hydrogen peroxide had the least effective sporicidal capabilities of the solutions tested reducing the population by less than 1 log. With respect to effect of storage on sporicidal activity of HOCl-containing products, samples stored up to 1 year past expiration were compared with those stored for 6 months prior to expiration and purchased fresh. There was no significant difference in the *in vitro* inactivation of *B. cereus* spores among the products. *B. cereus* spores were inactivated to below detectable levels after 1 min in 50% and 25% commercial strength solutions (>4.0 log CFU/ml). These findings were confirmed utilizing cantaloupe rind and spinach leaves treated with HOCl-containing products of the same storage times. *B. cereus* spores were inactivated to below the level of detection (>5.84 log CFU/cm^2, >5.15 log CFU/cm^2) in 10 min and 60 min for spinach and cantaloupe respectively, regardless of compound age.

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CHAPTER I

Literature Review

Food Defense

Bioterrorism may be defined as the use or threatened use of biological agents against a person or group with the main objective of causing illness and/or apprehension *(13, 27)*. In fact, the majority of bioterrorism events are hoaxes designed solely to generate fear rather than the actual use of a biological agent. With reports of arsenals of biological weapons in at least ten countries, there exists both the possibility of an attack and the potential for such biological weapons to become acquired or used by non-government organizations or persons *(10, 13)*.

Potential perpetrators of bioterrorism are incredibly varied, encompassing not only independent terrorist organizations, cult groups and lone offenders but also state-entities. There are very few cases in modern times of intentional contamination of a food supply. While there has been growing concern about biological weapons, the threat posed is nearly impossible to ascertain. Therefore, planning and implementation of appropriate intervention strategies and prevention of future attacks is a very difficult task.

In the few instances where food bioterrorism has occurred, the cases have been extremely well publicized. Such attention drawn to the incidents potentially contributes to the interest of those parties or persons who may be motivated to either acquire or use such

technologies *(10)*. These are just some of many reasons that the issues of food defense are so varied and difficult to address adequately.

While on first evaluation it may appear that food defense, or rather the actual concern itself is a relatively modern concern, there have been historical accounts since antiquity of contaminated food and other biological agents being used quite effectively as a weapons. As early as 184 BC, Hannibal ordered that pots filled with serpents be thrown onto the decks of enemy ships as a means of disseminating a weaponized biological agent. Carthaginian general Maharbal intentionally contaminated wine with mandagora (mandrake) and used it to intentionally contaminate water supplies. The Tartar army in 1346 utilized the bodies of plague victims, catapulting them into the Crimean city of Caffa (now Feodosija, Ukraine) in order to render the city defenseless prior to a subsequent attack *(10, 11, 46, 58)*. In modern times, only one bioterrorist attack on food has actually resulted in illness. In Oregon 1984, a cult group known as the "Rajneeshees" intentionally contaminated food with *Salmonella* Typhimurium sickening an estimated 751 people and resulting in 45 hospitalizations *(10, 46)*. In 1972, more than 140 countries signed the Biological and Toxin Weapons Convention which called for termination of all offensive biological weapons research and development and destruction of existing biological weapons stocks *(3)*.

Terrorist attacks on the United States on September 11, 2001 and subsequent attacks using *Bacillus anthracis* on the United States Capitol and post office facilities brought concerns about biosecurity sharply into focus. Because the United States and much of the world's developed nations now possess a highly organized and far more efficient means of

disseminating their food supply to their population, there is a growing concern that these very efficiencies may in fact provide a great risk to food security. Because there is now a truly global distribution network for food, the potential for individuals or organizations to intentionally inflict great harm on public health and confidence exists on a scale never encountered in earlier times. Effective intervention strategies to prevent and or contain biological attacks on food supply at all levels of distribution have become essential *(10, 11, 46)*.

Bacillus anthracis as a Biological Weapon

Anthrax is the disease caused by infection of humans or other mammals with endospores of the gram positive bacterium *Bacillus anthracis*. There are three potential forms that anthrax may take dependent on the mode by which an individual contracts the organism: pulmonary, gastrointestinal, and cutaneous. Cutaneous anthrax accounts for 95% of the cases of anthrax infections in the US *(15)*. In cutaneous anthrax, endospores are introduced through a cut or abrasion. Pulmonary or inhalational anthrax is rare and occurs through inhalation of the *B. anthracis* spores. This form of anthrax is often fatal, even with antimicrobial therapy *(15)*. Gastrointestinal anthrax, while rare, results primarily from the ingestion of undercooked contaminated meat.

There are two clinical forms that gastrointestinal anthrax may take following ingestion of contaminated food: oropharyngeal and intestinal anthrax. Oropharyngeal anthrax occurs when spores or vegetative cells enter a lesion in the upper portion of the gastrointestinal tract

whereas, intestinal anthrax occurs when spores or vegetative cells enter a lesion located in the intestines or lower portion of the gastrointestinal tract *(22)*. The infectious dose for gastrointestinal anthrax is not well established, however, the mortality is reported to be greater than 50% *(1)*. Because it is thought that anthrax infections occur as a result of vegetative bacteria or spores colonizing a pre-existing lesion, pre-existing health of an individual can impact the infectious dose. This has been demonstrated in outbreaks of pulmonary anthrax, such as the Sverdlovsk anthrax outbreak of 1979, where younger and presumably healthier individuals were not among the 66 deaths that occurred of 77 infected individuals *(35)*. One of the largest outbreaks of gastrointestinal anthrax occurred in 1770 called "*charbon"* in which 15,000 people died in Saint- Dominique or modern Haiti after a large-scale earthquake occurred rendering effective means of thermal inactivation of microorganisms in food unavailable *(36)*. The majority of recent cases of gastrointestinal anthrax have occurred in the Middle East, Africa and southeastern Asia and are also the result of ingestion of foods that had insufficient thermal processing *(10, 17, 36, 46)*. Incidence of *Bacillus anthracis* spores on foods has not been studies in depth. One study determined the incidence of *B. anthracis* contaminated meat at a slaughterhouse in Nigeria. It was found that 5% of cattle and 3.3% of sheep tested were found to be positive for anthrax *(39)*. During the period between 1978 and 1985 more than 10,000 cases of cutaneous and gastrointestinal cases of anthrax were reported in Nigeria *(39)*.

The potential distribution of *Bacillus anthracis* spores, used as a biological weapon, has received much attention in recent years and has prompted research and review of methods not only for detection of the spores and vegetative cells but also materials to inactivate them *(60)*.

In a report filed by the US Centers for Disease Control and Prevention (CDC) on biological and chemical terrorism preparedness and response, *Bacillus anthracis* was classified as a category "A" bioterrorism threat. CDC reports that *Bacillus anthracis* may pose a risk to national security due to its potential for large-scale morbidity and mortality and its potential to be easily disseminated in aerosolized form *(13, 46)*.

In 1998, the CDC received reports of a series of letters sent to health clinics in Indiana, Kentucky, and Tennessee that were purported to contain spores of *Bacillus anthracis*. In the same year three separate threats were made by telephone claiming anthrax contamination of ventilation systems. All these threats were found to be hoaxes after investigation by local law enforcement and the Federal Bureau of Investigations *(11)*. It wasn't until 2001, that a successful dissemination of *Bacillus anthracis* as a biological weapon took place at the US Capitol after the opening of spore-filled envelopes killing 5 and sickening 17 people. In September, 2008, the unique characteristics of the *B. anthracis* spores found in the letter sent to the capitol offices led investigators to two government scientists *(37)*. One, Steven Hatfill, was later found not to be involved, while Dr. Bruce Ivins was believed to have been responsible for perpetrating both the weaponization of the spores and sending out the letters to the capitol. The spores themselves were found to be 1.5-3 µm, many times smaller than the finest known grade of anthrax produced by either the U.S. or Soviet bioweapons programs. This raised questions about whether Dr. Ivins was capable of weaponizing these spores on his own as well as other concerns *(34, 37)*. These attacks and the accidental dissemination of *B. anthracis* during the Sverdlovsk outbreak of 1979 have underscored for many both the

potential for biological agents such as *B. anthracis* to be used against civilian populations and the potential vulnerability of our food supply.

Therefore, the need to understand, recognize and deal with biological agents not usually associated with foods in order to protect our population has become of utmost importance. In the event that a biological agent, such as *B. anthracis*, were distributed on food at the consumer level there is some question as to what options would be open to consumers and authorities to deal with those contaminated products. Disposal in solid waste facilities or municipal wastewater systems has the potential to overload such systems with the infectious agent. One strategy that may facilitate protection of both consumers and our waste treatment systems is partial or full decontamination of hazardous products in the home utilizing commercial household sanitizers. The purpose of this decontamination utilizing chemical household sanitizers would not be to make these products edible for the consumer or livestock, but rather to perhaps provide a greater degree of safety to consumers who are forced to handle biologically contaminated items. Furthermore, in the event that a large number of food products were to be distributed in the home, a partial or full decontamination might be the only way to convince municipalities to agree to dispose of such items from homes. As stated earlier the outcomes associated with overloading waste management systems or simply introducing an easily aerosolized biological spore into the environment en masse could have lasting, profound and as yet unforeseen effects at all levels of the population.

Methods of Inactivation of *Bacillus anthracis* Spores

A number of methods have been employed to inactivate either *Bacillus anthracis* or related sporeforming *Bacillus* species primarily involving heat or chemicals. Heat treatments including wet and dry heat have been utilized *(60)*. For example, in a study by Stein and others, it was shown that 8 logs of *B. anthracis* could be inactivated in boiling water in 5 min. Studies performed by Baweja and others *(6)* evaluated the influence of a variety of stresses during sporulation on the thermal sensitivity of *Bacillus anthracis* spores. *B. anthracis* spores were produced in a variety of media, at low or high pH, or exposed to low nutrient conditions and then later exposed to a heat or other denaturants to determine sensitivity. Nicholson suggested *(38)* that both low water content of the spore coat and its mineral content may play a role in the resistance of *B. anthracis* spores. While not proven, a lower water content in the proteins of the spore coat may impart a stabilizing influence and decrease the likelihood of denaturation at higher temperatures.

The extent of inactivation of bacterial endospores by chemicals is dependent upon many factors including the chemical used, form and concentration of the chemical, time and temperature of the treatment and genus, species and strain of the microorganism. Bacterial endospores are generally much more resistant to chemical sanitizing agents/sterilants than vegetative cells. One method for delivery of chemical sanitization is through gaseous treatments. This particular method has the advantage of dissemination, and therefore inactivation, over a large area. Gases utilized for inactivation of *Bacillus* species include ethylene oxide, methylene bromide, formaldehyde, hydrogen peroxide (H_2O_2) plasma/gas, and

chlorine dioxide (ClO_2) (Table 1). However, one must also realize all potential implications of releasing chemicals in any form. For example, formaldehyde is a known carcinogen thereby making it an unlikely substance to use as a household decontaminant. However, formaldehyde is very effective at neutralizing *Bacillus* spores, and when used as a gas it can be neutralized when reacted with ammonium carbonate resulting in a white powder, hexamethylene tetramine. The powder is benign and does give a visual indication that decontamination of an area has in fact occurred. However, if this reaction is incomplete, there is still the potential for the formaldehyde gas to leach into porous surfaces and pose a continuous toxic threat. A study by Rogers and others *(44)* evaluated the efficacy of the hydrogen peroxide gas generator. It was found that while hydrogen peroxide gas significantly reduced viable spores on various indoor surfaces, the efficacy was greatly affected by the porosity of the material itself, which poses a problem for decontamination of buildings as well as contaminated foods *(44)*.

Inactivation of *Bacillus anthracis* Spores by Chemical Sanitizers

A sanitizer may be defined as a chemical agent that reduces the number of vegetative bacteria to safe levels *(48)*. *Bacillus anthracis* spores are generally considered to be highly resistant to chemical agents. However, this resistance is dictated by a number of factors including type of chemical agent, genus, species, strain, pH, temperature and suspending medium among others. All of these factors must be kept in mind when selecting an appropriate chemical method to inactivate *Bacillus* vegetative cells and spores *(1, 17)*. The composition of a

food on which the spores are resident will lead to variability in the apparent effectiveness of sanitizers because of the varying influence of organic matter on activity as well as the ability of the agent to interact with the spore *(50, 55)*. Examples of the relative resistance of *Bacillus anthracis* and related *Bacillus* species are shown in Table 2.

Table 1. Treatment of Bacterial Spores Utilizing Gaseous Treatments

Gas	Conc./treatment applied	Bacterium, Inoculum	Surface	Efficiency	Reference
Ethylene oxide (C_2H_4O)	500mg/L, 30% to 50% RH 54.4°C , 30 min	*Bacillus globigii*, 10^6	Hygroscopic and non-hygroscopic	4 log reduction - non hygroscopic surfaces 6 log reduction - hygroscopic surfaces	*(19)*
Methylene Bromide (CH_3Br)	3.4-3.9 g/L, room temperature in the presence of some moisture, 24 h	*Bacillus anthracis*; 1 x 10^5 -5 x 10^7	dried onto sterile filter paper strips	100% killed	*(28)*
Hydrogen Peroxide Generator (H_2O_2)	≥1000 ppm, 20 min	*Bacillus anthracis*, 9.3 ±7.2 x 10^4	Industrial carpet	3.0 ± 2.1 log reduction	*(44)*
		3.3±2.9 x 10^3	Pine	3.7±0.67	
		1.5±2.6 x 10^3	Painted concrete	6.4±2.1	
		8.4±2.2 x 10^7	Glass	≥7.9±0	
		7.0±1.0 x 10^7	Decorative laminate	≥7.9±0	
		3.5±0.13 x 10^7	Galvanized metal ductwork	≥7.5±0	
		8.3±0.63 x 10^6	Painted wallboard	≥6.9±0	
Hydrogen Peroxide Plasma (H_2O_2)	0.208 mg/L, 1.5 Torr pretreatment for 10 min; 2.49 MHz, 150 W of pulsed plasma in a cycle of 0.5 ms plasma on 1.0 ms plasma off, 15 min	*Bacillus subtilis* ssp. *globigii* , 3.4 x 10^5	Paper disks packaged in spun-bonded polyethylene	100% killed	*(23)*
Chlorine Dioxide (ClO_2)	40 mg/L 60-80% RH 25°C, 1 hr	*Bacillus subtilis* ssp. *niger*, 1.4 X 10^6/0.2ml	Paper and Aluminum foil strips	100% killed	*(24)*[1]

Table 1, cont.

Chlorine Dioxide (ClO_2)	6-7 mg/L 20-40% RH, 23°C, 30 min	*Bacillus subtilis* ssp. *niger,* 10^6		10^1 CFU/biologic indicator estimated time to kill 90% 4.2 min	*(24)*[2]
Low Temperature Stream Formaldehyde (CH_2O)	12 µg/ml 63-83°C, 14 min treatment	*Bacillus stearothermophilus*, at 90°C		No lethality reported	*(61)*
Formaldehyde (CH_2O)	1100 ppm formaldehyde gas for 10 hr formaldehyde gas, Samples analyzed at 1 and 7 d post exposure	*Bacillus. anthracis*, *Bacillus. subtilis*, or *Geobacillus stearothermophilus,* 1.0×10^8	Dried on different indoor surfaces ranging from carpet, wood, painted surfaces	≥50% of biological indicators and spore strips (approx 1 x 10^6 CFU) were in activated on both porous and nonporous materials.	*(43)*

Table 2. Fluid Chemical Sanitizer Inactivation Parameters of Bacterial Spores

Method	Concentration, Time	Bacterium, Inoculum	Surface	Efficiency /Trend	Reference
Hydrogen Peroxide (H_2O_2)	Variable	*B. subtilis* subsp. *globigii*		D values (24°C): 10% – > 10 min 20% > 10 min 25.8% – 2.0 min 35% – 1.5 min 41% - 0.75 min	*(56)*
Hydrogen Peroxide (H_2O_2)	25.8% at 24°C	*Bacillus subtilis* (BS), *B. subtilis* subsp. *globigii* (BSG*), B. coagulans* (BC), *B. stearothermophilus* (BST)		D values (25.8% at 24°C): BS – 7.3 min BSG – 2.0 min BC – 1.8 min BST – 1.5 min z value = 40°C	*(56)*
Peracetic Acid (CH_3COOOH)	0.13 mol/L pH 5.0, 6.5, 8.0, < 30 min	*Bacillus subtilis* at 10^6 CFU/mL		100% killed	*(4)*
Peracetic Acid CH_3COOOH)	0.39 mol/L pH 4.0 7.0, 9.0, 24hr	*Bacillus subtilis* 10 mL spore suspension (approx 10^6 CFU/mL)	Stainless steel.	100% killed	*(4)*
Formaldehyde (CH_2O)	4% in sterile distilled water, 2hr	*Bacillus anthracis,* 10^8 CFU/mL		10^4 inactivation factor	*(47)*

Table 2, cont.

Formaldehyde (CH_2O)	400 mg/m^3, 30% RH, 22 min	*Bacillus globigii* NCTC 10073 at 1 x 10^2 - 3 x10^8	Sterile paper disks	1 log (90%) reduction at 23.5-25°C	*(12)*
	280 mg/m^3, 50% RH, 31 min			1 log (90%) reduction at 23.5-25°C	
	250 mg/m^3, 80% RH, 16 min			1 log (90%) reduction at 23.5-25°C	
	400 mg/m^3, 98% RH, 9 min			1 log (90%) reduction at 23.5-25°C	
Sodium Hydroxide (NaOH) In combination with peroxygen cleaning systems	1% solution, 20, 30, 40°C Pre-treatment, followed by peroxygen containing disinfectants, 10, 20, 30 min exposure	10^7 spores/mL *Bacillus cereus* ATCC 9139	Cellulose nitrate filters	Sporicidal effect increased with increase temperature and time. 2 log reduction at 40°C, 30 min. Alone peroxygen cleaning systems were not effective sporicidal agents.	*(30)*
Sodium Hydroxide (NaOH)	5% solution, 27.8^{o}C, 1.5hr	7 x 10^9 CFU/mL *Bacillus subtilis*		99%	*(59)*
	5% solution, 21.1^{o}C, 3.6hr			99%	

Table 2, cont.

Free Available Chlorine	Mean free chlorine concentration: 2.0±0.2 mg/L Mean decay rate: 0.0012/min	1 x 10^4 CFU/mL *Bacillus anthracis* Ames and Sterne (34F2), *Bacillus cereus* ATCC 7039, and *Bacillus thuringiensis* subsp. *israelensis* ATCC 35646 23°C at pH 7.0	CT value (mg x min/liter) for in activation): only high log red. reported: *B. anthracis A:*3 log 102 *B. anthracis* S:; 4 log, 90 *B. cereus:* 4 log, 82 *B. thuringiensis*: 4 log, 132	*(41)*
		23°C at pH 8.0	*B. anthracis A:* ND *B anthracis S:* 4 log, 254 *B. cereus:* log 4, 264 *B. thuringiensis*: log 4, 492	
		5°C at pH 7.0	*B. anthracis A:* log 3, 210 *B anthracis S:* 4 log, 280 *B. cereus:* log 4, 233 *B. thuringiensis*: log 4, 458	
		5°C at pH 8.0	*B. anthracis A:* ND *B anthracis S:* 4 log, 637 *B. cereus:* log 4, 680 *B. thuringiensis*: log 4, 961	

Table 2, cont.

Nanoemulsion of BCTP (w/o nano-emulsion soybean oil, tri butyl phosphate, triton X-100, 80% water)	1:1000 of stock solution, 4 hr	*Bacillus anthracis,* and three surrogates: *Bacillus cereus, Bacillus circulans, Bacillus megaterium* spores	>90%, 1 log*	*(21)*
BCTP 401 (w/o nano-emulsion soybean oil, tri butyl phosphate, triton X-100, 80% water)	1:1000 of stock solution, <30 min		>90%, 1 log*	

*Results were obtained through animal models and/or histological studies

There are many commercially available chemical sanitizers that may have activity against *Bacillus anthracis*. These include hypochlorous acid (HOCl), phenolics, formaldehyde (5%), glutaraldehyde (2% at a pH of 8.0-8.5), hydrogen peroxide (3%), peracetic acid, and chlorine compounds. While most or all of these are available for use in the food and pharmaceutical industries, fewer are available in consumer products. There is little data on the efficacy against bacterial spores of household sanitizers or disinfectants or products that could act as potential sanitizers because of the presence of a sporicidal substance. Table 3 lists a number of household products currently available and their corresponding sanitizer concentrations and pH.

Table 3. Commercial Name, Manufacturer, Active Compound, Concentration, and pH of Household Cleaners

Commercial Name	**Manufacturer**	**Active Compound**	**Concentration**	**pH**
Clorox	Clorox Company, Oakland, CA	NaOCl	6.00%	10.8
Clorox Clean-Up w/ Bleach	Clorox Company, Oakland, CA	NaOCl	1.84%	12
Tilex Mold & Mildew Remover	Clorox Company, Oakland, CA	NaOCl	2.40%	12.2
Lysol All Purpose w/ Bleach	Reckitt Benckiser Inc., Parsippany, NJ	NaOCl	2.00%	12
Hydrogen Peroxide	Kroger	H_2O_2	3.00%	4.2
The Works Drain Opener	HomeCare Labs, Lawrenceville, GA	HCl	20.00%	0.5

Chlorination is currently the most widespread method of sanitization. Its uses include disinfection of water supplies, wastewater, recreational water and sanitization of food contact surfaces and food processing environments. Chlorine is both easy to handle, very inexpensive, soluble in water and stable over a long storage time *(48)*. In a water solution there is an equilibrium between hypochlorite ions (ClO^-) and hypochlorous acid (HOCl) depending upon pH. The ratio of these two forms is highly sensitive to pH and temperature. Hypochlorous acid is an oxidant that is generally responsible for antimicrobial activity. The effectiveness of hypochlorites in water is dependent on the concentration of undissociated hypochlorous acid, the amount of organic matter present, pH, and temperature. Hypochlorites are thought to act on cell membranes disrupting or changing their permeability, inhibiting transport, fragmenting proteins, interfering with ATP production, inactivating certain enzymes and potentially degrading DNA thereby inhibiting cell division *(5, 16)*. In a study conducted by Rose and others *(45)* it was found that inactivation of *B. anthracis* spores could not be achieved at the levels of chlorine utilized currently in municipal water systems and there were slight differences in resistance between the Sterne and Ames strains used *(45)*.

Chlorine dioxide (ClO_2) is commonly used for disinfection of water and wastewater treatment. Chlorine dioxide is extremely reactive and unable to be produced in bulk, furthermore, is necessary to be prepared at the site of use *(48)*. Although more portable and less costly systems that generate chlorine dioxide have become more available, none are currently available at the consumer level. Therefore, the use of this particular sanitizing strategy at the consumer level in the event of widespread dissemination of a biological agent

would be both cost-prohibitive and impractical at this time. In comparison to hypochlorites, chlorine dioxide has the ability to degrade phenolic compounds, does not react with ammonia, and its bactericidal efficiency is less affected by pH and organic matter *(7, 42)*. The oxidation capacity of chlorine dioxide is about 2.5 times greater than hypochlorites. The mode of action for oxidizing compounds is to cause irreversible damage to phosphate groups located on fatty acids found in cell membranes or enzymes in microorganisms. Greater sporicidal activity has been shown by Ridenour et al. *(42)* and is thought to be explained by greater utilization of its oxidation capacity. Additionally, the use of concentrations up to 200 ppm of chlorine dioxide in water solutions is allowed by the Food and Drug Administration (FDA) in order to sanitize fruits or vegetables without necessitating a final rinsing step *(2)*. It was chlorine dioxide gas that was selected for the decontamination of government buildings after 2001 *B. anthracis* attacks. Cleanups at a Senate office building, and other contaminated facilities in the wake of 2001 attacks showed that decontamination of such sites, while possible, is both time-consuming and costly. Decontamination of the Senate office building cost $27 million, according to the Government Accountability Office. Cleaning the Brentwood postal facility outside Washington cost $130 million and took 26 months *(33)*.

Hydrogen peroxide is a substance found naturally at low concentrations in foods, such as milk and honey, as a result of normal cellular metabolism. It is also found in the mucous membrane of the oral cavity of animals and may function as an antimicrobial in the event that microorganisms are able to gain access to the bloodstream. The mechanism of antimicrobial activity of hydrogen peroxide has been suggested to include a series of enzymatic processes

that produce the hydroxyl radical, an oxidant which can attack membrane lipids, DNA and other cell components thereby inactivating bacteria *(48)*.

Sapers and others *(51)* investigated the decontamination of apples and cantaloupes containing *E. coli* O157:H7 utilizing 1% and 5% solutions of hydrogen peroxide. A population reduction approaching 4 log CFU/g was observed with a 5% rinse applied in conjunction with other treatment factors such as heat and agitation to apples. The 1% hydrogen peroxide treatment was found to be ineffective on cantaloupe melon rinds.

While hydrogen peroxide is Generally Recognized as Safe (GRAS), current regulations do not permit its use as a rinsing agent (21CFR184.1366). The Environmental Protection Agency (EPA) however, now allows ≤ 1% hydrogen peroxide to be applied to post harvest agricultural food (40CFR180.1197). The methodology used to apply these rinses was designed to be compatible with a fruit processing operation in which a wet dunk tank was used. While this may be practical in an industrial setting, the potential presence of pathogens remaining in the dunk tank post-application was not evaluated. If a consumer were to use this method, it has not yet been determined if true inactivation of the pathogen would occur or if it would remain viable afterwards on surface or in cleaning solutions. In addition, this method has not been studied using more resistant bacterial spores or using consumer grade hydrogen peroxide (3%) which is reported to be less stable *(48)*.

Although it is known that both strong alkali and strongly acidic solutions generally possess sporicidal capabilities, the mechanism by which this is achieved is not well understood in relation to *Bacillus* species. It has been proposed by Beuchat and others *(7)* that *Bacillus*

spores treated with strong acids release dipicolinic acid (DPA) while spores killed by alkali solution do not appear to release DPA until initiation of germination and degrade their spore cortex poorly. Furthermore, spores treated by alkali solution do not initiate metabolism. Thus, it may be that lethality caused by acid involves disruption of a spore permeability barrier while lethality attributed to the action of alkali is more related to the inactivation of certain lytic enzymes *(52)*. This supports the findings of Kulikovsky and others *(29)* where they observed that the lethality of *B. cereus* spores treated with 5% NaOH was in fact likely caused by modification of the outer spore coats and a disruption of permeability barriers of the spore *(29, 52)*.

A review of household sanitizers available to consumers and commercial sanitizers reveals that further research is needed in relation to their activity on specific food matrices in order to better understand their potential application in consumer food defense strategies.

Surrogate Species

To safely characterize and study the mechanisms of inactivation for spores of *B. anthracis,* researchers have often employed the use of a surrogate *Bacillus* species. To study pathogenic *Bacillus anthracis*, requires the use of a biosafety level 3 laboratory *(11)*. A surrogate may be defined as a microorganism that is non-pathogenic that behaves similarly when exposed to the same conditions. The distinguishing features of *B. anthracis* is that it is the causative agent of anthrax disease, it is a facultatively anaerobic, gram positive, non-motile, rod shaped bacteria. It is known that *B. anthracis* is closely related genetically to both *B. cereus* and

B. thuringiensis. To safely characterize and study the mechanisms of inactivation for spores of *B. anthracis,* researchers have often studied the more prevalent less toxic foodborne pathogen, *Bacillus cereus*.

Bacillus cereus is widespread in the environment and like *B. anthracis* it is a facultatively anaerobic, rod-shaped gram positive bacterium. Unlike its more virulent counterpart, *B. cereus* produces a hemolytic enterotoxin, is motile, and is not susceptible to penicillin. De Siano and others *(14)* compared growth characteristics of *B. anthracis* and *B. cereus* in brain heart infusion broth. All strains of *B. anthracis* and *B. cereus* demonstrated increased lag time and decreased cell density with decreasing pH. In contrast, the *B. cereus* strains had a longer generation time and a shorter lag time when compared to the virulent strains of *B. anthracis (14)*. The two microorganisms have similarities in spore coat proteins *(7, 14, 26)*. One of the principle differences between the species is the presence of the virulence genes on the plasmid pOX2 on *B. anthracis* that is lacking in other *Bacillus* species. Genomic comparisons of *Bacillus* species have revealed that these bacteria have highly conserved regions of similarity in 16S and 23S rRNA sequences. These sequences are often compared to establish a relatively recent evolutionary link between species *(25)*.

Sagripanti and others *(49)* compared the relative sensitivity to disinfecting agents of different strains of *Bacillus anthracis* and spores of other *Bacillus* species on different surfaces. Five virulent and three attenuated strains of *B. anthracis, B. atrophaeus, B. cereus*, *B. thuringiensis* and *B. megaterium* spores were deposited on glass, metal, or polymeric surfaces and were treated with peroxides, chlorine or other oxidants. All strains behaved similarly and

were inactivated to within 1 log among the 12 different *Bacillus* strains tested indicating similar sporicidal sensitivities between *Bacillus* species *(49)*. Thus, while some differences have been observed between virulent strains of *B. anthracis* and its potential surrogate *B. cereus*, the latter appears to be an acceptable surrogate based on genetic similarities and overall growth kinetics.

Produce and Surface Decontamination Factors

In 1997, cantaloupes were reportedly the most purchased melon in the United States accounting for half of the $836 million melon crop *(31)*. Per capita use of fresh-market spinach averaged 2.2 pounds during 2004-06 which was the highest since the mid-1940s. The value of fresh market spinach has more than doubled over the past decade and accounts for 94% of the $175 million valued at from 2004-2006 *(32)*. Because these are such popular produce items, they are potential targets for dissemination of biological terrorism agents such as *B. anthracis* spores. Additionally, both cantaloupe melon rind and leaf spinach provide surfaces that are challenging for application of sanitizing treatments.

The difficulty in sanitizing cantaloupe has been demonstrated in a number of studies. Park and others *(40)* found that a 2000 ppm chlorine dip reduced inoculated *Salmonella* by 1 log or less on the mesocarp of cantaloupe melon. They believed that low level of lethality was due in part to the high levels of organic material in the mesocarp of the cantaloupe which may have lead to neutralization of free chlorine prior to lethality *(40)*.

It has been shown that the spore of *B. cereus* and other *Bacillus* species are hydrophobic and in many cases possess pili or appendages that will favor attachment to a variety of surfaces. It is these same characteristics that may contribute to difficulty in removal of spores from solid surfaces and overall resistance to chemical, pH, and temperature related lethality treatments *(25)*. Ukuku and others *(57)* found a correlation between the negative surface charge and hydrophobicity of several bacterial pathogens and the strength of their attachment to cantaloupe rind surfaces. Additionally, it was noted in the same study by Ukuku, that the rind itself, with its rough texture and irregular surface favored bacterial cell attachment thereby reducing the effectiveness of sanitizing treatments *(57)*. The surface characteristics of the produce may also affect bacterial adhesion. For example, when materials possess pits and cavities on surfaces, as is observed in both cantaloupe and other living surfaces, spores are more likely to be able to penetrate and embed themselves in these areas affording themselves partial if not complete protection from inactivation treatments *(44)*. Reduced inactivation may also be in part due to the fact that the sanitizer may be neutralized by organic matter before it comes in contact with spores. This was supported by the finding of Sapers and Sites *(51)* who theorized that part of the variability in replication of inactivation studies of microorganisms on cantaloupe melons was due to differences between the aforementioned surface conditions of individual melons, the potential presence of bacteria in biofilms, and/or existing organic materials present prior to treatment. Sapers and Sites also suggested that the biofilms on produce surfaces are likely more difficult to remove from produce surface compared to other non-biological surfaces *(51)*. Biofilms are stable, three dimensional networks of living cells and

an inert network *(9)*. It has been well documented that bacterial cells on surfaces in a biofilm are far more resistant to toxic substances than their free-living or planktonic counterparts *(18)*.

The need to understand, recognize and deal with purposely added pathogenic biological agents not usually associated with foods in order to protect consumers is of the utmost importance. In the event that a pathogenic agent, such as *B. anthracis*, were distributed on food at the consumer level there is some question as to what options would be open to consumers and authorities to deal with those contaminated products. Disposal in solid waste facilities or municipal wastewater systems has the potential to overload such systems with the infectious agent. One strategy that may facilitate in protecting both consumers and our waste treatment systems is partial decontamination in the home. Therefore, the objective of this study was to determine the efficacy of commercial consumer products with antibacterial ingredients for inactivating *Bacillus* spores on surfaces of cantaloupe and on spinach leaves.

CHAPTER II

Materials and Methods

Preparation of Spores and Inoculum

Bacillus cereus ATCC 33018 and ATCC 49064 were obtained from American Type Culture Collection, Rockville, MD. Cultures were grown aerobically without agitation in brain heart infusion broth (BHI; Difco, Sparks, MD) for 24 h at 35°C for two consecutive transfers prior to spore preparation.

Spores were prepared using a previously published method with modification *(26)*. Briefly, this involved spread plating cells of both strains onto brain heart infusion agar (BHIA) containing 40 µg/ml manganese sulfate monohydrate (Sigma-Aldrich, St. Louis, MO) and 100 µg/ml calcium chloride dihydrate (Sigma) in order to induce sporulation. Following inoculation, plates were incubated aerobically at 35°C for up to 5 days to allow sporulation, the progress of which was monitored using phase contrast microscopy. Once sporulation was approximately 90%, the bacterial lawn was aseptically removed from the plate by scraping. The lawn was mixed with 10 ml of sterile distilled deionized (DI) water containing 1.0 mg/ml lysozyme (Sigma) in a 50 ml centrifuge tube (VWR, West Chester, PA). The lysozyme was used to ensure lysis of remaining vegetative cells. Tubes were held for 5 min at room temperature and centrifuged for 15 min at 10,000 x g in a Biofuge 17R centrifuge (Baxter Scientific Products, West Chester, PA). Following centrifugation, supernatants were discarded and the spore pellet resuspended in 10

ml of sterile, distilled DI water; the process was repeated three times. Final spore pellets were suspended at ca. 8 log CFU/ml in 10 ml sterile distilled DI water and 10 ml 95% ethanol (Sigma) for storage at 4°C prior to use.

Spore suspensions were agitated to resuspend and 0.7 ml of each strain were mixed in a sterile centrifuge tube. The tubes were then centrifuged for 5 min at 10,000 x g at 4°C . The supernatant was removed and the pellet was resuspended in 1.4 ml of sterile 0.1% peptone water in order to maintain 8 log CFU/ml in final spore suspension utilized in inoculation of produce.

Preparation of Neutralizer Buffers

Phosphate buffer was made using 34.0 g of KH_2PO_4 (Fisher Scientific, Fairlawn, NJ) dissolved in 500 ml DI water , adjusted to pH 7.2 with 1 N NaOH (Sigma), and then diluted to 1 l in DI water. 1.25 ml of 0.25 M phosphate buffer stock solution was added to 1 l DI water, dispensed into appropriate volumes as needed and sterilized (121°C, 15 min).

A neutralizer buffer was prepared for use in stopping the lethal action of the sanitizing agents at specified time points during each treatment. The neutralizer stock solution consisted of 40 g lecithin (Sigma), 280 ml polysorbate 80 (Tween 80) (Sigma), and 1.25 ml phosphate buffer stock (PB; pH=7.2) diluted with DI water to 1 liter and adjusted to pH 7.2 with 1.0 N NaOH (Fisher). Neutralizer working blanks (99 ml) were made by adding 100 ml of neutralizer stock solution, 25 ml of 0.25 M phosphate buffer stock, and 1675 ml of DI water; 99 ml blanks were sterilized by autoclaving (121°C, 15 min) and used within 14 days. For disinfectants containing hypochlorite, the standard neutralizer buffer solution was modified by the addition

of sodium thiosulfate (6 g/L; Sigma). The solution was brought to 99 ml for use in melon studies and 30 ml for use in spinach, dispensed into dilution bottles, capped, and sterilized prior to use (121°C, 15 min).

For hydrogen peroxide, catalase (source: *Micrococcus lysodeikticus*, Sigma) was added to the neutralizer buffer at 10,000 IU/ml and filter sterilized through a 0.2 µm cellulose acetate filter (Corning Inc., Corning, NY). The solution was then brought to volume using phosphate buffer. Sterile dilution bottles were prepared as above.

The neutralizer was shown to be effective in stopping the inactivation of all of the compounds tested against *B. cereus* spore suspensions in previous studies done in vitro.

Preparation and Inoculation of Produce

Cantaloupe melons and spinach were obtained from a local retail market. Cantaloupe melons free of or with the least visible blemishes and dirt were selected. The same brand of bagged spinach obtained at local retain store and was used throughout the study. Cantaloupe melons were rinsed upon receipt with sterile DI water in order to remove any dirt or debris on the rind surface. Melons were then placed into sterile stomacher bags and set aside at 25°C until use. Bagged spinach was maintained at 4°C until use. All produce was purchased the same day that it was used. Experiments were conducted during the during the spring, summer and fall of 2007 and winter of 2008, thus, it is likely that the melons and spinach were likely to have originated from several growing locations and growing conditions.

Inoculation of Produce

Initially, 25cm² sections of cantaloupe rinds were measured and marked utilizing a sterilized ruler. Cantaloupe rinds were then cut and removed and trimmed to remove the mesocarp using a sterile scalpel. Cut melon rinds were placed into sterile 100 mm diameter Petri dishes in a class II biological safety cabinet (Fisher Scientific; Pittsburgh, PA) until all samples were prepared. Similarly, spinach leaves were removed from the sealed bags, selected for uniform size and lack of visible blemishes and placed onto sterile 100mm diameter Petri dishes in a class II biological safety cabinet until all samples were ready to inoculate. Because of the potential of melon-to-melon and bagged spinach variability in microbiological quality and associated variance potentially produced, strict attention was paid to minimize these differences. Scalpel blades were resterilized using a solution of 95% ethanol and flamed over a Bunsen burner. In addition, to prevent cross contamination during sample preparation and treatment, all knives, cutting boards, and equipment were either sterilized using the aforementioned procedure or separate pieces of sterilized equipment were used across treatments.

Sections of untreated cantaloupe rind and untreated spinach leaf were set aside and used to determine background microflora present. Sections of produce were placed into a sterile filter stomacher bag (Fisher) using sterile forceps or a sterile spatula with 99 mL of the appropriate neutralizer buffer. Samples were then pummeled in a stomacher blender (Stomacher 400; Seward, England) for 2 min at 230 rpm and allowed to rest for 1 min. Pummeled suspensions were then serially diluted in 0.1% peptone water and spread plated on

solidified brain heart infusion agar (BHIA; Difco) plates. Plates were incubated for 24 h at 35°C before enumeration. One or two presumptive colonies were picked aseptically, stained with crystal violet and observed for cell morphology.

For inoculation of produce treated with sanitizers, sections were randomly spot inoculated with ten 10 µl aliquots across the produce surface to achieve a total inoculum of 0.1ml, or approximately 8 logs/25 cm^2. Immediately after produce was inoculated, Petri dish lids were replaced but propped open to allow movement of air and the dishes placed at room temperature (approximately 25°C) biological safety cabinet for 30 min to dry and allow for spore attachment prior to application of treatments.

Exposure of Inoculated Produce to Household Sanitizers

Sanitizer compounds were purchased in a retail market and chosen based on use-type (cleaner, disinfectant, etc.) and anti-microbial specification; pH measurements were taken on each full strength compound. Previous *in vitro* studies were used so that only compounds effecting a reduction were utilized in these studies. Compounds in pour bottles were transferred to sterile spray bottles and the nozzle adjusted so that each bottle would deliver 5 ml of sanitizer compound with 3 sprays. The compounds studied are listed with their manufacturer, active ingredient, concentration and pH in Table 3.

Retention of Sporicidal Activity of Household Bleach Cleaners

In order to determine whether household chlorine-containing cleaners that were past their manufacturer-reported expiration date were still able to inactivate spores, bleach

products containing 6.0% NaOCl were purchased with reported expiration dates of 11 November 2006, 6 July 2006, and 30 May 2007. The three products were then tested simultaneously for their ability to inactivate target spores *in vitro* and in milk.

Recovery of *Bacillus cereus* spores

Non-selective brain heart infusion agar (BHIA; Difco) was utilized for enumeration of all samples to ensure complete counts of recovered bacteria were obtained. No selective agents were used to ensure that injured cells were included in final enumeration.

Inoculated cantaloupe melon pieces exposed to commercial sanitizer for specified time were first removed from the incubator where they were held at 25°C. Melon pieces were then aseptically removed from Petri dishes using sterile forceps or a sterile spatula as needed and placed into a sterile filter stomacher bag (Fisher) with 99mL of neutralizer buffer appropriate to sanitizer being utilized. Samples were then pummeled in a stomacher blender (Stomacher 400; Seward, England) for 2 min at 230rpm and allowed to rest for 1 min. Pummeled suspensions were then serially diluted in 0.1% peptone water and spread plated on solidified BHIA plates prepared one day prior. Plates were incubated for 24 h at 35°C before enumeration of *B. cereus.* One or two presumptive *B. cereus* colonies were picked aseptically, stained and observed for cell morphology.

Inoculated spinach leaves exposed to commercial sanitizer for specified time were first removed from the incubator where they were held at 25°C. Spinach leaves were then aseptically removed from Petri dishes using sterile forceps or a sterile spatula as needed and placed into a sterile filter stomacher bag (Fisher) with 30mL of neutralizer buffer appropriate to

sanitizer being utilized. Samples were then pummeled in a stomacher blender (Stomacher 50) for one minutes at 230 rpm and allowed to rest for 1 one minute. Pummeled suspensions were then serially diluted in 0.1% peptone water and spread plated on solidified BHIA plates one day prior. Plates were incubated for 24 h at 35°C before enumeration of *B. cereus.* One or two presumptive B. cereus colonies were picked aseptically, stained and observed for cell morphology.

Data Analysis

All experiments were done in replicate three times. The statistical model consisted of a random block design, blocking on replication. Statistical analysis was conducted using the mixed models procedure (PROC MIXED) of SAS® 8.2 (SAS Institute Inc.; Cary, NC) and significance of factors set at $P<0.05$. Analysis of variance was used to determine differences in the survival of *B. cereus* on different food-contact surfaces. Analysis of variance ($P<0.05$) was conducted with SAS 8.2 and least squares means were compared by compound, with concentration and time points as fixed factors. Control population measurements were determined from untreated spore suspensions and from untreated pieces of inoculated produce.

CHAPTER III

Results and Discussion

Studies were conducted to test the efficacy of several common household products containing antimicrobial compounds to inactivate spores of *Bacillus cereus* (as a surrogate for *B. anthracis*) on fresh fruit and vegetable surfaces. Additionally, the effect of storage time on the utility of consumer products containing hypochlorite in this type of decontamination strategy was evaluated. The overall objective of this research project was to develop practical methods for decontamination of a potential bioterrorism agent on a solid food matrix at the consumer level using products readily available to the consumer. A related objective was to develop specific guidelines for consumers for handling food products that are potentially contaminated with infectious microbial agents.

Background microflora on the produce was enumerated, selected colonies picked and stained, and morphology determined for both cantaloupe melon rind and spinach leaves. On the cantaloupe and spinach, the background microflora levels were 2.36 log CFU/piece and 2.1 log CFU/leaf, respectively. This is lower than indigenous populations reported on produce surfaces reported in other studies which ranged from 2.5 to 3.0 Log CFU/g *(17, 20, 21)*). It is possible that the produce used in this study was exposed to some sort of rinse or sanitizing treatment prior to being offered for sale at the retail consumer level. Because the background microflora count was low and the primary goal of this study was decontamination rather than preservation of the produce, higher concentrations of sanitizer and longer exposure times were

utilized. Thus, in general most of the background microflora, predominately yeasts and molds, was eliminated at the initiation of testing and should not interfere with tests for the much more resistant *Bacillus cereus* spores. With respect to effect of storage on sporicidal activity of HOCl-containing products, samples stored up to 1 year past expiration were compared with those stored for 6 months prior to expiration and purchased fresh. Cantaloupe rind and spinach leaves were treated with HOCl-containing products of the various storage times. *B. cereus* spores were inactivated to below the level of detection (>5.84 log CFU/cm^2, >5.15 log CFU/cm^2) in 10 min and 60 min for spinach and cantaloupe respectively, regardless of compound age. These results were confirmed by Black et al. using *in vitro* inactivation tests with hypochlorites against *B. cereus* spores *(9)*. They found no significant difference in the *in vitro* inactivation of *B. cereus* spores among the products. *B. cereus* spores were inactivated to below detectable levels after 1 min in 50% and 25% commercial strength solutions (>4.0 log CFU/ml). Thus, it is unlikely that the age of sanitizers themselves plays an appreciable role in the efficacy of treatments at least in regards to chlorine-containing compounds.

The overall log reduction of viable *Bacillus cereus* spores after 180 min exposure for all compounds on cantaloupe surfaces is shown in Table 4. For the cantaloupe melon rind the control population mean was 7.15 $\pm$ 0.07 log CFU/cm^2. The log reduction on melon rind was greatest for undiluted sodium hypochlorite (Clorox®, 6.00% NaOCl). For the 1.84%-2.40% NaOCl-containing compounds, log reductions were between 2.75 and 3.40 over 180 min. Additionally, a 3.66 log CFU/cm2 reduction was noted in the sanitizer containing 20.% HCl. The kinetics of spore reduction on cantaloupe surfaces are shown in Fig. 1. Full strength Clorox® bleach was the

only product to reduce the spores to less than detectable level after 60 min. Products containing 1.84% -2.40% NaOCl (1.84-2.40%) showed an initial drop between 5-10 min followed by a plateau in the rate of reduction through 180 min. It is likely that lower reductions seen with reduced concentration NaOCl-containing products were a function of that lower concentration of NaOCl. Similarily, the efficacy observed in the HCl-containing sanitizer may have been due to the very high concentrations (20.0%) and resultant low pH (0.5) of the product. Additionally it is possible that the complex matrix and microstructure of the netting of the rind of the cantaloupe which has been shown to favor bacterial attachment further contributed to lower reductions observed in these products *(44, 51, 57).*

For spinach, the control population mean was 7.37 $\pm$ 0.01 log CFU/leaf. The overall log reduction of viable *Bacillus cereus* spores after 180 min exposure for all compounds on spinach surfaces is shown in Table 4. A greater than 5.84 log reduction in *B. cereus* spores was found for both Clorox® bleach and The Works Drain Opener (20.00% HCl). A 4.23 to 4.60 log reduction was seen with the 1.84%-2.40% sodium hypochlorite-containing products after 180 min. The kinetics of spore reduction on spinach leaves is shown in Fig. 2. As observed on the cantaloupe melon rind, the sharpest reduction was initially observed on treated spinach leaves treated with Clorox® bleach (6.00% NaOCl) and The Works Drain Opener (20.00% HCl). However,the former reduced the viable spore population to the lowest detection level in 10 to 60 min while the latter took approximately 3 h (Fig. 2). While final reductions and time to final counts was variable across all NaOCl-containing treatments, the same sharp initial drop was observed followed by a tailing effect. As discussed above, sanitizer concentration likely plays a role in the

final reductions observed. It has also been suggested by some that the efficacy of some sanitizers may be influenced by the presence of organic materials or the formation of biofilms. However, unlike cantaloupe rinds, fewer crevices and pits exist on the surface of spinach so it may not play as great a role in the tailing kinetics observed *(8, 51)*. The tailing effect was not seen with the HCl-containing product, the activity of which would not likely be as susceptible to organic matter as NaOCl.

Although a review of the literature and initial *in vitro* testing suggested that hydrogen peroxide might be an acceptable consumer product for use in decontamination *(8)*; its sporicidal capabilities were non-existent on solid food matrices. 3% Hydrogen peroxide were also evaluated in earlier studies both *in vitro* and in milk and were shown to have the least effective sporicidal capabilities of all the solutions tested reducing the population of *B. cereus* by less than 1 log. Based on the findings of Rogers and others *(44)* with a hydrogen peroxide generator, more success might be gained if gaseous treatments were applied to food surfaces.

In most cases, the produce surfaces had an negative impact on the activity of the various sanitizing treatments utilized in this study. As suggest above, it is likely that these differences were due in part to the produce surface, rougher in the case of the melons and waxier in the case of the spinach. The netted cantaloupe melon rind surface provides numerous sites for microorganisms and other contaminants to attach and evade sanitizing treatments. Sanitizing treatments in general were more apt to stay on spinach leaves for the duration of the treatment due to the decreased natural curvature of the spinach leaf. However, the spinach leaf had a tendancy to break down with longer treatment times and tended to disintegrate over

90 to 120 min of treatment. After spinach leaves were exposed to sanitizers beyond 120 min, they were completely degraded and not able to be handled for neutralization or plating so no data is recorded beyond this time point. This was particularly evident for leaves exposed to 6.0% NaOCl or 20.00% HCl. However, since maximum log reductions with these treatments was obtained before the leaf disintegrated (Table 4), this was not of primary concern.

Sapers and Sites *(51)* theorized that some of the variability in response of microorganisms on cantaloupe melon observed in separate repetitions was due to differences between individual melons, surface conditions, and presence of bacteria in biofilms in existence prior to treatment. Additionally, it was suggested by Rose and others *(45)* that biofilms on produce surfaces are likely present and may interfere with the sanitizing ability of chlorine. Sapers and Sites *(51)* found that biofilms are difficult if not impossible to remove completely from produce surface compared to other non-biological surfaces without destroying the biological surface itself though they were unable to confirm this through experimentation. In a study by Rogers et al. *(44)*, the efficacy of hydrogen peroxide gas to decontaminate porous wood surfaces was tested, which might be similar to the porous food surfaces used in the present study. The results indicated that, while the hydrogen peroxide gas significantly reduced viable spores on various indoor surfaces, the efficacy was greatly affected by the porosity of the material itself. They too found greater levels of variation with more porous materials. This poses a problem for decontamination of buildings as well as many types of contaminated foods. With materials possessing surfaces such as pits and crevices, as is observed in both cantaloupe and wood surfaces, spores are more likely to be able to penetrate and embed themselves in

these areas affording themselves partial if not complete protection from inactivation treatments *(44)*.

In addition to the influence of the product on spore inactivation, the bacterial spore coat itself contributes to resistance to inactivation treatments of any kind. Setlow and Setlow *(53)* found that in the spore coat of *Bacillus* species is a group of small, acid soluble proteins of the α/β type that are generally believed to be highly conserved and may play a role in the resistance to heat and certain sanitizing treatments, especially hydrogen peroxide. Its primarily function is simply to protect the DNA of the spore *(54)*. Nicholson et al. *(38)* suggested that spores may be resistant to certain to chemicals due in part to the following factors: impermeability of the spore coat to hydrophilic substances, low spore core water, protection of DNA by small acid soluble proteins, and due to the existence of the spore coat itself. Indeed, variability in resistance may also be due to the origin of the strains in that laboratory strains may or may not contain the same genomic information as certain wild type strains *(38)*. It is likely that these factors contributed to the requirement for the higher concentrations of sanitizers necessary to inactivate the *Bacillus cereus*..

In conclusion, two groups of commercial home-use disinfectant-sanitizer products were identified that have excellent potential for use as sporicidal agents for decontamination of foods or surfaces. Products containing NaOCl or HCl could be used by consumers to decontaminate food products that have been intentionally contaminated in a bioterrorism event so that they may be safer for disposal and handling. Development of safe decontamination procedures in the home will assist in preventing the spread of high

concentrations of pathogenic microorganisms in the solid waste or wastewater systems. It was also shown that the most effective product, 6.0% NaOCl, could be stored for at least 6 months past expiration without loss of activity.

In the event of a bioterrorist act involving foods at the consumer level of distribution, the findings of this study could be used to formulate guidelines for at least partial decontamination at the consumer level prior to disposal, thereby mitigating the overall threat posed to the community at large. Additionally information from this study could be developed into a plan for distribution to homeowners as to what products they need in a home security kit and procedures for handling those products in a bioterrorism event. This information should not be confused with a method to decontaminate food with household chemicals with the intended purposes of consuming those foods at a later date. For the purposes of this study, decontamination of food would hypothetically only take place to mitigate risk and decrease the microbiological contamination on those food surfaces and should not be interpreted as a recommendation to handle or consume food known to be contaminated with known biological or chemical agents.

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Appendix

Table 4. Mean Log reductions observed on cantaloupe and spinach following treatment with full strength sanitizer products in 180 min.

Commercial Sanitizer Name	Active Compound	Conc %	Mean Log Reduction[a]	
			Cantaloupe Melon (log CFU/cm^2)	Spinach (log CFU/leaf)
Clorox	NaOCl	6.00	>5.15[b]	>5.84[b]
Clorox Clean-Up w/ Bleach	NaOCl	1.84	3.23[cd]	4.4[c]
Tilex Mold & Mildew Remover	NaOCl	2.40	2.75[b]	4.23[cd]
Lysol All Purpose w/ Bleach	NaOCl	2.00	3.4[cd]	3.6[d]
The Works Drain Opener	HCl	20.0	3.66[cd]	>5.84[b]
Hydrogen Peroxide	H_2O_2	3.00	0	0

[a]Initial counts: cantaloupe - 7.15 ± 0.07 log CFU/cm^2; spinach - 7.37 ± 0.01 log CFU/leaf

[b-d] Means within a column that are followed by the same letter(s) are not significantly different (P>0.05); n = 6 (duplicate samples, 3 replications).

Figure 1. Inactivation of *Bacillus cereus* on the surface of cantaloupe mesocarp by various commercial products containing antimicrobial compounds applied at full strength.

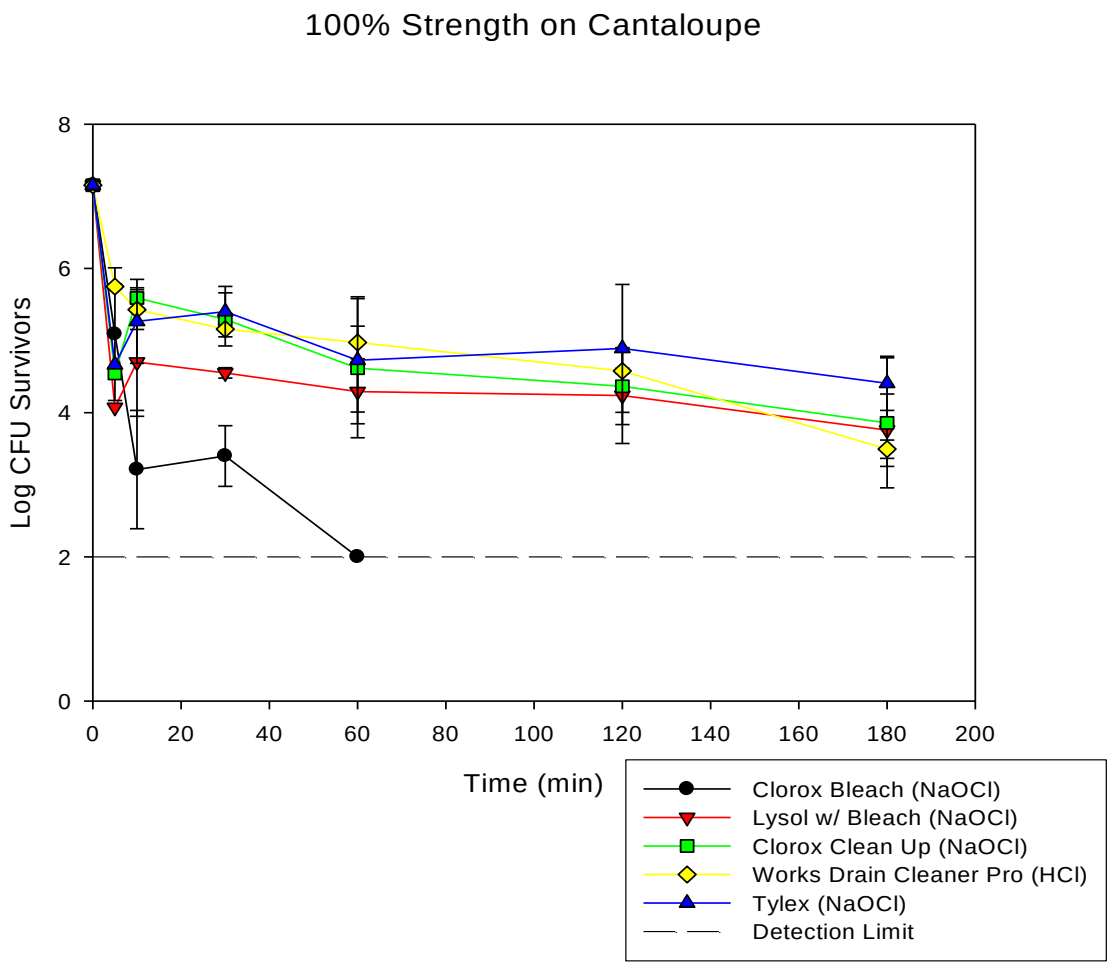
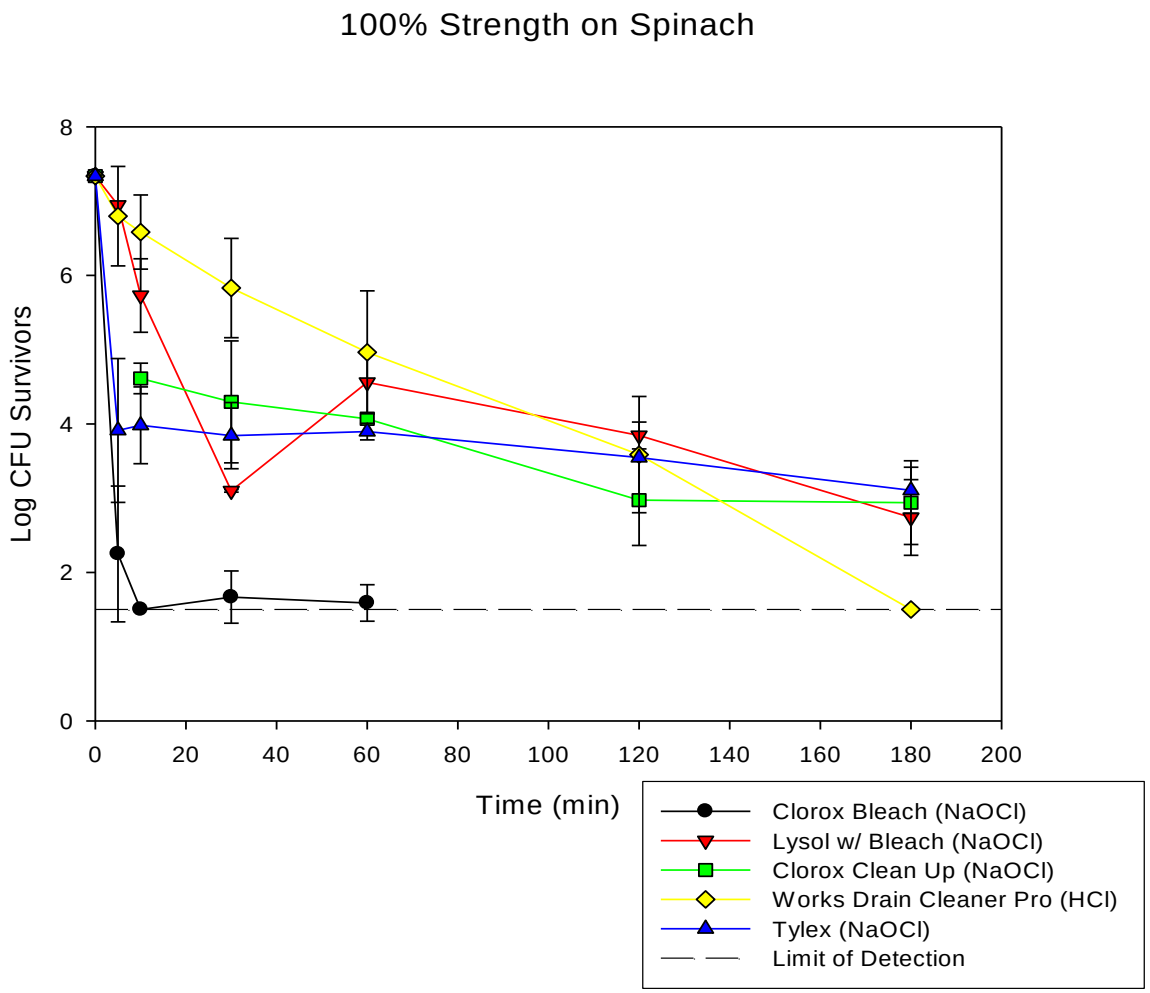


Figure 2. Inactivation of *Bacillus cereus* on the surface of spinach leaves by various commercial products containing antimicrobial compounds applied at full strength.



SAS Commands

Analysis of variance was used to determine differences in the survival of *B. cereus* on different food-contact surfaces. Analysis of variance (P<0.05) was conducted with SAS 8.2 and least squares means were compared by compound, with concentration and time points as fixed factors. Tukey adjustment was added to calculations due to its appropriate power and experiment-wise error control thus controlling type I error.

SAS programming: *B. cereus* ATCC 33018 and ATCC 49064 exposed cantaloupe melon rind recovered on BHI per treatment rep at each time

```
data melon;
input time $ CFU $ rep;
datalines;
%include 'C:\Documents and Settings\Helyn\571\sas macros\danda.sas';


%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

SAS programming: *B. cereus* ATCC 33018 and ATCC 49064 exposed spinach leaf recovered on BHI per treatment rep at each time

```
data spin;
input time $ CFU $ rep;
datalines;
%include 'C:\Documents and Settings\Helyn\571\sas macros\danda.sas';


%mmaov(one, CFU, class=rep time, fixed=time, random=rep, adjust=tukey);
```

Vita

Helen Kerr was born in St. Paul, Minnesota on October 27th, 1980 to parents Jo Anne Minter and David Kerr. Helen grew up in the Twin Cities area with her older siblings Mara and Caryn Jacob. She graduated from Convent of the Visitation School for Girls in 1999. She then moved onto the University of Wisconsin in Madison where she received a B.S. degree in Animal Sciences. In 2004, she began working for Organic Valley Family of Farms in La Farge, WI in their research and development department focusing on new product development. In August of 2006 Helen continued her education at the University of Tennessee at Knoxville where she began the M.S. program in Food Science focusing in Food Microbiology and graduated from this program in May, 2009. Helen is continuing her education at the University of Tennessee at Knoxville pursuing her doctorate in Food Science and Technology focusing in Food Microbiology.