Efficacy of a Food-Grade Mixture of Volatile Compounds to Reduce Salmonella Levels on Food Contact Surfaces

by

Laurie Leveille

(Under the Direction of Mark Harrison)

Abstract

A novel antimicrobial solution called Flavorzon was evaluated for its ability to reduce Salmonella contamination levels on food contact surfaces. The food contact surfaces evaluated include stainless steel, HDPE cutting board, PVC conveyor belt, and HDPE tote plastic, which were inoculated by spot inoculation or by growing attached cells on the coupons for 48 h. The effectiveness of Flavorzon was evaluated by comparing it to other common sanitizers including chlorine, peracetic acid, and quaternary ammonium compounds. There was no difference in the effectiveness of Flavorzon compared to the other common sanitizers. All sanitizers were more effective on spot inoculated cells than on attached cells. The food contact surface tested also affected the ability of sanitizers to reduce Salmonella. Flavorzon shows potential to be used as a natural alternative to other common sanitizers.

Index Words: Food contact surfaces, Salmonella, sanitizers, natural antimicrobial
EFFICACY OF A GOOD-GRADE MIXTURE OF VOLATILE COMPOUNDS TO REDUCE 
*SALMONELLA* LEVELS ON FOOD CONTACT SURFACES

by

LAURIE LEVEILLE

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LAURIE LEVEILLE

Major Professor: Mark A. Harrison
Committee: Jose Reyes De Corcuera
William Kerr

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this to my mom, dad, stepmom, and stepdad. I am so lucky to have four role models who have helped me become the person I am today, and four people always cheering for my success. You have all been so incredibly supportive throughout my entire college career, and I could not have done this without you.
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CHAPTER 1

INTRODUCTION

Decreasing the incidence of foodborne illnesses is a major goal in the U.S. and worldwide. The U.S. Food and Drug Administration (FDA) aims to reduce the number of Salmonella foodborne illness cases in the U.S. from an average of 15.0 cases per 100,000 people per year in 2006-08 to 11.4 cases per 100,000 people per year in 2020 (41). Cross-contamination from food contact surfaces is a major cause of foodborne illness, including that caused by Salmonella. In 2008, only 58.3% of foodservice operations properly cleaned and sanitized food contact surfaces, thus one of the tactics for achieving this goal is to increase the proportion of foodservice operations where food contact surfaces are properly cleaned and sanitized to 64.1% by 2020 (41). Cross-contamination can occur almost immediately upon a food product touching a contaminated surface, so surfaces must be thoroughly cleaned and sanitized (14). If cells present on a surface become attached, they can persist in the facility for years, creating risk to all products produced in that facility during that time (48).

A novel sanitizer called Flavorzon was evaluated in this effort to reduce Salmonella contamination levels on food contact surfaces. Flavorzon is based on the volatile substances released from an endophytic fungus, Muscodor crispans. The volatile compounds released from Muscodor crispans were identified by gas chromatography/mass spectrometry (GC/MS) and re-combined in similar proportions to create the commercial product Flavorzon (36). Flavorzon is considered a natural product and contains all substances on the FDA Generally Recognized as
Safe (GRAS) list. Preliminary work determined a 1% concentration to be bactericidal to many serovars of *Salmonella*; therefore, that was the concentration used in this study.

An increasingly popular method for cleaning food contact surfaces is through use of a disposable wipe saturated with a sanitizer, providing convenience and the reliable application of the correct sanitizer dose to a surface (56). In this study, Flavorzon was applied to contaminated food contact surfaces through a saturated, disposable wipe. Food contact surfaces were inoculated by either spot inoculation or by submerging the coupons in a growth medium to allow cells to attach for 48 h prior to the sanitizing challenge. Four common food contact surfaces were tested including stainless steel, polyvinyl chloride (PVC) conveyor belt material, high-density polyethylene (HDPE) tote plastic, and HDPE cutting board. To determine the effectiveness of Flavorzon, the microbial reduction achieved was compared to common food industry sanitizers including chlorine, peracetic acid, and quaternary ammonium compounds.
CHAPTER 2

LITERATURE REVIEW

Contamination of Food Contact Surfaces

The presence of microorganisms on food processing surfaces increases the risk of cross-contamination to food, creating the potential for foodborne illnesses. Microorganisms can enter a food processing facility through contaminated ingredients, employees, water and pests (30). Once in a facility, the microorganisms may find a niche that is difficult to clean, such as a crevice in a machine or a crack in the floor (30). These microorganisms can persist for years within the facility, possibly leading to contamination of the product (48).

According to a 2008 report from the U.S. Food and Drug Administration (FDA), improper cleaning of food contact surfaces and equipment is a common issue with eight out of the nine facility types observed in that report having contamination levels that are out of compliance (19). The FDA Food Code requires that food contact surfaces be cleaned a minimum of every 4 h, or any time there is a change from working with one raw material to another, or one raw material to a ready-to-eat food (21). However, the 2008 report shows that frequency may not be sufficient and more emphasis is needed on training managers and employees on the importance of preventing contamination of food contact surfaces (19).

Microbial cells can attach to food processing surfaces with or without an abundance of nutrients present (61). These attached cells become more resistant to cleaning and sanitizing methods than their unattached counterparts, making them a serious concern for the food industry (38). When cells first contact a surface, they become reversibly attached with weak electrostatic
forces (44). The reversibly attached cells form a biofilm when, over time, the cell produces a
glycocalyx composed of extracellular binding polymers (22). Bacteria within a biofilm can be up
to 1,000 times more resistant to sanitizers than free-flowing cells in a solution (33). Studies
suggest that using a detergent before sanitizing will make attached cells equally as susceptible to
sanitizers as unattached cells, which emphasizes the importance of cleaning a surface before
sanitizing it (13).

**Food Contact Surfaces**

Direct food contact surfaces have immediate physical contact with the product, and indirect food contact surfaces are contacted by splashed product, condensate, or solid particles
that may run off, drop off, or fall off onto the product (24, 37). When selecting food contact
surfaces for a plant, it is important that materials are homogenous, hygienic, chemical resistant,
physically durable (37). The material may encounter high temperatures, acidic ingredients, harsh
detergents and cleaning agents, as well as fat and salt components of food. It is important that
these conditions do not wear down the material or cause substances to migrate from the food
contact material into the product (37).

Stainless steel is the most widely used material in direct contact with food in the food
industry, and is commonly used to fabricate processing equipment and utensils (16). It is made
from iron, chromium, molybdenum, and nickel, none of which are harmful to consumer health.
Stainless steel is susceptible to corrosion in the presence of high chlorine levels, acidity, and high
temperatures near 150°C. The finish of the stainless steel can impact its resistance to chloride,
heat, and pH (37). Silicon coatings can also be applied to the stainless steel to increase corrosion
resistance, decrease fouling, or chemical reactivity (52).
Polyvinyl chloride (PVC) is widely used in the food industry because it is tough, strong, versatile and resistant to some chemicals. It is able to maintain desirable properties in cold temperatures, but does not retain mechanical performance above 80°C. It is often used in piping, sinks, drains, tanks and conveyor belting. A wide variety of products with different properties can be produced by varying the composition of stabilizers and plasticizers used in the formulation of PVC (37, 47).

High-density polyethylene (HDPE) is a strong plastic approved for use in the food industry due to its resistance to moisture absorption, staining, chemicals and corrosion. HDPE has strong mechanical resistance and is able to withstand temperatures up to 130°C (45, 55). HDPE can be formed into various products such as liquid food bottles, crates, trays, totes, and cutting boards. The thickness and shape of HDPE can vary greatly, giving it a wide range of characteristics to fit many intended uses (55).

Sanitizers

Sanitizers are used to reduce or eliminate the presence of microorganisms to an acceptable level. To be considered effective, a sanitizer should produce 99.999% kill of 75 million to 125 million *Escherichia coli* and *Staphylococcus aureus* in suspension within 30 s after application at 25°C (34, 57). The EPA regulates sanitizers and classifies them as no-rinse food-contact surface sanitizers and non-food-contact surface sanitizers. Common no-rinse food-contact surface sanitizers include chlorine, peracetic acid, and quaternary ammonium compounds (33).

Chlorine bleach is an oxidative sanitizer consisting of sodium hypochlorite and water, and is often used for sanitizing food contact equipment. Equipment does not have to be rinsed if 200 ppm chlorine or less is applied, which is a sufficient strength to kill most microorganisms on
smooth surfaces (34). Chlorine is relatively inexpensive and effective against all types of microorganisms, giving it an advantage to other sanitizers. The effectiveness of chlorine decreases above a pH of 9.0 and a harmful gas is created as the pH becomes more acidic, therefore it is most effective at a pH range of 6.5-7.0. Some disadvantages of chlorine include that it quickly loses activity in the presence of organic matter and it is highly corrosive to common food processing materials, including stainless steel (49).

Quaternary ammonium compounds (quats) are a class of non-oxidizing sanitizers that consist of four organic groups linked to a nitrogen ion (34). There are many advantages to using quats in a food processing environment. They are tasteless and odorless, non-corrosive to food processing equipment, non-irritating to skin and can leave a bacteriostatic coating on surfaces to inhibit microbial growth. Quats are effective against many microorganisms including viruses, however they have limited activity against most gram-negative bacteria except Salmonella and E. coli. Some disadvantages of using quats are that high concentrations are required to achieve the antimicrobial effect and activity may be limited by water hardness or anionic surfactants (34, 49).

Peracetic acid is a type of peroxycarboxylic acid-based oxidizing sanitizer that is effective against both gram-positive and gram-negative bacteria, as well as viruses, fungi, and yeast (40). Peracetic acid is effective at 100 ppm and a contact time of less than five minutes, however if organic matter is present, then 200-500 ppm may be required (40). Advantages associated with peracetic acid include that it breaks down into non-hazardous by-products, it is non-corrosive to most food processing materials, and it is effective at relatively low concentrations when compared to other sanitizers (49). Disadvantages include an acrid odor, and harm to employees if inhaled, swallowed, or in contact with skin (40).
The effectiveness of these sanitizers is dependent on the temperature, sanitizer concentration, and contact time with the surface (5). The type of surface the sanitizer is applied to and whether or not the cells are planktonic or attached to the surface also impacts the effectiveness of the sanitizer (7). The quality of the water used to prepare the solutions can also impact the efficacy of the sanitizer, with increasing hardness or pH of the water decreasing the bactericidal activity of some sanitizers (43). Due to this variation, it is important to validate cleaning procedures within a facility to make sure they are sufficient for reducing contamination (50).

Flavorzon Solution

Volatile organic compounds (VOCs) released from endophytic fungi of the Muscodor genus have been shown to exhibit antimicrobial activity against many fungal and bacterial species (23, 36). Muscodor crispans is found in the stem tissues of Ananas ananassoides, a wild pineapple plant that inhabits the Bolivian Amazon Basin (23, 35). Muscodor crispans is molecularly similar to other members of the Muscodor genus, however this isolate does not contain the harmful compounds naphthalene or azulene derivatives, making it the only one safe for use with food (23, 35). Analysis done by gas chromatography/mass spectrometry (GC/MS) showed that most of the compounds in Muscodor crispans are Generally Recognized As Safe (GRAS) (36). The GRAS compounds released from Muscodor crispans have been synthesized into an antimicrobial solution called Flavorzon.

Flavorzon consists of a variety of esters, alcohols, and small molecular weight acid substances, (Table 1) (35). Propionic acid was substituted for the isobutyric acid that occurs naturally from Muscodor crispans to improve the odor of Flavorzon. Propionic acid is a compound known to have antimicrobial activity against molds and some bacteria, including
Salmonella (1, 42). Propionic acid is the most abundant compound in Flavorzon, and since it has shown antimicrobial activity at a minimum of 0.1% concentration, it may be a large contributor to the antimicrobial activity of the solution (17). One study has suggested that isobutyl isobutyrate may also have some antimicrobial activity against Escherichia coli, Salmonella spp. and others (3). Many of the remaining compounds are small molecular weight esters, which likely enhance the antimicrobial activity of the other compounds through synergistic effects (51, 54).

The antimicrobial activity of Flavorzon has been tested against a variety of human and plant pathogens (23). The vapor phase of Flavorzon inhibited growth of Staphylococcus aureus ATCC 6538, Salmonella choleraseuis ATCC 10708, Mycobacterium tuberculosi, and several other pathogens when incubated at 23°C for 2 days (36). The effect of the vapor phase of Flavorzon has been shown to have a bacteriostatic effect, halting the growth of microorganisms while present, but not fully eliminating the viability of the organisms to grow after Flavorzon is removed (18).

Faith et al. (18) showed that Flavorzon has a dose-dependent effect for reducing Salmonella in experimentally inoculated ground turkey and ground beef. In ground turkey, a 2.84 log₁₀ reduction and 3.25 log₁₀ reduction in Salmonella were shown over a 5-day period at 8°C for 0.25% and 1% Flavorzon solutions, respectively. In ground beef, a 0.70 log₁₀ reduction and a 2.45 log₁₀ reduction in Salmonella was shown over a 5-day period at 8°C for 0.25% and 1% Flavorzon solutions, respectively. The background microflora in ground turkey showed a 5.91 log₁₀ CFU reduction while no reduction was seen in the background microflora of ground beef over five days (18).
Flavorzon is a commercially produced product that contains compounds in the same proportion as they are released from *Muscodor crispans*, omitting a few that are not GRAS or were deemed unnecessary (27, 36). To account for the natural variation that will occur in the volatiles released from the fungus, the compounds are obtained or produced through fermentation and then combined into Flavorzon to form a consistent product (53). The cost of Flavorzon is competitive with other surface disinfectants on the market (53).

**Salmonella spp.**

*Salmonella* is a motile, gram-negative, rod-shaped bacterium in the family *Enterobacteriaceae*. The *Salmonella* genus contains two species and 2,579 serotypes identified as of 2007 (20). *Salmonella enterica* is the species of major concern for foodborne outbreaks. Two of the most commonly encountered serovars responsible for foodborne illnesses are *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis. *Salmonella* has been found on a variety of food products including animal products, produce, and low-moisture foods such as spices (10). The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* accounted for over 1 million illnesses, nearly 20,000 hospitalizations, and 378 deaths in 2011, making it the most common bacterial cause of foodborne illnesses that year (8).

*Salmonella* infection often causes diarrhea, fever, and abdominal cramps 12 to 72 h after infection. In some patients, the symptoms will be severe enough to require hospitalization. The illness usually lasts 4 to 7 days, and most patients will experience a full recovery. Some patients are susceptible to a more severe illness when the infection spreads from the intestines to the bloodstream and to other organs. This occurs most commonly in the elderly, infants, or immunocompromised patients and can lead to death. A small number of patients will develop
reactive arthritis as a result from their *Salmonella* infection, even if the infection was fully cured (9).

*Salmonella* is a resilient and adaptive organism, making it a major concern for the food industry. Exposure to stress conditions may result in adapted *Salmonella* that are able to grow at temperatures from 2-54°C, a pH range of 3.99 to 9.50, and water activity as low as 0.93 (31). *Salmonella* has demonstrated cross-protection against multiple stresses, where the existence of one stress enhances the organism’s ability to survive subsequent stresses (31). Since *Salmonella* is able to survive many traditional processing methods, it is important that food processing facilities take measures to reduce *Salmonella* on food contact surfaces so that it does not contaminate the product. With an infectious dose as low as one cell, it is imperative for food processors to have a zero tolerance policy for the presence of *Salmonella* in their facility (31).

If present on a food contact surface, *Salmonella* can remain in high enough numbers to contaminate other food products for up to four weeks (14). *Salmonella* can easily attach to cutting boards and may form a biofilm (46). If a *Salmonella* biofilm has formed on a surface which product passes over continuously, the outer, weaker layers of biofilm will break off from the shear force, contaminating product as it passes through (33). A study of a past outbreak suggests that *Salmonella enteritica* serovar Agona was able to persist behind the wall in a food processing environment for ten years, causing contamination of the product with identical strains ten years apart (48). *Salmonella* has a high rate of transfer from the food processing surfaces to food when contact occurs, so it is important for processors to make sure it does not become present in their facility (29, 46).

**Inactivation of Salmonella on food contact surfaces.** There are many different methods for reducing *Salmonella* contamination levels. A study by Yang et al. (58) determined the
effectiveness of common household measures to reduce *Salmonella* Typhimurium contamination levels by a suspension test. Exposure to water for 1 and 10 min at 55°C decreased planktonic *Salmonella* Typhimurium levels by approximately 1.0 and 1.2 log\(_{10}\), respectively. Household bleach with 0.0314% sodium hypochlorite concentration decreased planktonic *Salmonella* Typhimurium levels by greater than 6 log\(_{10}\) after exposure for 1 min at 25°C. Undiluted vinegar with 5% acetic acid was able to reduce planktonic *Salmonella* Typhimurium levels by greater than 5 log\(_{10}\) after treatment for 1 min at 25°C and 55°C (58).

The effectiveness of common sanitizers for reducing *Salmonella* contamination levels on retail deli slicers was observed by Yeater et al. (60). Rifampin-resistant *Salmonella* Typhimurium was allowed to attach to seven distinct sites of the retail deli slicer for 30 min before being treated with quaternary ammonium chloride-based wet foam (WF) or dry foam (DF), or 200 ppm chlorine with a 10 min contact time. The effectiveness of all sanitizer treatments differed from each other, with chlorine being the least effective and WF being the most effective. The mean reduction of chlorine, DF, and WF were 2.3 log\(_{10}\), 2.6 log\(_{10}\), and 3.2 log\(_{10}\), respectively. The difference in reduction was most likely due to the increased surface adherence of quat sanitizers due to their surfactant nature, and the washing effect of the wet foam application increasing the removal of cells (60).

Cells that have formed a biofilm on a surface are more resistant to sanitizers than their planktonic counterparts (38). Sodium hypochlorite (500 ppm) and benzalkonium chloride (0.02%) failed to eradicate early (48 h) and mature (168 h) *Salmonella* biofilms on several food contact surfaces even at a 90 min contact time (11). Sodium hydroxide (1 M) was able to eradicate the early biofilm after 10 min, but only had approximately a 2.0 log reduction on the mature biofilm after 90 min (11). A recent study showed that combining steam and sanitizers
produces an additional 0.01-2.78 log_{10} reduction in *Salmonella* Typhimurium biofilm on stainless steel than either treatment alone, suggesting the combination creates a greater effect on cell membrane integrity (4).

**Recovery of Bacteria from Surfaces**

Disposable wipes saturated with a sanitizing solution provide a convenient way to sanitize surfaces while minimizing the risk for cross-contamination by disposing of them after a single use (26). The wipes are pre-saturated with the appropriate amount of sanitizer, making it easy to apply the correct dose of sanitizer to the surface. The demand for saturated wipes in the U.S. is projected to rise 3.6% yearly through 2018 to $2.9 billion, with the industrial market projected to increase more than the consumer market (56). With the rising demand of this type of product, a tool such as the Swiper Automated Machine (SAM; Engineerable LLC, Atlanta, GA) was created to provide a consistent method for testing the efficacy of disposable sanitizing wipes.

SAM was designed to provide a repeatable method for wiping the surface of a stationary coupon with consistent speed and pressure (Figure 1). A study by Bolton et al. (6) demonstrated the effective use of SAM for applying sanitizers with a saturated, disposable wipe to reduce murine norovirus contamination on stainless steel coupon surfaces. The machine consists of a two-axis robotic arm with a removable custom wipe adapter, temporarily held in place on the horizontal arm using neodymium magnets. The downward force delivered by the wipe adapter, while wiping across the coupon, is digitally programmed and displayed using a weigh scale panel meter. If using the machine in a horizontal wiping motion, the user sets the distances traveled by the wipe adapter manually by end stop microswitches. The machine also offers a touch and lift-off feature where there is only a downward force applied, without the horizontal
motion. Coupons are held in place on the base of the machine using two silicon suction cups and a connection to vacuum (6).
CHAPTER 3
MATERIALS AND METHODS

*Salmonella enterica* Serovars

*Salmonella enterica* serovars were obtained from the culture collection in the Department of Food Science and Technology, University of Georgia, Athens. Cultures were stored at -80°C and activated by transferring at least twice in tryptic soy broth (TSB; Becton Dickinson and Company, Franklin Lakes, NJ) at 24 h intervals at 35°C. *Salmonella* presence was periodically confirmed using triple sugar iron (TSI; Becton Dickinson and Company) and lysine iron agar (LIA; Becton Dickinson and Company). Prior to the experiment, 0.1 ml of each serovar was transferred to an individual test tube containing 9 ml TSB and incubated at 35°C for 18-24 h. On the day of the experiment, 3 ml of each serovar were pooled together as a cocktail in a sterile, 15 ml conical centrifuge tube (ThermoScientific, Rochester, NY). The cocktail was centrifuged (Eppendorf Centrifuge 4810, Hauppauge, NY) at a relative centrifugal force of 2,300 x g for 5 min. The supernatant was removed and the pellet was resuspended in 9 ml 0.1% peptone water (Becton Dickinson and Company). The cocktail was centrifuged for another 5 min at 2,300 x g, the supernatant was removed again and the pellet was resuspended in 9 ml 0.1% peptone water for final use. Resuspended cultures contained approximately $10^8$ CFU *Salmonella*/ml as determined by plating onto trypticase soy agar (TSA; Becton Dickinson and Company).
Flavorzon Solution

In a previous study, the vapor phase of the fungus *Muscodor crispan* was analyzed by GC/MS to determine the relative proportions of volatile compounds released from the fungus (36). The compounds that are Generally Recognized as Safe (GRAS) are combined to form a commercial mixture called Flavorzon (Jeneil Biotech, Saukville, WI). To account for the variation that occurs from naturally produced volatile compounds from *Muscodor crispan*, the components were obtained or produced through fermentation individually and purified before being combined to produce Flavorzon. Several compounds released from *Muscodor crispan* that are not GRAS or were deemed unnecessary were not included in Flavorzon.

**Flavorzon Antibacterial Activity Determination.**

The antimicrobial activity of Flavorzon was determined using the broth dilution method adapted from the American Society for Microbiology (12). The minimum inhibitory concentration (MIC) is the lowest concentration in which there is no visible growth of *Salmonella* in broth, but it is still viable when plated on TSA. The minimum bactericidal concentration (MBC) is defined as the lowest concentration in which there is no visible *Salmonella* growth in the broth or when subsequently plated on TSA. The MIC and MBC were determined for Flavorzon with eight serovars of *Salmonella enterica* and the most resistant were selected for the cocktail used in the subsequent challenge experiments.

**Antibacterial Solution Preparation**

Peracetic acid (PAA; VigorOx LS-15; PeroxyChem, Philadelphia, PA), quaternary ammonium compounds (Quat; Pacto Quat Clean IV; Brulin, Indianapolis, IN), chlorine bleach (The Clorox Company, Oakland, CA), and Flavorzon (Jeneil Biotech, Saukville, WI) were mixed with sterile distilled water to create the antimicrobial solutions. On each day of the
experiment, solutions of 200 ppm PAA, 200 ppm quat, 200 ppm free chlorine and 1% Flavorzon were prepared by mixing the sanitizers with deionized water. The PAA and chlorine solutions were tested to verify a 200 ppm concentration using a photometer test kit (V-2000; CHEMetrics, Midland, VA).

**Coupon Fabrication and Cleaning**

Coupons (2x5 cm) were fabricated from stainless steel (type 304, 4B finish), polyvinyl conveyor belt material (PVC; #120 white; W.L. Deckert Co., Inc., Milwaukee, WI), high-density polyethylene tote plastic (HDPE; 0.08 cm thick, United States Plastic Corporation, Lima, OH), and high-density polyethylene cutting board (HDPE; 0.635 cm thick, Cutting Board Company, Norcross, GA). All coupons were cut to 2x5 cm by the University of Georgia Instrument Shop. Before use, coupons of all food contact materials were placed in a sonicating water bath (Aquasonic Model 550HT; VWR, Radnor, PA) with a 1% solution of alkaline detergent (Micro90; International Products Corporation; Burlington, NJ) and sonicated for 60 min at 55°C. Coupons were then rinsed with deionized water, dried with KimWipes (Kimberly-Clark; Roswell, GA), and sonicated for 20 min in a 200 ppm solution of acid sanitizer (Formula 386L; Zep; Atlanta, GA) at room temperature. Coupons were then rinsed with deionized water and dried with KimWipes. Stainless steel and HDPE cutting board coupons were placed in self-sealing autoclave pouches (Propper Manufacturing Co., Long Island City, NY) and autoclaved for 30 min at 121.1°C. PVC conveyor belt and HDPE tote plastic coupons could not be autoclaved, so after sonicating, rinsing, and drying, they were soaked in 70% ethanol for 30 min, placed in a covered, sterile tray and left in the biosafety hood until ready for use.
Saturated Wipe Preparation

Wipes (Refillable Wiping System; Best Sanitizers Inc.; Penn Valley, CA) were cut to 9x10 cm using sterile scissors and autoclaved in sealed pouches for 30 min at 121.1°C. The day of each experiment, wipes were removed from the autoclave pouches using sterile tongs and placed in the bottom of a sterile, conical 50 ml centrifuge tube (ThermoScientific, Rochester, NY). A 2.4 ml volume of 200 ppm chlorine, 200 ppm PAA, 200 ppm quat, or 1% Flavorzon was pipetted into each centrifuge tube containing a wipe. The tubes were closed with a screw-top until they were ready to be used.

Coupon Inoculation

Coupons were inoculated by either allowing Salmonella to grow and attach on coupons submerged in a growth medium or by spot inoculation. To prepare coupons prior to allowing Salmonella attachment, waterproof tape (2.5 cm wide, Thomas Scientific; Swedesboro, NJ) was cut slightly longer than the length of the coupons and placed on one side of a cleaned coupon. This was done so Salmonella would only grow on one side of the coupon since only one side was subsequently treated with the antimicrobial and sampled. The taped coupons were placed in a 50 ml autoclaved beaker containing a 10:1 solution of sterile, distilled water and TSB, and 0.1 ml Salmonella cocktail. The beakers were covered with autoclaved aluminum foil and incubated at 35°C for 48 h before the challenge experiment. Coupons were removed from the beaker using autoclaved tongs and the tape was removed using autoclaved tweezers. Coupons were rinsed with 25 ml sterile distilled water to dislodge loose cells, placed in a sterile petri dish (100x15 mm, Fisher Scientific; Pittsburgh, PA), and used in antimicrobial trials within 60 min.

To prepare the spot inoculated coupons, cleaned coupons were spot inoculated with 100 μL Salmonella cocktail using a micropipette (Eppendorf Research Plus; Cole-Palmer; Vernon
Hills, IL) and 200 μL filter pipette tips (USA Scientific; Ocala, FL). Approximately 25 spots were placed on each coupon. Coupons were allowed to dry for 30 min in the biosafety hood and used in the antimicrobial trials within the following 30 min.

**Saturated Wipe Application**

Wipes were applied to coupons with the Swiper Automated Machine using a method adapted from Bolton, et al. (6). Wipes saturated with the antimicrobial solutions were attached to a removable wipe adapter (Delta Micro Factory Corp., Beijing, China) using a plastic twist tie. The inoculated coupon was held onto the machine by two silicon suction cups and a vacuum. Wipes were applied to the surface of the stationary, inoculated coupon at a constant 9.8 Newton downward force (imposed by a 1,000 g ± 10 g mass load) over the entire area of the coupon. Coupons were wiped two times in the same direction and then allowed a five-minute contact time with the antimicrobial solution, which was determined to be the most effective wiping method in preliminary trials. After the five-minute contact time, the coupons were placed into individual stomacher bags (VWR, Randnor, PA) containing 10 ml D/E Neutralizing Broth (Becton Dickinson). Coupons were rubbed by hand in the stomacher bag for 30 s to dislodge attached cells.

**Microbiological Analysis**

Dilutions were made from the stomacher bag using 9 ml 0.1% peptone water and plated on TSA using a spiral plater (Autoplate 4000, Spiral Biotech, Norwood, MA). Plates were incubated at 35°C for 24 h and then manually counted using the segment pair counting method. In the event the presence of *Salmonella* was below the detection limit, 1 ml was transferred immediately after plating from each stomacher bag containing the coupons to 9 ml buffered peptone water (BPW; Becton Dickinson) and incubated at 35°C for 24 h for enrichment.
Subsequently, 1 ml was transferred from the BPW to 9 ml Rappaport-Vassiliadis *Salmonella* enrichment broth (RV; Becton Dickinson) and incubated at 35ºC for 24 h. A loopful of the enrichment was streaked onto xylose lysine deoxycholate agar (XLD; Becton Dickinson) that was incubated at 35ºC for 24 h before being checked for presumptive *Salmonella* colonies.

**Statistical Analysis**

The response variable for each value was calculated using the formula

\[ Y = \log_{10} \left( \frac{\text{Count}}{\text{Initial Count}} \right). \]

Three replications of the experiment were conducted for each set of factors, with duplicate samples within each replication. A mixed effect model was fit with three factors (antimicrobial, surface, and inoculation) as the fixed effects and with replication as the random effect using R software (www.r-project.org). ANOVA analysis was done on the mixed effect model to determine the efficacy of Flavorzon compared to other sanitizers and the effect of the food-contact surface and inoculation method. The interaction between food contact surface and inoculation method was also evaluated. The baseline variables used were Flavorzon as the sanitizer, conveyor belt as the surface, and biofilm as the inoculation method. In the case that the value was below the detection limit, CFU/ml=10 was used as the value before performing the log transformation. After calculating the response variables, values greater than 0.5 were replaced with 0.5 to account for experimental errors, since the antimicrobials did not increase the contamination levels. The value of significance was reported as p= 0.05.
CHAPTER 4
RESULTS

Flavorzon Antibacterial Activity Determination

The minimum inhibitory concentration (MIC) of Flavorzon ranged from 0.25-0.5% for the eight serovars of *Salmonella*. A 1% solution of Flavorzon was determined to be the minimum bactericidal concentration (MBC) for all *Salmonella enterica* serovars tested. *Salmonella enterica* serovars Baildon, Muenster, and Typhimurium H661 were determined to be the most resistant serovars with a 0.5% MIC, therefore they were selected for the cocktail used in the subsequent challenge experiments.

Sanitizer Effectiveness

Wiping with Flavorzon showed no significant difference (p>0.05) in bacterial reduction when compared to wiping with 200 ppm chlorine, 200 ppm peracetic acid, or 200 ppm quaternary ammonium compounds (Figures 2-5). Wiping with sanitizers was significantly (p<0.05) more effective at reducing *Salmonella* contamination levels on spot inoculated coupons compared to coupons with attached cells (Table 2). The food contact surfaces that were treated also had a significant effect on the reduction of *Salmonella* contamination levels. Contamination levels for attached cells on HDPE tote plastic, stainless steel, and PVC conveyor belt were significantly more reduced than attached cells on HDPE cutting board coupons (p<0.05). Contamination levels for spot inoculated cells were significantly more reduced on HDPE tote plastic and stainless steel than spot inoculated cells on PVC conveyor belt and HDPE cutting board (p<0.05) (Table 3).
CHAPTER 5

DISCUSSION

In this study, we investigated the use of a novel sanitizer called Flavorzon to reduce Salmonella contamination levels on food contact surfaces using a saturated, disposable wipe. Wiping with Flavorzon was similar in effectiveness ($p>0.05$) in reducing Salmonella contamination levels compared to wiping with 200 ppm chlorine, 200 ppm peracetic acid, or 200 ppm quaternary ammonium compounds for all food contact surfaces and inoculation methods evaluated. The antimicrobial activity of Flavorzon is most likely due to the propionic acid and isobutyl isobutyrate that have shown some antimicrobial activity in previous studies $(1, 3, 42)$. The low molecular weight esters in Flavorzon may have a synergistic effect that increases the overall antimicrobial activity of the other compounds $(51, 54)$. One benefit to using Flavorzon over other common sanitizers is that it is a natural product that contains FDA GRAS compounds $(27)$.

A significant difference ($p<0.05$) was observed in sanitizer effectiveness dependent on the food contact surface tested. For coupons with attached cells, a significantly greater reduction was shown on PVC conveyor belt, HDPE tote plastic, and stainless steel coupons than on HDPE cutting board coupons. For spot inoculated coupons, greater reduction was seen on HDPE tote plastic and stainless steel coupons than on HDPE cutting board and PVC conveyor belt coupons. The tote plastic and stainless steel are relatively smooth surfaces compared to the cutting board and conveyor belt, which in comparison are rougher. This follows an observation by Yang et al. $(59)$ that sanitizers are significantly more effective on smooth surfaces than on rough surfaces.
This effect is most likely due to the rough surfaces providing more surface area for bacterial attachment and thus the potential for bacterial biofilms to mature faster, increasing its protection against sanitizers (59). Yang et al. (59) showed that sanitizer efficacy was greater on smooth HDPE cutting boards than rough HDPE cutting boards with *Listeria monocytogenes* biofilms grown for 7 and 14 days. If the biofilms were treated after 21 days, there was no longer a significant difference, suggesting that the biofilm had matured more quickly on the rough surface cutting boards, giving them an increased resistance to sanitizers at 7 and 14 days (59). The average initial counts of attached cells in the current experiment were approximately 0.6 log higher on the HDPE cutting board and PVC conveyor belt material than on HDPE tote plastic and stainless steel, supporting the concept that rough surfaces may provide more surface area for bacterial attachment.

Wiping with sanitizers was significantly (p<0.05) more effective at reducing spot inoculated cells than attached cells on all coupon materials. This finding agrees with several other studies that show an increased resistance of attached cells to sanitizers when compared to their planktonic counterparts (7, 32). This is most likely caused by the increased protective resistance that attached cells create, as well as the inability of sanitizers to penetrate the extracellular polymeric substance surrounding attached cells (15, 22). A study by Carballo and Araujo (7) compared the effectiveness of sanitizers on planktonic and attached cells on food contact surfaces and found that sanitizers used at concentrations two and four times greater than recommended by the manufacturer were ineffective at reducing attached cells. The attached cells in their experiment were grown on coupons for one hour with agitation (7), suggesting that the attached cells grown for 48 h in the current experiment may be more mature and have an even greater resistance to the sanitizers. The sanitizers in the current experiment were used at the
concentration suggested by the manufacturer, so a higher concentration may have been necessary to achieve a greater reduction. Carballo and Araujo also observed that adding heat in combination with the sanitizer at the concentrations suggested by the manufacturer was sufficient to remove attached cells (7).

Mosteller and Bishop (38) suggested that a 3 log$_{10}$ reduction in cells adhered to a surface is a reasonable goal for testing the effectiveness of sanitizers. None of the attached cell treatments tested reached this goal and only the chlorine and peracetic acid applied to HDPE tote plastic coupons and peracetic acid and quat applied to stainless steel coupons achieved a 3 log$_{10}$ reduction for spot inoculated cells. While SAM applied a consistent concentration of the sanitizers, the method may not have applied enough sanitizer for an effective contact time to achieve the desired microbial reduction. Preliminary tests done before this experiment suggested that the mechanical removal of cells during wiping is approximately 0.7 log$_{10}$, which contributes to the overall reduction seen in the wiping trials. Therefore, the reduction from the sanitizers alone may have been less than what the data from the current study shows.

Overall, this study suggests that Flavorzon is a potential alternative sanitizer for use in the food industry. Although the results showed less than a 3 log decrease in the contamination levels as a result of sanitizer application, use of the SAM provided a means to conduct a comparative study using a device that eliminated the potential variation in the application of sanitizers that would occur from a hand application method. In order to make sanitizers more effective, other studies suggest that using chemical cleaners prior to sanitizing will greatly improve the removal of spot inoculated and attached cells (28). The effect of other application methods and combination treatments involving Flavorzon should also be investigated to achieve a greater level of microbial reduction. Flavorzon could possibly be used as a rinse or a spray,
which may allow greater contact of the Flavorzon to the surfaces, increasing the effectiveness over wiping. Using heat (7) or UV (25, 39) in combination with sanitizers has been shown to increase the reduction of bacterial contamination levels in previous studies, so these combinations should also be investigated with Flavorzon.
REFERENCES


53. Strobel, G. 26 February 2016. Personal Communication [E-mail: uplgs@montana.edu] Available from: [E-mail: laurlev7@uga.edu]


Figure 1: (A) Swiper Automated Machine used for wiping across the surface of a coupon with a disposable wipe saturated with a sanitizer. (B) The disposable wipe is attached to a removable wipe adapter, which attaches to the horizontal arm of SAM with neodymium magnets (6).
Figure 2: Population reduction of *Salmonella* on stainless steel coupons shown with 95% confidence interval. Coupons had either attached cells or spot inoculated cells and were treated with one of four sanitizers: 1% Flavorzon, 200 ppm chlorine, 200 ppm peracetic acid (PAA), or 200 ppm quaternary ammonium compounds (Quat). Values are averaged from three replications, with two samples of each treatment within each replication (n=6).
Figure 3: Population reduction of *Salmonella* on HDPE cutting board coupons shown with 95% confidence interval. Coupons had either attached cells or spot inoculated cells and were treated with one of four sanitizers: 1% Flavorzon, 200 ppm chlorine, 200 ppm peracetic acid (PAA), or 200 ppm quaternary ammonium compounds (Quat). Values are averaged from three replications, with two samples of each treatment within each replication (n=6).
Figure 4: Population reduction of *Salmonella* on PVC conveyor belt coupons shown with 95% confidence interval. Coupons had either attached cells or spot inoculated cells and were treated with one of four sanitizers: 1% Flavorzon, 200 ppm chlorine, 200 ppm peracetic acid (PAA), or 200 ppm quaternary ammonium compounds (Quat). Values are averaged from three replications, with two samples of each treatment within each replication (n=6).
Figure 5: Population reduction of *Salmonella* on HDPE tote plastic coupons shown with 95% confidence interval. Coupons had either attached cells or spot inoculated cells and were treated with one of four sanitizers: 1% Flavorzon, 200 ppm chlorine, 200 ppm peracetic acid (PAA), or 200 ppm quaternary ammonium compounds (Quat). Values are averaged from three replications, with two samples of each treatment within each replication (n=6).
Table 1: Compounds identified by GC/MS in *Muscudor crispans*, reproduced from Mitchell (36), which are used in the composition of Flavorzon and their common food industry uses. The total area from GC/MS analysis is given, which is an indicator of the relative abundance of each compound used in Flavorzon.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Industrial Uses</th>
<th>Total Area (GC/MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl ethyl ketone</td>
<td>Solvent; Flavoring (2)</td>
<td>2.83</td>
</tr>
<tr>
<td>Methyl isobutyrate</td>
<td>Flavoring (fruity) (2)</td>
<td>30.56</td>
</tr>
<tr>
<td>Isobutyl acetate</td>
<td>Solvent, Flavoring (sweet apple, banana-like) (2)</td>
<td>2.29</td>
</tr>
<tr>
<td>Isobutyl isobutyrate</td>
<td>Flavoring (fruity, grape-like) (2); Shows some inhibition against <em>Salmonella</em> and other bacteria/fungi (3)</td>
<td>1.09</td>
</tr>
<tr>
<td>Isobutyl alcohol</td>
<td>Flavoring (sweet, fruity); solvent (2)</td>
<td>1.78</td>
</tr>
<tr>
<td>2-Butenal, 2-methyl-, (E)-</td>
<td>None</td>
<td>1.51</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>Flavoring (banana oil) (2)</td>
<td>4.79</td>
</tr>
<tr>
<td>Propanoic acid, 2-methyl-, 2-</td>
<td>None</td>
<td>4.78</td>
</tr>
<tr>
<td>methylbutyl ester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>Flavoring (pungent taste) (2)</td>
<td>5.38</td>
</tr>
<tr>
<td>Propionic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Inhibits growth of mold and some bacteria (1, 42)</td>
<td>351.18</td>
</tr>
<tr>
<td>Acetic acid, 2-phenylethyl ester</td>
<td>Flavoring (sweet, rosy-fruity, honey-like odor) (2)</td>
<td>1.31</td>
</tr>
</tbody>
</table>

<sup>a</sup>Propionic acid was substituted for the Propanoic acid, 2-methyl- that is naturally produced by *Muscudor crispans* to improve the odor of the solution.
Table 2: Average *Salmonella* reduction for coupons inoculated by spot inoculation or by allowing cells to attach to the coupon or 48 h.

<table>
<thead>
<tr>
<th>Inoculation Method</th>
<th>Attached Cells</th>
<th>Spot Inoculated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.87 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.56 ± 1.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>ab</sup>Mean values (*n*=96) within a row with different superscripts are statistically different (*p*<0.05).

<sup>x</sup>The values shown are the mean reduction by all sanitizers and food contact surfaces tested for that inoculation method plus standard deviation.
Table 3: Average *Salmonella* reduction for stainless steel, HDPE tote plastic, PVC conveyor belt, and HDPE cutting board coupons inoculated by spot inoculation or by allowing cells to attach to the coupon or 48 h.

<table>
<thead>
<tr>
<th>Inoculation Method</th>
<th>Attached Cells</th>
<th>Spot Inoculated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless Steel</td>
<td>-1.17 ± 0.76a</td>
<td>-3.15 ± 1.07a</td>
</tr>
<tr>
<td>HDPE Tote Plastic</td>
<td>-0.95 ± 0.58a</td>
<td>-3.45 ± 1.60a</td>
</tr>
<tr>
<td>PVC Conveyor Belt</td>
<td>-1.10 ± 1.32a</td>
<td>-1.87 ± 1.26b</td>
</tr>
<tr>
<td>HDPE Cutting Board</td>
<td>-0.26 ± 0.77b</td>
<td>-1.78 ± 1.04b</td>
</tr>
</tbody>
</table>

ab Mean values (n=24) within a column with different superscripts are statistically different (p<0.05).

The values shown represent the mean values plus standard deviations for *Salmonella* counts on coupons with attached cells and spot inoculated cells after being treated with 1% Flavorzon, 200 ppm chlorine, 200 ppm peracetic acid, and 200 ppm quaternary ammonium compounds for each coupon material.
APPENDIX A

TESTING THE ANTIMICROBIAL ACTIVITY OF THE VAPORS FROM FLAVORZON

Objective

To determine if the vapors from Flavorzon have antimicrobial activity against coupons, and tomatoes inoculated with Salmonella in a closed chamber.

Procedure

1) Transferred loopful of Salmonella enterica subsp. Typhimurium to 9 ml TSB and incubated for 24 h at 35°C.
2) Spot inoculated stainless steel coupons with 100 μL of Salmonella culture.
3) Grew Salmonella attached cells on stainless steel coupons at 35°C for 48 h.
4) Spot inoculated tomatoes with 100 μL Salmonella.
5) Spot inoculated TSA plate with 100 μL Salmonella.
6) Filled 10 ml beakers with 10 ml undiluted Flavorzon and placed in center of two desiccators.
7) Filled 10 ml beakers with 10 ml distilled water and placed in the center of the other two desiccators for the control.
8) Placed spot inoculated coupons, attached cell coupons, tomatoes, and TSA plate in a desiccator around the beaker in the center.
9) Closed and sealed desiccator with vacuum grease.
10) Placed two desiccators (one with Flavorzon and one with control) in 20°C incubator and two desiccators (one with Flavorzon and one with control) in 4°C incubator for 72 h.
11) After 72 h, removed desiccators and placed items in individual stomacher bags containing 1% peptone.

12) Prepared dilutions from stomacher bag and plate on TSA.

13) Incubated plates for 24 h at 35°C prior to enumerating CFUs.

Results

The reduction of *Salmonella* on the coupons, tomatoes, and spot inoculated TSA plates was dependent on the proximity to the Flavorzon beaker in the closed desiccator. A slight reduction was observed in the desiccators at 20°C, however no reduction was seen in the desiccators at 4°C.
APPENDIX B

ASSUMPTIONS FOR RUNNING AN ANOVA

To run an ANOVA, there should be normal distribution of standard deviations. The tables below show the standard deviations for each set of factors tested (antimicrobial, inoculation method, and food contact surface). The standard deviations showed some variance, however it was determined that ANOVA was still appropriate to use due to the equal sample sizes and the sample sizes were large enough with n=6 for each treatment.

<table>
<thead>
<tr>
<th></th>
<th>Attached Cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDPE Cutting Board</td>
<td>PVC Conveyor Belt</td>
<td>HDPE Tote Plastic</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>Flavorzon</td>
<td>-0.48 ± 1.26</td>
<td>-0.48 ± 0.18</td>
<td>-1.06 ± 0.39</td>
<td>-1.14 ± 1.11</td>
</tr>
<tr>
<td>Chlorine</td>
<td>-0.19 ± 0.57</td>
<td>-1.65 ± 1.42</td>
<td>-0.85 ± 0.54</td>
<td>-1.21 ± 0.49</td>
</tr>
<tr>
<td>PAA</td>
<td>-0.04 ± 0.44</td>
<td>-1.74 ± 2.01</td>
<td>-1.08 ± 0.74</td>
<td>-1.04 ± 0.68</td>
</tr>
<tr>
<td>Quat</td>
<td>-0.34 ± 0.68</td>
<td>-0.54 ± 0.51</td>
<td>-0.81 ± 0.72</td>
<td>-1.28 ± 0.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Spot Inoculated Cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDPE Cutting Board</td>
<td>PVC Conveyor Belt</td>
<td>HDPE Tote Plastic</td>
<td>Stainless Steel</td>
</tr>
<tr>
<td>Flavorzon</td>
<td>-1.90 ± 0.82</td>
<td>-2.15 ± 0.61</td>
<td>-2.59 ± 1.30</td>
<td>-2.88 ± 0.99</td>
</tr>
<tr>
<td>Chlorine</td>
<td>-1.66 ± 0.77</td>
<td>-1.65 ± 1.68</td>
<td>-3.77 ± 1.31</td>
<td>-3.30 ± 1.13</td>
</tr>
<tr>
<td>PAA</td>
<td>-2.30 ± 1.50</td>
<td>-1.41 ± 1.55</td>
<td>-4.49 ± 0.85</td>
<td>-3.38 ± 1.26</td>
</tr>
<tr>
<td>Quat</td>
<td>-1.25 ± 0.87</td>
<td>-2.26 ± 1.06</td>
<td>-2.93 ± 2.23</td>
<td>-3.02 ± 1.13</td>
</tr>
</tbody>
</table>