CONTROL OF *LISTERIA MONOCYTOGENES* ON CONTACT AND NON-CONTACT SURFACES BY ELECTROSTATIC SPRAYING OF QUATERNARY AMMONIA

By

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A THESIS

IN

FOOD SCIENCE

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCE

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December 2008

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Christine Alvarado, Dr. Mindy Brahsears, and Dr. Pawan Takhar whose support and guidance has been essential to me during the time I have spent as a master's student. They have shared their knowledge of food science as well as the skills needed to endure even the most complex and frustrating situations. Each of them has spent time guiding and enlightening me and for that I am very grateful. I would like to especially thank Christine Alvarado for her encouragement and faith in me as a student.

I would also like to thank my friends and family who encouraged me during my research. Desirae Culp, my best friend, I thank you for grabbing coffee with me at odd hours of the day, helping me study for my thesis, and practicing my defense with me. I thank you all for you love and support during this time in my life.

Ana Marie Luna and Tanya Jackson the two best lab technicians any graduate student could hope for, I thank you sincerely. Ana, thank you for your help with running my data and helping me understand my statistics. Tanya, I appreciate all the ordering of supplies, overlaying of agar, and all the lab knowledge that you shared with me. I appreciate the hours the both of you spent helping me finish my work and complete this goal. Thank you!

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ABSTRACT

The attachment of bacteria on food processing equipment and in the environment after sanitation can cause potential cross- contamination, which can lead to food spoilage, possible food safety concerns, and surface destruction. The purpose of this research was to determine if electrostatic spraying of quaternary ammonium compounds would provide a more efficient means for sanitizing food contact and environmental surfaces to reduce bacterial attachment and prevent biofilm formation. Ceramic tile, FRP (plastic wall board), polypropylene conveyor belt- mesh top (24% open mesh) and stainless steel conveyor – single loop (80% open mesh) were inoculated with a *Listeria monocytogenes* cocktail with a final concentration of 10^6 cfu/ ml and then subjected to either an air pressure spray or electrostatic spray treatment using 200 ppm of ala-quaternary ammonium. There were significant ($P< 0.05$) reductions in the amount of LM that remained on the surfaces after being treated with both the electrostatic spray and the air-pressure spray, but no significant differences between the two treatments ($P< 0.05$). To determine which treatment could prevent biofilm formation, the ceramic tile, FRP, stainless steel coupons (306 food grade), and polyethylene (plastic cutting board) were treated with either an air pressure spray or electrostatic spray treatment using 200 ppm of ala-quaternary ammonium. Biofilms were allowed to form onto the surfaces for 24 hrs. The biofilms were measured by crystal method analysis and scanning electron microscopy (SEM). The crystal method analysis indicated that electrostatic spray significantly ($P< 0.05$) reduced the biofilm formation on all the surfaces and the SEM confirmed the absorbency reading.

CHAPTER I

INTRODUCTION

Sanitation is an important pre-requisite to any successful Hazard Analysis Critical Control Point (HACCP) program (3). Equipment surface type and soil can affect the type of sanitation program and type of sanitizers that are chosen in an operation (3). Another aspect that must be considered when choosing a sanitizer and application system is the type of pathogenic bacteria that are common to that specific operation (9). The selection of a sanitizer is critical to a successful HACCP program because if bacteria are left on a surface they can proliferate, cross contaminate, and potentially cause illness if ingested (7). Another potential problem with bacteria that are not properly removed after cleaning and sanitation is that they can form into a biofilm (7).

Bacterial adhesion and biofilm formation is very problematic because biofilms are resistant to chemicals and sanitizers (5). Therefore chemicals that are developed to eliminate bacteria are not effective against biofilms because they are formulated to work against bacteria in their planktonic form (5). This sanitation problem is a concern for the food industry because biofilm forming bacteria persist and grow after sanitation thus making biofilms a potential threat to food safety (8). One particular pathogenic bacteria of interest is *Listeria monocytogenes* (4). *Listeria monocytogenes* forms biofilms. This pathogen is known to cause serious illness if ingested by those with immunocompromised systems and pregnant women (1). The ability for *Listera monocytogenes* to survive sanitation by forming biofilms and the severity of the disease it can potentially cause has led to develop a sanitation programs to remove this bacteria

from the food environment. Electrostatic spray is one possible means of application that needed to be studied to determine if it could provide a more effective sanitation program.

Electrostatic sprayers are being investigated because they allow for a more effective distribution of sanitizers (10). Electrostatic sprayers produce electrically charged spray particles that are carried in an air stream towards their intended target, which is oppositely charged (6). The charged particles are attracted to their intended target (6). This technology has been effective in many industries such as painting and pesticide application (2). Several studies have been conducted to determine the efficacy of pesticide application on various crops. The positive findings obtained in these studies gave a better understanding of the mechanisms and limitations of chemical application by electrostatic spray. These studies involving applying pesticides electrostatically laid the foundation for further research to determine if electrostatic spray could be a means of sanitation application in the food industry. Russell (2003) found that when applying electrolyzed (EO) water with an electrostatic sprayer, pathogenic bacteria from the surfaces of eggs were eliminated (10). This research led to future research involving the possibility that the electrostatic sprayer could be used as part of a sanitation system. Therefore, this study was conducted to determine the effect of EO water applied using electrostatic spraying on Salmonella Typhimurium, *Staphylococcus aureus, L. monocytogenes,* and *E.coli* on eggshell surfaces (10).

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CHAPTER II

REVIEW OF LITERATURE

Listeria monocytogenes

Listeria monocytogenes (LM) is a gram positive bacteria with a negative cell surface charge and is ubiquitous in nature therefore it is commonly found in the food industry (29). *Listeria* has been isolated from stream water, silage, drains, soil, humans, and food processing environments (46). LM is a food borne pathogen that accounts for less than 1% of food borne illness, but is responsible of 28% of deaths caused by food borne diseases (41). *Listeria monocytogenes* is found in a variety of food products such as soft cheeses, dairy products, raw foods, ready to eat products, and equipment surfaces (28, 37).

Listeriosis is the disease that is caused by LM and is contracted through the consumption of contaminated foods (50). Once the pathogen has entered the body, it will spread from cell to cell infecting many soft body tissue sites, such as the liver (20). Listeriosis generally affects pregnant women and immuno-compromised people (14). Infections caused by LM lead to flu- like symptoms, meningitis, septicemia, and spontaneous abortions in pregnant women (22). Due to the serious effects of the pathogen, the USDA has imposed a “zero tolerance” policy for any “ready to eat” products that test positive for LM (18). This has made eradicating the bacteria from the production environment a major concern, but *Listeria* has a variety of survival mechanisms, which has made removal very difficult (49).

One of LM’s unique characteristics is its ability to adapt and survive harsh environments (20). Though it grows best at a neutral to slightly alkaline pH, it has been

known to grow at pHs as low as 5.0 and as high as 9.6 (42). *Listeria monocytogenes* has been known to survive temperatures ranging from 2 to 45°C, but it grows best at 37°C. (42) *Listeria mmonocytogenes* has been isolated from acidic foods, foods with high salt contents, and foods kept at 2-4°C (refrigeration) (20, 40). The ability to survive at different pH gradients and varying temperatures allows the pathogen to survive the manufacturing and ripening of many cheeses and fermentation of milk attributing to its presence in dairy products. (5, 19) *Listeria's* pH tolerance has attributed to it being isolated from various meat samples, with chicken being the best host for growth of the pathogen (3).

This high tolerance to extreme environments and ability to adapt to changing pHs has also allowed the organism to survive commercial sanitizers that have been formulated specifically to destroy LM (49). The high occurrence of food recalls due to the presence of LM attests to the organism's ability to survive current cleaning and sanitation methods used in the industry (5, 36). It has been determined that LM's ability to adhere to surfaces not only allows it to persist even after sanitation has occurred, but increases the possibility of cross contamination (35, 52).

Biofilm formation

It has been determined that in all natural habitats, bacteria prefer to reproduce on the surfaces rather than in the liquid phase (6). All surfaces, whether hydrophobic, hydrophilic, metal, plastic and/ or glass are sites where biofilms can develop (6). Within biofilms, microcolonies are formed with 'water channels' between them and these colonies can be hetero or homogeneous in species, structure, and chemical composition

(6). The formation of a biofilm on a surface is a bacteria's ideal strategy for survival because of the protection and availability of nutrients (9).

Biofilms were first identified in the scientific community in 1702, when Van Leeuwenhoek first described them as "animalcules," but it was not until Costerton defined them in 1978 did they become more widely studied (16). Costerton stated that bioflims are a community of microorganisms encased in an exospolysaccarhide (EPS) matrix and are attached to each other or to a surface (15).

Biofilm formation occurs in five stages; 1) initial, irreversible attachment, 2) formation of EPS, 3) mircocolony formation, 4) biofilm maturation, 5) sloughing off of cells. Stage one and stage two work together in helping the cells to bind to the surface by using exopolysaccharide glycocalyx polymers as adhesion and capture mechanisms and then as a food source to allow for cell division (9).

The EPS matrix around the biofilm acts as a reservoir and a layer of protection against adverse environmental conditions and biological and chemical agents (10). The EPS consists largely of water (98-99%) and the remainder is comprised of nutrients such as polysaccharides and glycoproteins (6). The production of EPS allows bacteria to irreversibly attach to a surface (9, 13). It acts against antibacterial agents by trapping the molecules and preventing them from entering into the system (9). This protection gives biofilms the ability to resist antibacterial agents and has allowed biofilms to survive on surfaces even after cleaning and sanitation (6, 13).

Biofilms have the ability to readily form in the food industry environment because of the availability of water, nutrients and surfaces for attachment (21, 38). This is a problem in the food industry because the hygiene of the surfaces affects the overall

quality and safety of the food product (21, 27). The transfer of attached bacteria to a food product can lead to food spoilage or the transmission of diseases (7,46). Microbial contamination can also lead to mechanical blockage, corrosion of equipment, and overall impairment of the intended functions of equipment such as heat transfer (46).

Improper cleaning and sanitizing of food contact surfaces may lead to direct contamination of the product, through the product physically touching the contaminated surface (21). Environmental surfaces that are improperly cleaned and sanitized can lead to the transfer of microorganisms through the air, human contact, and contact with other equipment (21). The major sources for microbial contamination have been recognized as the areas used for food handling, storage, or processing (51). Currently the most effective way of preventing biofilm formation in the food industry is to develop an effective cleaning and sanitizing regime (21). An effective cleaning program should eliminate any soil and detach the microorganisms on the surface; while the sanitation should remove any remaining unwanted bacteria, the purpose of sanitation is to destroy bacteria that cleaning did not remove (6, 21). An ineffective sanitation program can lead to problems concerning the quality and safety of the food product (21).

Biofilms are known to be resistant against chemical cleaners and sanitizers and thus survive and proliferate on processing surfaces after cleaning and sanitizing. This allows the biofilm to contaminate food products and further proliferate and possibly cause illness once ingested. The ability to resist chemical agents has made the need for new and more effective sanitation methods more important in processing environments.

Good sanitation practices are current methods for controlling the growth of *Listeria* in the food industry (42). The effect of different sanitizers on the effectiveness of

killing *Listeria* has been researched and it has been concluded that chlorine, acid anionics, quaternary ammonium, and iodophors are all effective against the pathogen, but that the areas of application need to be devoid of organic material or the detergents will become neutralized (42). For an effective system, sanitation needs to be conducted immediately after cleaning has taken place (6). Another aspect that needs to be considered when constructing a sanitation program is exposure time, temperature, concentration, pH, soil content, water hardness and the possibility of bacterial attachment (11). Bacterial attachment is a large problem because once the bacteria attaches to a surface it can then form into a biofilm, which is more resistant to sanitizers than planktonic cells (46). *Listeria monocytogene*'s ability to adhere and form biofilms on surfaces regardless of temperature makes it a very serious sanitation problem (4). Various sanitizers are known to inactivate *Listeria* spp. when they are planktonic, but the ability of these sanitizers to inactivate a *Listeria* biofilm is a current topic of study (12, 28).

Several studies have been conducted to determine the ability of *Listeria monocytogenes* to adhere to food-contact surfaces all of which determined that LM can attach to industry surfaces including plastic and stainless steel. This knowledge has made it evident that surface selection is important to help control contamination, but it is not as effective as once believed. Proper surface selection can help in reducing the ability of microorganisms such as LM in forming biofilms.

A study was conducted to determine what effect sanitizers and contact surfaces had on the reduction of *Listeria* biofilms. *Listeria* was allowed to adhere to stainless steel coupons and plastic coupons for two days at 30°C (28). The coupons were then rinsed in PBS and dipped in either hypochlorite solution of 100- 200 ppm or a 10- 20 ppm

iodophor solution or a combination of both sanitizers (hypochlorite 10 ppm and iodophor 1 ppm) for five minutes. After treatment, the coupons were placed in a neutralizing solution for 30 seconds. For cell enumeration, the coupons were then swabbed and serial dilutions were plated on tryptic soy agar. Overall reduction by either sanitizer was most effective on the stainless steel coupons. A reduction was seen on the plastic coupons, but it was significantly less when compared with the stainless steel reductions. Hypochlorite was more effective at inactivating biofilm cells than iodophor on either material. The combination of chlorine and iodophor provided complete inactivation of biofilm cells. Therefore stainless steel and the combination of the sanitizers would provide the best possibility for the reduction of LM biofilms (28).

Another study was conducted to determine the ability of LM to adhere to different types of materials commonly found in the food- processing industry. Beresford and others tested assayed the adhesion capabilities of LM after submerging the materials in a planktonic culture for two hours at 30°C. All the materials were cut into flat coupons (0.8 X 0.8 cm) before inoculation. The materials tested were, stainless steel (types 304,430, and 316), aluminium, polycarbonate, polypropylene, polyurethane, PETG, PTFE, Lexan, Nitryl rubber, silicone rubber, natural white rubber, natural white rubber type CAN-70, and EPDM rubber. To recover the attached cells, the material coupons were sonicated for one minute and the recovered bacteria were suspended in PBS and plated for enumeration. From this study it was determined that stainless steel had the highest level of attachment, but that LM was able to adhere to all of materials that were tested. Overall Beresford and others found that material selection might not help in the reduction of food contamination (1).

From studying the adherence abilities of LM it was deduced that not only does LM adhere to surfaces, but that this adhesion can turn into biofilm formation (34). Chavant and others studied LM's ability to form biofilms on stainless steel and polyetrafluoroethylene (PTFE) at 8, 20, and 37°C. The surfaces were cut into 3 X 1 cm coupons and were then sterilized with a commercial surfactant and rinsed with both tap water and demineralized water before being autoclaved. The materials were placed in a petri dish containing 0.7 milliliters of LM bacterial suspension with a final concentration of 10^7CFU ml^{-1}. The surfaces were tested at two hours, six hours, and one, two, five and seven days. For enumeration, the coupons were washed with sterile tryptone salt (TS) to remove any nonadherent cells. The surfaces were then places in five ml of TS and sonicated for three minutes. The recovered cells were made into serial dilutions and counted on tryptic soy agar. Along with cell enumeration, scanning electron microscope was used to visualize the bacterial growth on the surfaces. This study determined that the initial attachment of LM was greater on stainless steel regardless of the temperature, though at the low temperature growth was slowed on both surfaces. Also regardless of temperature, the stainless steel chips were completely covered in biofilms at the end of five days. For PTFE, 100% biofilm coverage was only seen in the samples that were incubated in 20°C. Biofilm formation occurred on all surfaces after two hours at both 20 and 37°C and detachment was seen on these surfaces after two days. This study showed that LM readily forms biofilms regardless of temperature, but the lower temperatures do slow the process and stainless steel showed a greater allowance for attachment (8).

Sanitation

Several studies have been conducted to try and determine what sanitizers and concentrations are the most effective against *Listeria.* Though in these studies it was also determined that the type of material, the exposure time, and the temperature of the sanitizer had an effect on the overall reduction in the pathogen. Sinde and Carballo compared the attachment of LM on stainless steel, rubber, and polytetrafluorethylene and the effect that two commercial sanitizers had on the final attachment. The two commercial sanitizers were quaternary ammonium and diethylenetriamine, but it was determined that regardless of the material they found a significant reduction in attachment when either of the sanitizers was used in conjunction with standard cleaning and sanitizing methods. This study found that the more hydrophobic a surface enabled the bacteria to adhere in higher numbers. This meant that polyetrafluorethylene allowed for a greater amount of attachment and stainless steel allowed for the least. This suggests that material selection is important and that stainless steel is the more optimal choice. Also this study indicated that the use of a mixture of sanitizers might be more efficient at controlling bacterial adherence than the use of one sanitizer (47).

Mafu and others determined if temperature, concentration, and type of surface had an effect on the efficacy of four different sanitizing solutions used to destroy LM. They used stainless steel, glass, polypropylene, and rubber for their surfaces and sodium hypochlorite, iodophor A, Iodophor B, and quaternary ammonium sanitizers at 20° and 4° C. At low temperatures, the sanitizers were less effective regardless of the surfaces qualities. At 4°C, higher concentrations of the sanitizers were needed to be as effective as the lower concentrations that were used for at 20°C. At 20°C, inactivation of LM occurred for all of the sanitizers and the efficacy of the sanitizer was determined by the

surface qualities. It was determined that the less porous the surfaces were, the less resistance the bacteria became to the sanitizers. On these surfaces, such as stainless steel, quaternary ammonium was more effective than sodium hypochlorite (37).

Another study was conducted to determine the effectiveness of sanitizers on LM cells that had been exposed to cleaning solutions. The cells were suspended in 1% solutions of eight commercial cleaners and then incubated at 4°C for 30 minutes. The cells were exposed to Sodium hypochlorite (NaOCl) or benzalkonium chloride and cetylpyridinium chloride (two major components of quaternary ammonium sanitizers). The effect of the chlorine on the cells was dependent on the cleaning chemicals used prior to sanitation. The cells that had been exposed to low alkaline or alkaline cleaning products were displayed a log reduction of 7.28 after being sanitized with chlorine concentrations of either four or six mg 1^{-1}. The cells that had been exposed to heavy alkaline or water cleaning treatments showed a minimum of a one log reduction after being treated with either four or six mg 1^{-1}. Cells previously exposed to cleaner solutions were also sensitive to benzalkonium chloride and cetylpyridinium chloride. When the sanitizers were administered with benzalkonium chloride at 50 and 100 mg 1^{-1} a 3.48 to 4.80 log reduction was observed respectively. When cetylpridinium chloride was administered at 50 mg and 100 mg 1^{-1} and the populations were reduced by 5.64 and 7.23 logs respectively. This study helped explain the impact cleaners have on the effectiveness of sanitizers. It also showed that quaternary ammonium (benzalkonium chloride and cetylpyridinium chloride) is effective even after exposure to cleaners (50).

Based on the studies discussed in this section, proper sanitation of equipment is the only method of control for LM. For a sanitation program to be effective four factors

need to be achieved prior to sanitation: chemical energy, mechanical energy, temperature, and time. Chemical energy is employed in the cleaning phase when chemicals are used to break down soils and remove them from surfaces. Mechanical energy is further removal of soils from surfaces by physical means; for example spraying and scrubbing. The temperature of the chemicals is important because it needs to hot enough to melt the fats and oils that need to be removed, but not too hot that it bakes the soil onto the equipment. The contact times for chemicals vary, but there are several methods of increasing this time such as foams and gels. If these four steps are conducted properly, then sanitizers can be used to remove micro-organisms, product residues, foreign bodies, and cleaning chemicals left behind. If these steps are not carried out prior to sanitation, soil deposits can remain on the equipment and reduce the effectiveness of the sanitizer. Therefore, it is necessary that proper sanitation programs are developed and executed to ensure that no residual bacteria remain on the equipment (21).

It is also necessary to pick the appropriate sanitizer for the operation. The studies discussed have shown that the type of surface and bacterial species used have an impact on the effectiveness of sanitizers. Quaternary ammonium was very effective in these studies and is also known to work well on most surfaces (11). Acid- quaternary ammonium (ala- quat) is one form of quaternary ammonium that is recommended for use on stainless steel and walls (11). A characteristic of ala- quat is that it is combative against bacteria that produce biofilms (11). This quality is an important component to consider if the problem bacterium is LM (11). *Listeria* has a polysaccharide coating or film that helps protect it from bactericidal compounds. (20) Ala- quat is one compound that is able to disrupt the coating, penetrate the cell and cause death (11). Ala- quaternary

ammonium also has the ability to inhibt of enzymes that are required for bacterial cell function (11). Due to causing cell wall disruption and enzyme inhibition ala- quaternary ammonium has been shown to be effective in controlling *L. monocytogenes* (11). For manufacturers of ready to eat food that generally use stainless steel equipment and are focused on eliminating LM, ala- quat could be the optimum sanitizer for that operation.

Electrostatic spray

Electrostatic spray application technology has been utilized by the painting and agricultural industries, specifically pesticides (7,39). This technology has also been used extensively and effectively in the automobile industry for over 25 years (26). Electrostatic spray has been advantageous to these industries because it provides an overall better quality paint job by causing the particles to self- disperse (48). It achieves this by dispersing less paint over the surfaces, reducing the amount of overspray, and makes the operation safer for workers (26).

There are many variations of the electrostatic sprayer, but they all have a similar mechanical procedure (45). The general principle behind an electrostatic sprayer is to apply a charge to liquid droplets as they are sprayed through a nozzle (17). The solution (paint or chemical) passes through an electrostatic field at high speeds, which applies the charge onto the liquid medium (26). To impart the charge onto the liquid, a method called induction charging is utilized (17). This method produces a very high charge within the nozzle without using high voltages (17).

Specialized nozzles are used in electrostatic sprayers so that induction charging can occur (45). In these nozzles, a charge is placed on the cylinder by an electrode that is supplied with energy from a source such as a generator (34). When the liquid passes

through this charged cylinder, the charge then transfers the free electrons that have formed to the liquid (24). The highly charged liquid then undergoes a shearing process, which results in the formation of atomized droplets (34). When the liquid is placed in this electric current the solution is broken up into small charged droplets (24). The force of electrostatic attraction is strongest if the droplets that are formed are small (17). Tang and others (1994) determined that droplets with an average size of 32 μm would allow for optimal velocity and charge distribution (48). Law (1982) also stated that the small droplet size allowed for optimal spray deposition density on the leaves of broccoli plants (32). It has been established that these charged droplets have a force of attraction 75 times greater than gravity (17). This means that when the droplets are sprayed from the nozzle they have the ability to move upwards thereby overcoming gravity and coat surfaces that are hidden (17).

Outside of the spray nozzle, the droplets travel in what is called a “spray cloud” and carry the charge to the nearest grounded surface (26). This grounded surface magnetically attracts the now charged particles and causes them to land and “wrap around” the target (26). The “wrap around” effect causes the charged droplets to disperse over the areas of the surface that are non- charged, which results in a more even coverage of the surface (39). Studies have concluded that electrostatic sprayers provide four to ten times better coverage than other conventional sprayers (17).

This electrostatic spray technology has been used in agriculture for the application of pesticides increasing the deposition of these chemicals onto plants from two to seven fold (45). This type of application has been successful in many different areas of agriculture, but the efficacy of the electrostatic sprayer has been found to be dependant

on the type plant that is being treated (31). Plants with little waxy surface coatings (cuticles) and plants that grow in drier climates have a higher success rate than many other plants (26). A good example of a type of plant that follows the description above are apple trees (26). The trees are grounded, the leaves of the plant have very little cuticle covering, and the climate in which these plants are grown all contribute to the success of electrostatic spray to effectively distribute pesticides (26).

Dry harvested peas, spring wheat, winter wheat, and spring beans also have all been determined to be good receptors for electrostatic spray pesticides. With all of these crops, it was found that the deposit of pesticides was greater when applied electrostatically than with conventional methods. It was also determined that when pesticides were applied with the electrostatic sprayer, the chemicals were more effective at killing their intended targets. When applied electrostatically at lower concentrations these pesticides were still more effective than those applied by conventional methods. Therefore, on these particular crops, electrostatic spray was a superior means of application and was also cost effective because less pesticide could be used to produce satisfactory results (7).

Electrostatic sprayers have also been successful on some plants when the orientation of the sprayer has been adjusted so that the spray is directed away from the ground. Normally, when pesticides are sprayed, the charge on the pesticide naturally attract to the soil since the soil is grounded. To overcome the charge's natural attraction to the ground, the spray pattern is directed towards the crop at either a horizontal or an upward angle at close distances. This helps to ensure that the charge particles do not have far to travel before they are attracted to the plant, which helps control the possibility of

drift as well as ensuring that the droplets make contact with the plant instead of the soil. With the adjustment of the angle and distance application, there has been an increase in efficacy of usage with pesticide application (26).

Laboratory studies using electrostatic spray as a pesticide application yielded a 1.9 to 4.4 fold increase in pesticide deposit on the surface of various plants (34). This study also found that coverage of the lower foliage, undersides of leaves and fruiting forms was improved when electrostatic spray was implemented. This improvement was possible because the charged droplets have the capability of over coming gravity and can thus travel upwards in order to reach the underside of the leaves. When the pesticides were applied at one half the recommended amount, the control of insects was equal to that of conventional methods. Therefore, there was an overall improvement in the uniformity of the deposition of the chemical and the amount of chemical needed to treat the crops. This study determined that with the electrostatic sprayer, insect control costs could be reduced up to $50/hectacre (34).

One specific area of interest is the use of electrostatic spray on crop vegetation. Cotton is a low-lying crop that has made the need for new methods of pesticide application crucial to help combat the increase in pesticide costs and loss of product due to increased insect populations. The overuse of these expensive pesticides has lead to the realization that the previous spray systems were inefficient and wasteful. Thus, there was a need to research electrostatic spray as a means of application and control. A study was conducted to determine if electrostatic spray could be a possible means of controlling infestations by two insects common to cotton: the bollworm and the tobacco budworm. Over a three-year period, crop squares were monitored biweekly to determine if

electrostatic spray yielded a better control over the conventional hydraulic sprayer. Upon the determination that in laboratory settings the electrostatic spray could be effective at controlling insect damage, a new study was conducted to determine the efficacy of the electrostatic sprayer in the field. This study indicated that pesticides could be applied at half the normal rate when applied with an electrostatic sprayer and yield better control. On the crops that were treated with the electrostatic sprayer less damage to the leaves of the plant were observed due to the increased control over the insect populations. The overall control of insects was determined to be equal or greater than conventional sprayer methods (23).

A study was done to determine the efficacy of an electrostatic sprayer as a pesticide application in cabbage. Cabbage is a crop that is plagued by the insect *Plutella xylostella* more commonly known as the Diamondback moth. This species of moth is responsible for a majority of the economic loss that cabbage growers are faced with each year. The larvae of the Diamondback moth tends to feed on the underside of leaves and hidden areas of the plant, making inefficient spray coverage a very serious issue for cabbage farmers. It has been reported that in the 80's and early 90's over 90% of cabbage growers were using conventional hydraulic nozzle sprayers (43). The sprayers were inefficient at inhibiting the insects because they could not penetrate through the folded leaves of the cabbage plant. With the improvements made in electrostatic application, various studies were conducted with the focus of determining the efficacy of the application at controlling the Diamondback moth. Perez and others tested the electrostatic sprayer verses two commonly used application systems, the knapsack and the drop nozzle on cabbage at early head formation (43). This study determined that the overall leaf

coverage was significantly better when the electrostatic sprayer was utilized because the charged droplets were able to migrate to the hidden portions of the plant. An increase in the overall mortality of the insects was achieved with the electrostatic sprayer. It was concluded that with the use of electrostatic spray a "low dose" resistance management strategy could be implemented. This means that if the pesticides are applied electrostatically the number of treatments needed per season can be reduced; saving both time and money (43).

A more recent study of the use of electrostatic spray on crops was conducted on blueberries. Unlike the previous studies, the application was not used to combat insects, but a fungal plant pathogen *Monilinia vaccinii-corymbosi*. This pathogen causes mummy berry disease in blueberries and enters the plant through the flower's stigmas. This disease causes considerable economic loss to growers because there is a near-zero tolerance for mummified fruit in the commercial industry. For commercial growers, the infection is generally controlled with the use of repeated fungicide applications. The need for alternative methods of management of the pathogen has arisen amongst the pick-your- own and organic producers. These producers have tried applying biofungicides with standard sprayer methods and have found that disease suppression was unsatisfactory. This has lead to the interest in using the electrostatic sprayer technology as a means of application. In this study two biocontrol agents, *Pseudomonas fluorescens* and *Bacillus subtilis* were applied to the blueberry plants via hydraulic sprayer, electrostatically charged sprayer, and non-electrostatically charged sprayer (45). Overall there was a two to seven fold increase of deposition of both of the biocontrol agents on the flower stigmas when applied via the electrostatic sprayer. This study only determined

that the electrostatic sprayer was a more efficient means of application, it did not measure whether or not the disease was inhibited in the plants that were treated (45).

The success of electrostatically charging chemicals to control insects and plant diseases has lead to an increased interest in improving this technology and expanding its use into other industries. One industry that has recently taken an interest in this technology has been the poultry industry. A study has been conducted using electrostatic spraying as a means of applying sanitizers specifically electrolyzed water (44).

Electrolyzed water (EO) is a nontoxic and inexpensive sanitizer that has been proven to eliminate pathogens like *Escherichia coli* 0157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* after 10 minutes of exposure (18). The only chemical used in the production of EO water is sodium chloride (18). Electrolysis in conjunction with the weak sodium chloride solution form an acidic EO water with a pH range of 2.4 to 2.7 (2). The acidic EO water requisitions electrons from surrounding cellular membranes rendering the cell unstable (18). This gives the antimicrobial the ability to move into the cell and inactivate it, thus making EO water effective at inactivating pathogens (2). It has also been determined to inactivate pathogens that have adhered to surfaces (44). Due to the EO's ability to effectively kill pathogens without being toxic to the product, the poultry industry has taken an interest in using it as a possible application in the hatchery and harvesting industries (44).

In 2003, Russell conducted a study to determine if electrolyzed water sprayed electrostatically could reduce the presence of pathogens on the surface of eggshells (44). The eggshells were inoculated with one of four common pathogens found in the poultry industry; Salmonella Typhinmurium, *Staphylococcus aureus, Listeria monocytogenes,*

and *Escherichia coli* 0157:H7. Using an electrostatic sprayer, the electrolyzed water was applied for 15 seconds every hour for a 24-hour period. At the end of the application time, it was determined that the sanitizer, in conjunction with the electrostatic sprayer, was highly effective at eliminating the adhered pathogens for most of the eggs. Electrolyzed water was able to eliminate Salmonella Typhimurium , from 53 % of eggs. For eggs inoculated with *Staphylococcus aureus* a higher percantage of elimination was seen in the range of 73- 80%. The range elimination between replications for the eggs inoculated with *Listeria monocytogenes* was 53- 93%. The range of elimination for the eggs inoculated with *E. coli* was between 60- 100%. On the eggs that did not exhibit elimination a reduction was observed. The Salmonella Typhumurium inoculated eggs showed a minimum five log cfu/ mLreduction after treatment. In the eggs that remained positive for *Staphlococcus aureus,* a minimum reduction of three log cfu/ mL was displayed. The eggs that remained positive for *Listeria monocytoges* had a minimum reduction of one to two log cfu/ mL reduction. The eggs with remaining *E.coli* displayed a minimum of four log cfu/ mLreduction. The elimination and the reduction of these pathogens from the surfaces of the eggs demonstrate the efficacy of EO water when applied electrostatically (44).

In 2004, another study was conducted to determine if EO could be used as part of two- step washing process for commercial table eggs. In this study the comparison between EO water and commercial detergents were used both in vitro and using a pilot-scale egg washer. All eggs used in this study were cleaned with a commercial detergent prior to inoculation. The eggs were inoculated with either *Salmonella enteritidis or E.* coli. The eggs were then soaked in EO water for three minutes at 45°C or in Diversey (a

commercial sanitizer). This study also measured the albumen height, presence of cuticle, and eggshell strength to evaluate whether or not the electrolyzed water had an effect on the overall shell quality. This study determined that EO was complimentary to current commercial sanitizers used in two- step washing processes. The reductions of pathogens and the effects on egg quality were not significantly different between treatments. This study only determined that EO could be used as a cheaper, alternative to the current sanitizer (2).

Electrolyzed water as a possible spray treatment in killing *Campylobacter jejuni* and removing fecal contamination from poultry carcasses has also been studied. This study compared the use of EO water as a spray treatment (in a commercial style spray cabinet), an immersion treatment, and a pre-spray on carcasses to be harvested (30). As a spray (and a pre- spray) treatment, EO water was compared to treatment with TSB using a commercial spray cabinet. To simulate immersion treatment, a chiller tank was filled with 90L of EO water or chlorinated water and the carcasses were dipped in the tank for 40 minutes. It was determined that as a pre-harvesting spray, the alkaline form of EO was most effective at reducing the possibility of cross contamination. It was also concluded that alkaline EO water was effective at removing feces attached to the surfaces of chicken carcasses (30).

Justification of objectives

The purpose of this study was to determine the efficacy of the use of electrostatic spraying ala- quaternary ammonium sanitizer onto contact and non- contact surfaces to control LM. This study also was used to determine if LM biofilm formation could be controlled if contact and non- contact surfaces were electrostatically pre-treated with ala-

quaternary ammonium sanitizer. This research will give the industry insight into alternative means of sanitation in the hopes of finding new, more effective ways of eradicating LM and thus providing a safer food product for the consumer.

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CHAPTER III

COMPARISON OF APPLICATION METHODS OF SANITATION TREATMENTS ON THE REDUCTION OF *LISTERIA MONOCYTOGENES*

Introduction

Listeria monocytogenes (LM) is a gram positive bacteria that is ubiquitous in nature and is commonly found in the food processing environments. *Listeria monocytogenes* is a food borne pathogen that accounts for less than 1% of food borne illness, but is responsible of 28% of deaths caused by food borne diseases (16). LM is found in a variety of food products such as soft cheeses, dairy products, raw foods, ready to eat products, and equipment surfaces (10, 13).

Listeriosis is the disease that is caused by LM and is contracted through the consumption of contaminated foods (22). Infections caused by LM are characterized by flu- like symptoms, meningitis, septicemia, and spontaneous abortions in pregnant women (7). Due to serious affects of the pathogen, the USDA has imposed a “zero tolerance” policy for any “ready to eat” products that test positive for LM (5). This has made eradicating the bacteria from the production environment a major concern, but *Listeria* has a variety of survival mechanisms that has made removal and zero tolerance very difficult to achieve (20).

Several studies have been conducted to determine which sanitizers and concentrations levels are the most effective against *Listeria.* Sinde and Carballo (2000) compared the attachment of LM on stainless steel, rubber, and polytetrafluorethylene and the effect that quaternary ammonium and diethylenetriamine had on the final attachment

(19). This study indicated that the use of a mixture of sanitizers might be more efficient at controlling bacterial adherence on LM than the use of one sanitizer (19).

Mafu (1990) investigated if temperature, concentration, and type of surface had an effect on the efficacy of four different sanitizing solutions used to destroy LM (14). They used stainless steel, glass, polypropylene, and rubber as tests surfaces and sodium hypochlorite, iodophor A, Iodophor B, and quaternary ammonium as sanitizers. It was determined that the less porous the surfaces, the less resistance the bacteria became to the sanitizers. Additionally, on these surfaces such as stainless steel quaternary ammonium was more effective than sodium hypochlorite (14).

Quaternary ammonium compounds were very effective in these studies and is traditionally known to effectively reduce pathogens on most surfaces (3). Acid-quaternary ammonium (ala- quat) is one form of quaternary ammonium that is recommended for use on stainless steel and walls (3). A characteristic of ala- quat is that it is combative against bacteria that produce biofilms (3). This quality is an important component to consider if the problem bacterium is LM (3). *Listeria* has a polysaccharide coating or film that helps protect it from bactericidal compounds (6). Ala- quat is one compound that is able to disrupt the coating, penetrate the cell and cause death (3). Ala-quaternary ammonium also has the ability to inhibit of enzymes that are required for bacterial cell function and by causing cell wall drisruption and enzyme inhibition (3).

It is clear from these studies that choosing the appropriate sanitizer is essential to establish an effective program. Another key element that needs to be addressed is application of the sanitizer. These studies have also determined that current sanitation

programs are inefficient at removing bacteria from surfaces and therefore new technology needs to be developed in order to ensure elimination.

Electrostatic spray is application technology that has been utilized by the painting and agricultural industries, specifically pesticides (2,15). The general principle behind an electrostatic sprayer is to apply a charge to liquid droplets as they are sprayed through a nozzle (4). Specialized nozzles are used in electrostatic sprayers so that induction charging can occur (18). In these nozzles, a charge is placed on the cylinder by an electrode that is supplied with energy from a source such as a generator (12) When the liquid passes through this charged cylinder, the charge then transfers the free electrons that have formed to the liquid (8). The highly charged liquid then undergoes a shearing process, which results in the formation of atomized droplets (2). When the liquid is placed in this electric current the solution is broken up into small charged droplets (8). The force of electrostatic attraction is strongest if the droplets that are formed are small (4). Law (1982) also stated that the small droplet size allowed for optimal spray deposition density on the leaves of broccoli plants (11). It has been established that these charged droplets have a force of attraction 75 times greater than gravity (4). This means that when the droplets are sprayed from the nozzle they have the ability to move upwards thereby overcoming gravity and coat surfaces that are hidden (4).

A study has been conducted using electrostatic spraying as a means of applying sanitizers, specifically electrolyzed water (EO). In 2003, Russell conducted a study to determine if electrolyzed water sprayed electrostatically could reduce the presence of pathogens on the surface of eggshells. It was determined that the sanitizer, in conjunction with the electrostatic sprayer, was highly effective at eliminating the adhered pathogens,

Salmonella Typhinmurium, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* 0157:H7 from over 53% of the eggs (17).

Experimentation is required to determine if the use of electrostatic spray is a potential means for sanitation application. Therefore, the objective of this study was to determine if applying ala- quaternary ammonium sanitizer electrostatically to four different commonly used food industry materials. The materials evaluated were, tile, FRP (plastic wall board), plastic belting, and wire belts. (Table 3.1) Stainless steel belting and plastic belting are two common materials that are used to make conveyor belts, which are often used in the food production industry and serve as the food contact surfaces for this study. Tile and FRP are materials used in the food production industry as part of the environmental surfaces and therefore are used in this study as the non-contact surfaces.

Materials and Methods

Preparation of surfaces for attachment

The types of materials used and dimensions are provided in table 3.0. Twenty pieces of each material were used for each replication. Two parallel holes were drilled on the sides of the tile and FRP pieces to allow for 15.24 cm wire strips to be attached. Wire strips were added to two parallel sides of the materials, allowing for the pieces to be suspended from the end of the sprayer stand. Once the strips were attached, the pieces were then submerged in a water soap bath and allowed to soak for five minutes to allow for any organic matter to be removed. Then the pieces were scrubbed with a sterile scrub brush and rinsed with warm tap water for five minutes and then rinsed in distilled water to ensure that soap residue was removed from the materials. The pieces were placed under a hood and allowed to air dry for 30 minutes. After the pieces were dried, five

pieces of each material were randomly selected and swabbed (entire surface front and back) to validate that there were no bacteria present on the surfaces prior to inoculation. The swabs were then placed in 10 ml of sterile letheen broth in a glass test tube. Letheen broth is nutritious media that is used for neutralizing quaternary ammonium compounds.

Frozen cultures

Three strains of Streptomycin resistant *Listeria monocytogenes* were used which were determined in previous studies to form good biofilms. The strains used to make the LM cocktail were Scott A, Brie 1, and ATCC 7644, all of which have been associated with outbreaks of Listeriosis. Each strain was grown separately in tryptic soy broth (TSB) with Streptomycin at a concentration of 1000 μg/ml, incubated for 24 hours at 37 C. This process was repeated three times, after which 2.4 ml of the fresh culture were added to 240 mL of TSB with Streptomycin and incubated for 24 hours at 37 C. Centrifugation was used to separate the LM from the TSB (4,000 x *g*, 20 min, 1 C), and the broth was discarded in order to obtain the concentrated LM pellet. This process was conducted and repeated for each strain of LM. The LM pellets were then re- suspended together in 20 mL of fresh, sterile TSB with 1000 ug Streptomycin. 1 mL of the culture was pipetted into cryogenic vials and maintained in a freezer box at -80°C until the cultures were used.

To determine the total plate count of the cocktail, the cultures were removed from the -80°C freezer box and 1 ml of the culture was pipetted into 10 ml of sterile tryptic soy broth (TSB) (Oxoid Ltd., Hampshire, UK) then incubated at 37°C for 24 hours. After incubation the tube was removed and three replicate aliquots of 1 mL were pipetted out of the culture tube and transferred into 9 ml of TSB. The TSB culture tubes were also

incubated for 24 hours at 37°C. The cells were enumerated by spread plating onto duplicate tryptic soy agar (TSA) plates (Difco, Fisher Scientific Co. LLC, Pittsburgh, PA) and then incubated for 24 hours at 37°C. The cell suspension was determined to be 7.1 X 10^8 log cfu/ mL.

Inoculation

The LM frozen concentrated cocktail was added to 1000L of BPW to give a final concentration of 10^6 log cfu/ mL and poured in a sterile stainless steel tub. The materials were immersed for one minute, removed, and placed on racks under a hood to dry for one hour to allow the LM to attach to the materials. After one hour, five pieces of each material were randomly selected and swabbed to determine LM adherence. These pieces were not subjected to any treatment and therefore served as the control.

Treatment

The remaining pieces were then separated into two groups. Each group contained 5 pieces of each material. One group was sprayed with ala- quaternary ammonium (Birko Corp., Henderson, CO) via a pressure sprayer and the other group via an electrostatic sprayer (Electrostatic Spraying Systems, Watkinsville, GA) for ten seconds. The sprayer stand was an apparatus that held the sprayer in place and kept the nozzle three feet from the material being treated. At the end of the apparatus was a window in which a plexi glass could be inserted and removed to ensure that exposure to the ala- quaternary ammonium was accurate. Behind the plexi glass window was a hollow 40.64 X 25.40 cm metal square and this is where the metal strips could be inserted to secure the materials to the apparatus. Before spraying the pieces were suspended from the sprayer stand by attaching the metal strips to the apparatus. Prior to treatment application, the sanitizer

solution was tested with Serim Monitor for quaternary ammonium compounds ppm test strips (Serium Manufactoring Corp., Elkhart, IN) to determine the overall concentration of the sanitizer. The pieces were then sprayed with the 200 ppm ala- quaternary ammonium solution for ten seconds. The materials were then removed and suspended from a holding container for ten minutes to allow the ala- quaternary ammonium adequate time to disrupt the polysaccharide coating of the cell and eradicate the bacteria. After the 10 minutes had lapsed, the materials were then swabbed and the swabs were placed in 10ml letheen broth (BD Difco, Sparks, MD) tubes. Letheen was used to inactivate the quaternary ammonium used so that after the swab testing was conducted there was no further disruption of the cells.

Enumeration of micro-organisms

From the letheen tubes, the bacteria were plated onto modified oxford plates (MOX) (Difco, Fisher Scientific Co. LLC, Pittsburgh, PA) with a letheen overlay. Letheen agar (BD Difco, Sparks, MD) was used an overlay to help with the recovery of cells that were treated with quaternary ammonium. The LM were enumerated by spread-plating 100µl onto respective plates and incubating at 37^{o} C for 48 hours.

Statistical analysis

The experiment was performed in triplicate, and data was analyzed by a two-way analysis of variance for treatments and materials, which showed no interaction between the replication and treatment. Therefore, replications were pooled by treatment and type of material, means were separated using Duncan's multiple range tests of the Statistical Analysis System (SAS Institute Inc., Cary, NC). A significance level of $P < 0.05$ was employed to separate the means.

Results and Discussion

Listeria monocytogenes is commonly isolated from food surface areas (both contact and non- contact) (16). When LM is allowed to persist on equipment and surfaces within the processing environment, it can then lead to cross contamination, which has lead to outbreaks, and costly recalls (2, 8). The increases in the incidence of listeriosis outbreaks has lead to the USDA's zero tolerance policy on ready to eat foods making LM a very serious and costly problem for the food industry (9).

Listeria monocytogenes has been determined to have a high negative charge due to the presence of bound acidic compounds on the cell wall (1). Consequently, on the cell wall of LM there are several electron acceptor sites (1). These acceptor sites have a high affinity for positively charged compounds and therefore, the LM would readily take up positively charged compounds (1). Electrostatically spraying quaternary ammonium compounds would result in a more positively charged compound (10). The increase in positive charge could improve the overall effectiveness of the sanitizer at destroying bacteria with a negative cell wall. This study was conducted to investigate the efficacy of electrostatic spraying of ala- quaternary ammonium against Streptomycin resistant LM that have been attached to materials common to the food industry. The effectiveness of the treatment was determined by analysis of LM reduction after 10 minutes of exposure to the ala- quaternary ammonium.

Table 3.1 shows the effect the treatments had on the reduction of LM for each material. The data was compared to determine significant differences ($P < 0.05$) between the replications for each material. It was determined that no significant difference existed between the replications and therefore the data could be pooled together as one

replication. The pooled data was then analyzed to determine if there was a significant difference between the treatments per material. There was a significant difference between the treatments for the materials.

For the materials FRP, stainless steel, and tile it was observed that the electrostatic treatment was not significantly different from the air- pressure treatment, but both treatments were significantly less than the control. Therefore, regardless of the treatment it was the quaternary ammonium used that provided the overall reduction in LM on the treated surfaces. The ala- quaternary ammonium on FRP reduced the amount of LM present on the surface from 5.29 log CFU/ ml to 3.06 log CFU/ ml. On stainless steel the ala- quaternary ammonium reduced the amount of LM present on the surface from 3.57 log CFU/ ml to 2.92 and 2.74 log CFU/ ml. The ala- quaternary ammonium reduced the amount of LM present on the tile surface from 4.15 log CFU/ ml to 2.82 and 2.76 log CFU/ ml. Therefore on these materials the electrostatic spray was compatible with current application practices. One explanation for the electrostatic spray not out performing the air- pressure treatment could be the surface quality of these materials.

Due to FRP being made of a plastic wallboard, it reacted to the charged particles as an insulator. Charged particles will look for other grounded surfaces to land on before they land on the insulated surface (8). If the charged spray is directed at the insulated target, when it comes in contact with the surface it will lose it charge and therefore do not disperse as effectively (8). The uncharged particles no longer drifted in a spray cloud and could have been carried away from the plastic and towards another surface in the area (8). The drifting and inactivation of the particles could account for decreased effectiveness of the treatment.

The stainless steel belting used in this research was an 80% single loop belt. The design of the belt could have attributed to the poor conductivity of the material. The stainless steel loops had a very small diameter and large gaps between each connected loop. The small diameter of the stainless steel loops in conjunction with the dielectric constant of the free space (air) decreased the electric force of the atomized droplets. The materials interaction with the air dampened the electric field created by the electrostatic spray, therefore the spray was unable to overcome the kinetic energy of the material and produce the "wrap around" affect. This is possibly why there was no significant difference between the treatments.

Ceramic tile is commonly used as an electrical insulator in chemically and electrically hostile environments. One of the components of ceramic tile is lead. Lead is added to promote bonding of other constituents used to make ceramics. The use of lead to bind the materials in ceramics together creates an insulation effect. Also the ceramic tile that was used for this study had a hard glaze finish which is commonly used on tile and acts as a protective barrier. The hard glaze in this case acted the same way the heavy cuticle covering of a plant would (8). As studies have shown plants with greater amounts of waxy surfaces (cuticles) form a barrier to the charged particles (8). Therefore, the insulated ceramic tile induced the inactivation of the droplets and caused them to be dispersed in the same manner as the droplets that were dispersed via the air- pressure sprayer.

On the plastic belt the electrostatic spray was less effective than the air – pressure treatment. This resulted in less reduction in the amount of LM present on the surfaces after treatment. The tile treated with electrostatic spray and ala- quat reduced the amount

of LM present from 6.13 log CFU/ ml to 3.68 log CFU/ ml where as the air- pressure spray and ala- quat achieved a lower reduction of 3.27 log CFU/ ml. This effect can be attributed to the inert qualities of the plastic. Plastic acts as an insulator and causes the electrolyzed particles to inactivate and therefore lose the ability to over coming gravity (4). Another factor that could have lead to decreased impact of the treatment was the surface characteristics of the plastic belting. Studies have shown that LM grows and survives in niches and joints that are formed by equipment (17). The plastic belting had several niches and holes that acted as reservoirs for LM to potentially hide in and thus survive the treatment. The uncharged particles drifting away from the insulated material and the materials cosmetic characteristics are potential explanations for why electrostatic spray was less effective reducing LM on the surface of the plastic belting.

This study did determine that ala- quaternary ammonium was effective at reducing the number of LM that were attached to surfaces despite the application that was used. From table 3.1 it is evident that the electrostatic spray performed at the same level as the air- pressure sprayer so for a sanitation application it would not provide a more effective alternative program; although the changes that need to be made would be more directed to the materials studied and not the electrostatic sprayer itself. Further studies are needed to determine what physical characteristics of processing equipment could be changed to accept charged droplets, without compromising the surface.

Conclusion

For the purpose of this study, the use of electrostatic spray as a sanitizer application did not out perform the current methods of application. Though reduction was seen in the overall viable cell counts, this reduction was attributed to the efficacy of the

quaternary ammonium that was used not to the means of application. Electrostatic spray is a unique technology and if it could be formatted to overcome the surface qualities of equipment that are commonly found in the food industry, then it might be an invaluable sanitizing mechanism.

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Appendix A

Table 3.0 Different dimensions of materials with their composition and design

Materials	Design- % Open	Dimensions
Tile	Ceramic- 0%	10.16 cm X 10.16 cm
FRP	Plastic Wall Board- 0%	10.16 cm X10.16 cm
Stainless Steel	Single Loop- 80%	6.99 cm X 6.35 cm
Plastic Belt	Mesh Top- 24%	6.99 cm X 6.35 cm

Table 3.1: Effects of electrostatic spray and air- spray treatments on the reduction of *Listeria monocytogenes* on different materials measured in Log CFU/ mL.

Material	Control	Electrostatic Spray	Air- Pressure Spray
FRP	5.29^{a} +/- .18	3.06^{b} +/- .18	3.06^{b} +/- .18
Stainless Steel Belt	3.57^{a} +/- .18	2.92^{b} +/- .20	2.74^{b} +/- .19
Tile	4.15^{a} +/- .12	2.82^{b} +/- .12	2.76^{b} +/- .13
Plastic Belt	6.13^{a} +/- .11	3.68^{b} +/- .11	3.27^{c} +/- .11

^{a,b,c} superscripts within columns are different if $P < 0.05$

Means of triplicate measurements ± standard deviations

FRP n= 13, Stainless Steel n= 12, Plastic Belt n= 9, Tile n= 10

CHAPTER IV

COMPARISON OF SANITATION TREATMENTS ON THE REDUCTION OF BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES*

Introduction

Surfaces that have attached bacteria pose a serious problem for the food industry (7). Attached bacteria have the possibility to continue to grow and spread through the environment and can lead to biofilm formation (5). Biofilms are more resistant to cleaning and therefore if allowed to form on industry surfaces, they become a serious issue and can lead to virulent bacteria (5).

Biofilms have the ability to readily form in the food industry environment because of the availability of water, nutrients and surfaces for attachment (9, 14). This is a problem in the food industry because the hygiene of the surfaces affects the overall quality and safety of the food product (9, 11). The transfer of attached bacteria to a food product can lead to food spoilage or the transmission of diseases (3,15). Microbial contamination can also lead to mechanical blockage, corrosion of equipment, and overall impairment of the equipments intended functions such as heat transfer (15).

From studying the adherence abilities of LM it was deduced that not only does LM adhere to surfaces, but that this adhesion can turn into biofilm formation (12). Chavant and others (2002) studied LM's ability to form biofilms on stainless steel and polyetrafluoroethylene (PTFE) at 8, 20, and 37°C. The materials were placed in a petri dish containing 0.7 milliliters of LM bacterial suspension with a final concentration of 10^7CFU ml^{-1}. The surfaces were tested at two hours, six hours, and one, two, five and seven days. Biofilm formation occurred on all surfaces after two hours at both 20 and

37°C. This study showed that LM readily forms biofilms regardless of temperature, but the lower temperatures do slow the process and stainless steel showed a greater allowance for attachment (4).

A study was conducted to determine what effect sanitizers and contact surfaces had on the reduction of *Listeria* biofilms. *Listeria* was allowed to adhere to stainless steel coupons and plastic coupons for two days at 30°C (10). The coupons were then rinsed in PBS and dipped in either hypochlorite solution of 100- 200 ppm or a 10- 20 ppm iodophor solution or a combination of both sanitizers (hypochlorite 10 ppm and iodophor 1 ppm) for five minutes. Overall reduction by either sanitizer was most effective on the stainless steel coupons. . Hypochlorite was more effective at inactivating biofilm cells than iodophor on either material. The combination of chlorine and iodophor provided complete inactivation of biofilm cells. Therefore stainless steel and the combination of the sanitizers would provide the best possibility for the reduction of LM biofilms (10).

One option for reducing biofilms is using pre-treated materials, which can be sanitized before they have the potential to be contaminated by foodstuffs. In a prior study, the ala- quaternary ammonium was dispersed at concentration levels of 200 ppm or lower so that they will not affect the quality of any food they might come in contact with, but still be effective against LM (10). Pre- applying sanitizers might help in reducing a bacteria's ability to attach to survive and thus attach to the surface (6).

Electrostatic spray is application technology that has been utilized by the painting and agricultural industries, specifically pesticides (3,16). The general principle behind an electrostatic sprayer is to apply a charge to liquid droplets as they are sprayed through a nozzle (8). Specialized nozzles are used in electrostatic sprayers so that induction

charging can occur (15). In these nozzles, a charge is placed on the cylinder by an electrode that is supplied with energy from a source such as a generator (14) When the liquid passes through this charged cylinder, the charge then transfers the free electrons that have formed to the liquid (10). The highly charged liquid then undergoes a shearing process, which results in the formation of atomized droplets (3). When the liquid is placed in this electric current the solution is broken up into small charged droplets (10). The force of electrostatic attraction is strongest if the droplets that are formed are small (8). Law (1982) also stated that the small droplet size allowed for optimal spray deposition density on the leaves of broccoli plants (13). It has been established that these charged droplets have a force of attraction 75 times greater than gravity (8). This means that when the droplets are sprayed from the nozzle they have the ability to move upwards thereby overcoming gravity and coat surfaces that are hidden (8).

Listeria monocytogenes has been determined to have a high negative charge due to the presence of bound acidic compounds on the cell wall (2). Consequently, on the cell wall of LM there are several electron acceptor sites (2). These acceptor sites have a high affinity for positively charged compounds and therefore, the LM would readily take up positively charged compounds (2). Electrostatically spraying quaternary ammonium compounds would result in a more positively charged compound (10). The increase in positive charge could improve the overall effectiveness of the sanitizer at destroying bacteria with a negative cell wall. The objective of this study was to determine if electrostatically applying ala- quaternary ammonium sanitizer to food surfaces prior to bacterial interaction would help prevent biofilm formation.

Materials and Methods

Preparation of surfaces for attachment

The types of materials used and dimensions are given in table 4.0. Twenty- five pieces of each material were used for each replication. The pieces were then submerged in a water soap bath and allowed to soak for five minutes to allow for any organic matter to be removed. Then the pieces were scrubbed with a sterile scrub brush and rinsed with warm tap water for five minutes and then rinsed in distilled water to ensure that soap residue was removed from the materials. The pieces were placed under a hood and allowed to air dry for 30 minutes. After the pieces were dried, they were all mounted onto a white poster board. To suspend the pieces from the board and allow for maximum exposure, a small amount of adhesive was placed in the middle of the surface and attached to metal stubs. The metal stubs served as extra grounding for the materials as well as means to anchor the materials to the white poster board. There were two sets of boards in which the pieces were placed in rows making a 12.7 cm X 12.7 cm square in the middle of the board. Two parallel holes were made in the boards so that metal strips could be attached. The metal strips were used to fix the boards vertically in front of the sprayer apparatus so that the spray was directed horizontally towards the materials. The sprayer stand was an apparatus that held the sprayer in place and kept the nozzle three feet from the material being treated. At the end of the apparatus was a window in which a plexi glass could be inserted and removed to ensure that exposure to the ala- quaternary ammonium was accurate. Behind the plexi glass window was a hollow 40.64 X 25.40 cm metal square and this is where the metal strips could be inserted to secure the materials to the apparatus.

Frozen cultures

Three strains of Streptomycin resistant *Listeria monocytogenes* were used which were determined in previous studies to form good biofilms. The strains used to make the LM cocktail were Scott A, Brie 1, and ATCC 7644, all of which have been associated with outbreaks of Listeriosis. Each strain was grown separately in tryptic soy broth (TSB) with Streptomycin at a concentration of 1000 μg/ml, incubated for 24 hours at 37 C. This process was repeated three times, after which 2.4 ml of the fresh culture were added to 240 mL of TSB with Streptomycin and incubated for 24 hours at 37 C. Centrifugation was used to separate the LM from the TSB (4,000 x *g*, 20 min, 1 C), and the broth was discarded in order to obtain the concentrated LM pellet. This process was conducted and repeated for each strain of LM. The LM pellets were then re- suspended together in 20 mL of fresh, sterile TSB with 1000 ug Streptomycin. 1 mL of the culture was pipetted into cryogenic vials and maintained in a freezer box at -80°C until the cultures were used.

To determine the total plate count of the cocktail, the cultures were removed from the -80°C freezer box and 1 ml of the culture was pipetted into 10 ml of sterile tryptic soy broth (TSB) (Oxoid Ltd., Hampshire, UK) then incubated at 37°C for 24 hours. After incubation the tube was removed and three replicate aliquots of 1 mL were pipetted out of the culture tube and transferred into 9 ml of TSB. The TSB culture tubes were also incubated for 24 hours at 37°C. The cells were enumerated by spread plating onto duplicate tryptic soy agar (TSA) plates (Difco, Fisher Scientific Co. LLC, Pittsburgh, PA) and then incubated for 24 hours at 37°C. The cell suspension was determined to be 7.1 X 10^8 log cfu/ mL.

Treatment

One board was sprayed with ala- quaternary ammonium via a pressure sprayer and the other board via an electrostatic sprayer. The sprayer stand apparatus held the sprayer in place and kept the nozzle 3 ft from the material being treated. Prior to treatment application, the sanitizer solution was tested with ppm strips to determine the overall concentration of the sanitizer. After the boards were secured to the apparatus, the pieces were then sprayed with the 200ppm ala- quaternary ammonium solution for ten seconds. The boards were removed and placed in a vertically in a holding container for 30 minutes. This allowed the ala- quat adequate time to dry onto the materials. The materials were then collected in labeled zip lock bags.

Inoculation

A LM frozen concentrated cocktail was added to 1000L of BPW to give a final concentration of 10^6 log cfu/ mL. All five pieces of each material per treatment were added to a well and five mls of the inoculated solution was pipetted into each well. The well plates used were six- well plates with a growth area of 9.6 cm^2. Controls of each material were made by placing non- treated materials in wells with the inoculation solution. The wells were then placed in an incubator for 24hrs at 37°C.

Enumeration of micro-organisms

After the 24 hours of incubation the wells were removed for crystal violet assay to determine the amount of biofilm formation. For crystal violet assay non- treated, non-inoculated pieces of the materials were also analyzed to determine the amount of crystal violet absorbed that was absorbed by each material. Under a hood, the inoculated solution was pipetted out of the wells and the materials were removed with sterile forceps. The

materials were rinsed with distilled water and placed in clean wells. Then five mls of 0.1% crystal violet solution was placed in each well and allowed to sit for 30 minutes. At the end of 30 minutes the pieces were removed with sterile forceps and rinsed by a two-dip method. They were first dipped in a beaker of distilled water and then in another beaker of distilled water to remove any remaining crystal violet. The rinsed materials were then placed in clean wells. Once the materials were placed in the clean wells, three mls of a 95% ethanol solution was added to the wells to remove any remaining crystal violet. The ethanol/ crystal violet solution was pipetted out of the wells and placed in cuvettes. The cuvettes were read using spectrophotometer at an absorbency of 595 nm. Final absorbency was determined by subtracting the amount of crystal violet that each non- treated, non- inoculated material absorbed from the absorbency of the experimental readings. This was done to separate the amount of crystal violet that was absorbed by the material and by the biofilm.

Scanning electron microscopy

Two pieces of each material, for each treatment were placed in a well with five mL of inoculated solution and incubated for 24 hours at 37°C. The inoculated medium was removed from the wells by pipetting the medium out of the wells. The test surfaces were rinsed with distilled water and then placed in new wells. Then five mls of a mixed fixative of 1% glutraldehyde, 1% paraformaldehyde and 4% osmium tetraoxide was added to the wells and allowed to sit for 30 minutes at room temperature. The fixative was then pipetted out and the materials were dehydrated in ascending concentrations (70%, 80%, 95%, and 100%) of ethanol for five minutes at each concentration. The materials were then placed under a hood and allowed to dry completely. The materials

were mounted on aluminum stubs and sputter-coated for 2 minutes with gold-palladium in a sputter coating machine (Technics Hummer V sputter coater). The samples were examined under a Hitachi S-570 scanning electron microscope (SEM). SEM was used to quantify the absorbency readings from the crystal violet assay.

Statistical analysis

The experiments were performed in triplicate, and data were analyzed by a two-way analysis of variance for absorbency and materials, which showed no interaction between the two. Means were separated using Duncan's multiple range tests of the Statistical Analysis System (SAS Institute Inc., Cary, NC). A significance level of $P < 0.05$ was employed to separate the means.

Results and Discussion

Bacterial attachment is a large problem in the food industry because after the bacteria attach to a surface it can then form into a biofilm, which is more resistant to sanitizers than planktonic cells (15). The ability of *Listeria monocytogene* to adhere and form biofilms on surfaces regardless of temperature makes it a very serious sanitation problem for food processing plants (1). Biofilms have the ability to readily form in the food industry environment because of the availability of water, nutrients and surfaces for attachment (9, 14). This is a problem in the food industry because the hygiene of the surfaces affects the overall quality and safety of the food product (9, 11). The transfer of attached bacteria to a food product can lead to food spoilage or the transmission of diseases (3,15). Microbial contamination can also lead to mechanical blockage, corrosion of equipment, and overall impairment of the equipments intended functions such as heat transfer (15). Due to the frequency of biofilm formation on industry surfaces and in

industry environments, this study was conducted to investigate the possibility of a pre-treatment of equipment by spraying ala- quaternary ammonium against Streptomycin resistant LM strain on food industry materials prior to bacterial exposure. The effectiveness of the treatment was determined by crystal violet assay and direct observation of biofilm formation using SEM.

The treatments and the materials showed a significant difference in the amount of biofilms that formed (Table 4.1). Electrostatic spray with ala- quaternary ammonium was effective at preventing biofilm formation on all materials. As illustrated in Table 4.1, electrostatic spray was able to inhibit biofilm formation on all the materials significantly better than the air- pressure spray treatment. The FRP that was pre- treated with air-pressure spray and ala- quat resulted in a significant reduction in the controlled absorbency from 1.09 nm to 0.88 nm. The FRP that was pre-treated with electrostatic spray and ala- quat, achieved a greater reduction of 0.19 nm. After the stainless steel was pre- treated with air- pressure spray and ala- quat there was significant reduction in the controlled absorbency from 0.39 nm to 0.29 nm. The pre- treatment with electrostatic spray and ala- quat on stainless steel produced a greater reduction of 0.19 nm. After the plastic was pre- treated with air- pressure spray and ala- quat, there was not a significant reduction in the controlled readings; where as the pre- treatment with electrostatic spray and ala- quat on plastic did significantly reduced the controlled absorbency from 0.23 nm to 0.16 nm. After the tile was pre- treated with air- pressure spray and ala- quat there was not a significant reduction in the controlled absorbency; where as the pre- treatment with electrostatic spray and ala- quat on tile did significantly reduced the controlled absorbency from 1.95 nm to 1.08 nm.

From the SEM images it can be determined that not only was biofilm formation minimal, but that the LM remained planktonic. An example of cells remaining planktonic can be seen in figures 4.3 and 4.6. These cells remain single celled in suspension and do not aggregate to form a biofilm. In the figures 4.3 and 4.6 the cells remained as single rod cells can in suspension. This is visual evidence that the cells remained planktonic after treatment. This is important because as a biofilm, LM is protected from sanitizers (1). The ability to prevent biofilm formation on surfaces in the industry would help the efficacy of current sanitation procedures because planktonic cells are not protected from sanitizers like biofilms.

Tile and FRP had the greatest amount of control over biofilm formation as compared to the other materials. The images from the SEM confirm the absorbency readings from the crystal violet assay. This indicates that, a pretreatment of electrostatic spray would be beneficial on non- contact surfaces. Therefore on clean floors and walls, the use of electrostatically spraying ala- quaternarny ammonium could help prevent LM from forming into a biofilm. If LM is prevented from forming a biofilm, then it can more easily be removed by cleaning and sanitation procedures. Therefore, the incidence of environmental cross contamination could significantly be decreased. The decrease in environmental cross contamination could lead to a safer food product and benefit both the producer and the consumer.

The control on biofilm formation on the stainless steel and plastic materials was significant and the images from the SEM confirm the control. This indicates that, pretreatment with electrostatic spray would be beneficial on contact surfaces. Therefore on conveyor belts, plastic cutting boards, and stainless steel equipment the use of

electrostatically spraying ala- quaternary ammonium could help prevent LM from forming into a biofilm. This would make cleaning and sanitizing of equipment more efficient and help prevent LM from coming in direct contact with food products. This could help reduce the amount of LM outbreaks and recalls, saving the industry millions of dollars and their reputation.

The success of the electrostatic spray as a pretreatment in this study was attributed to increased droplet distribution of the ala- quaternary ammonium over the surfaces. The increase in droplet distribution also increased the amount of the material's surface that came in contact with the ala- quaternary ammonium. The increase in the amount of surface that was covered in ala- quaternary ammonium resulted in more control of biofilm formation of LM than was observed in the materials that were not treated with electrostatic spray.

The only aspect of this study that should be changed is the addition of other materials. This would give a broader spectrum of ability of the electrostatic sprayer application of ala- quaternary ammonium in control the ability of LM to form biofilms. This change would help industry individuals determine whether or not this application would be a benefit to their specific environment.

Conclusion

The use of the electrostatic sprayer as a pretreatment was shown to be very effective at controlling biofilm formation. All of the materials treated electrostatically had significantly less biofilm formation and therefore it can be concluded that if used prior to contamination the electrostatic sprayer could essentially control biofilm formation on industry equipment.

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Appendix B

Table 4.0 Different dimensions of materials with their composition and design

Materials	Design- % Open	Dimensions
Tile	Ceramic- 0%	2.22 cm X 2.22 cm
FRP	Plastic Wall Board- 0%	2.22 cm X 2.22 cm
Stainless Steel	Food Grade Sheet Metal- 0%	2.54 cm X 2.54 cm
Polyethylene	Plastic Cutting Board- 0%	2.22 cm X 2.22 cm

Table 4.1: Effects of pre-treatments applied electrostatically and via an air- pressure sprayer on the control of LM biofilm formation on different materials measured as absorbency on a spectrophotometer at 595 nm.

Material	Control	Electrostatic Spray	Air- Pressure Spray
FRP	1.09^{a} +/- .05	0.21^{c} +/- .05	0.89^{b} +/- .05
Stainless Steel	0.39^{a} +/- .01	0.19^{c} +/- .01	0.29^{b} +/- .01
Plastic	0.23^{a} +/- .01	0.1642^{b} +/- .01	0.2272^{a} +/- .01
Tile	1.95^{a} +/- .06	1.08^{b} +/- .06	1.8^{a} +/- .06

^{a,b,c} superscripts within columns are different if $P < 0.05$

Means of triplicate measurements ± standard deviations

FRP n= 15, Stainless Steel n= 14, Plastic Belt n= 15, Tile n= 15

SEM Images

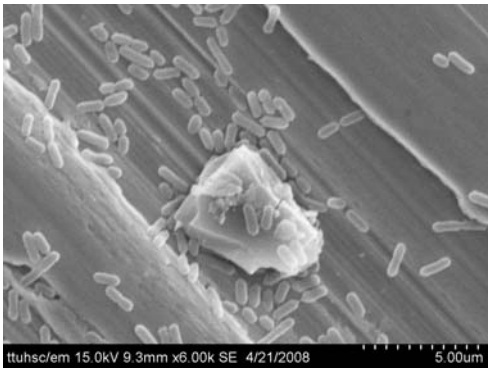

Figure 4.1 Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on untreated stainless steel. Magnification was at 6.00 k.

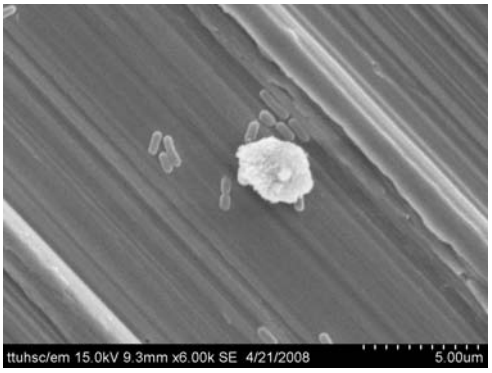

Figure 4.2. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on stainless steel treated with ala-quaternary ammonium via air pressure sprayer. Magnification was at 6.00 k.

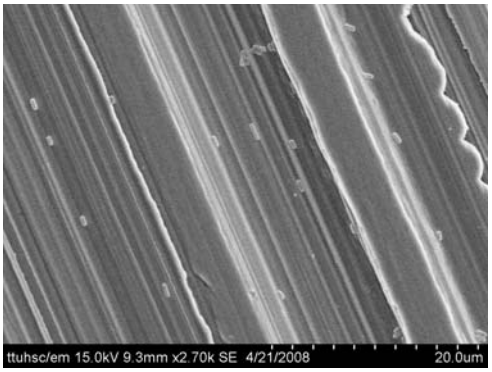

Figure 4. 3. Scanning electron micrograph showing *Listeria monocytogenes* colonies formed on stainless steel treated with ala-quaternary ammonium via electrostatic sprayer. Magnification was at 2.70 k.

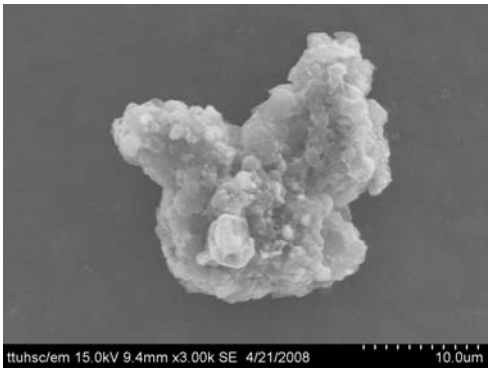

Figure 4. 4. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on untreated plastic. Magnification was at 3.0 k.

Figure 4. 5. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on plastic treated with ala-quaternary ammonium via air pressure sprayer.. Magnification was at 6.0 k.

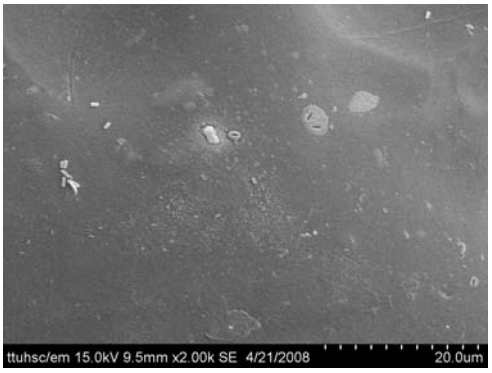

Figure 4. 6. Scanning electron micrograph showing *Listeria monocytogenes* colonies formed on plastic treated with ala-quaternary ammonium via electrostatic spray. Magnification was at 2.0 k.

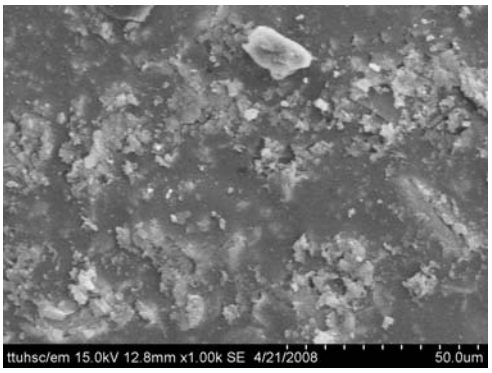

Figure 4. 7. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on untreated tile. Magnification was at 1.0 k.

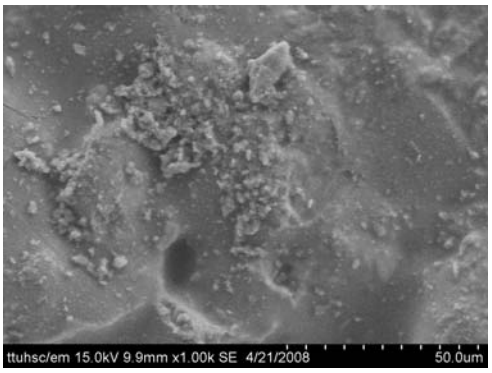

Figure 4. 8. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on tile treated with ala- quaternary ammonium via air pressure sprayer.. Magnification was at 1.0 k.

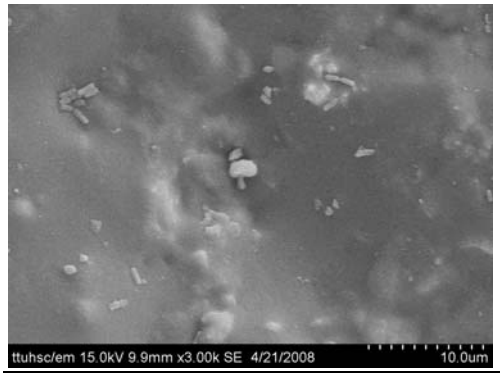

Figure 4. 9. Scanning electron micrograph showing *Listeria monocytogenes* colonies formed on tile treated ala- quaternary ammonium via electrostatic sprayer. Magnification was at 3.0 k.

Figure 4.10. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on untreated FRP. Magnification was at 6.0 k.

Figure 4.11. Scanning electron micrograph showing *Listeria monocytogenes* biofilm formed on FRP treated with quaternary ammonium via air pressure sprayer. Magnification was at 1.0 k.

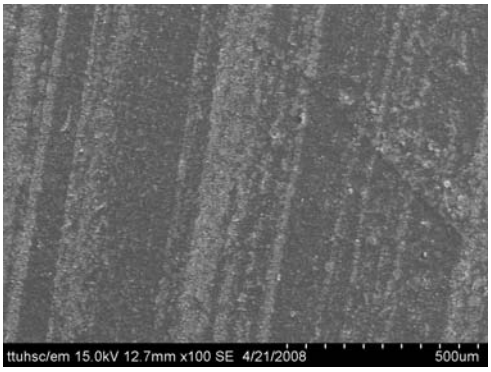

Figure 4.12. Scanning electron micrograph showing *Listeria monocytogenes* colonies formed on FRP treated with ala- quaternary ammonium via electrostatic sprayer. Magnification was at 1.0 k.

CHAPTER V

Conclusion

This study determined that the electrostatic spray could potentially be implemented into a sanitation program. Electrostatically spraying ala- quaternary ammonium resulted in reductions of LM similar to those obtained with the air- pressure sprayer. Therefore as a sanitation application the electrostatic sprayer provides the similar results as the air- pressure sprayer.

It can only be inferred from previous studies that the reason the electrostatic sprayer did not out perform the air- pressure sprayer is attributed to the inert qualities of the materials being treat. Further studies would have to be conducted to determine if this was in fact the reason electrostatic spray did not increase ala- quaternary ammonium's ability to reduce the number of LM present on the surfaces.

The electrostatic sprayer was determined to be effective as a pre-treatment. With the use of the electrostatic sprayer and ala- quaternary ammonium, the ability of LM to form biofilm was significantly reduced. It was determined that with this pretreatment many of the LM cells remained planktonic on the surfaces. Keeping the cells from forming biofilms would help improve the efficiency of cleaning and sanitizing programs. The use of electrostatic spray as a pretreatment would greatly benefit the industry.

This study determined that the electrostatic sprayer is comparable to current means of sanitation application and that further study needs to be done to improve this technology. It also determined that the electrostatic sprayer was a superior pretreatment and controlled biofilm formation of LM.

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