

Quantitative Evaluation of Recovery Methods for *Listeria monocytogenes* Applied to
Stainless Steel

Suk-Kee Kang

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Joseph D. Eifert, Committee Chair
Robert C. Williams
Steven Pao

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ABSTRACT

The ability of *Listeria monocytogenes*, to attach to various food contact surfaces such as stainless steel, polypropylene, and rubber compounds is well documented. The retention of these or other pathogenic bacteria on food contact surfaces increases the risk of transmission to food products. The objective of this study was to compare several methods for quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces. A cocktail of four serotypes of *Listeria monocytogenes* (Scott A (4b)), 1/2b, 3b, and 4b) were mixed in equivalent concentrations and inoculated onto type 304 stainless steel coupons in a 2cm x 2cm area. After a one hour exposure, coupons were sampled by one of the following methods: 1) swabbing using a pre-moistened Dacron swab, 2) rinsing with phosphate buffered saline, 3) direct contact onto a Tryptic Soy Agar containing 0.6% yeast extract (TSA+YE) plate surface for 10 seconds, 4) sonication in an ultrasonic water bath (40 kHz), 5) contact with the bristles of a sonicating brush head for 1 min, and 6) indirect contact (2-4 mm) with the bristles of a sonicating brush head for 1 min. Coupon rinses were plated onto TSA containing 0.6% yeast extract and incubated for 24 hours at 35°C. The three sonication methods yielded higher recovery than the other three methods ($p < 0.05$). Brushing the coupons with the sonicating brush head yielded a recovery level of 58% and indirect exposure to the sonicating brush head permitted a recovery level of 65% from the initial microbial load. The lowest cell recovery (~20%) was observed with the swab and direct agar contact methods.

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DEDICATION

I dedicate this body of work to my family and friends, for without their support and encouragement, I would not have been able to complete this journey.

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CHAPTER I

INTRODUCTION AND JUSTIFICATION

The retention of bacteria on food contact surfaces increases the risk of potential transmission of pathogens to food products. Exposure to pathogenic bacteria on surfaces may occur either by direct contact with contaminated surfaces or indirectly by atomization. As a group, bacteria are well adapted to life on surfaces and may have a selective advantage when attached (Marshall, K.K, 1992). *Listeria monocytogenes* is a prominent foodborne pathogen known for its ability to withstand less than favorable environments. It is ubiquitous and psychotropic in nature, making control of it especially difficult. This organisms' ability to attach to various food contact surfaces such as stainless steel, polypropylene, and rubber compounds is well documented (Sinde and Carbello, 2000). The presence of *Listeria monocytogenes* in RTE foods is widely believed to occur due to post process procedures rather than being due to survival during the processing itself (McLauchin, 1987). In the food plant, it can survive on almost every surface (Besse, 2002; Wang, *et al.* 2003). In the onset of *Listeria monocytogenes* contamination in the processing environment, control of the organism has proven to be difficult (Smoot and Pierson, 1998). The recovery pathogenic organisms such as *Listeria monocytogenes* from specific food manufacturing environments strongly suggests that contamination may occur during the production of other foods in the processing plant and it may be a hygiene / cleaning protocol problem. Foodborne illness could be reduced through increased efforts to prevent these organisms from attaching to food contact

surfaces and improved methods for the removal of these organisms from food contact surfaces.

Accurate detection and enumeration of microbial contaminants, either by use of conventional sampling techniques or novel methods employing sonication or ultrasonics, relies initially upon mechanical action to effectively dislodge and remove the contaminant from the surface. It is also imperative that the method employed also releases the microorganisms from potential devices used for subsequent recovery and cultivation. Some methods are inherently more effective at the removal from the soiled surface and recovery is optimized.

As Moore, *et al.* (2002a,b) states, in the use of swabs for recovery of microorganisms from surfaces, there exists a lack of standardization of both the swabbing pattern and the pressure applied to the swab during sampling, meaning, technician- to- technician variation in the sampling procedure may potentially play a significant role in the recovery and enumeration of the sampled surface. This can lead to misleading determinations of the initial cleanliness of the surface or the effectiveness of the cleaning procedure used in processing plant. Such risks play a major factor in cross contamination, which can ultimately lead to premature spoilage of food products and in certain cases, lead to foodborne diseases. While many surface sampling techniques are available, their effectiveness as a reliable quantitative technique varies greatly.

The purpose of the study is to make a contribution to the methodology in the field of quantitative microbiological sampling of food contact environments, using *Listeria monocytogenes* attached onto stainless steel. It will also consider effectiveness of experimental design, particularly monitoring for results that may not be as sensitive in

enumeration as other sampling techniques and also for results that may be invalidated due to the presence of excessive, uncontrolled variability and data scattering due to technician to technician variability.

LITERATURE REVIEW

Listeria monocytogenes

Characteristics of the organism

The genus *Listeria* has six species currently recognized: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (Gasarov, *et al.* 2005). *Listeria* species are Gram positive, non-sporeforming short rods (0.5 x 1-2 μm) occurring singly or in short chains that are widely distributed in the environment. It is catalase positive, oxidase negative, and shows β -hemolysis on blood agar. *Listeria* species are characterized as being motile, facultative anaerobes which prefer microaerophilic conditions. *Listeria monocytogenes* grows well on nutritionally rich media such as brain heart infusion agar (BHIA) and tryptic soy agar (TSA). It can propagate up to 10% NaCl, however, *L. monocytogenes* is able to survive in salt concentrations as high as 25% at 4°C (Hitchens, 1996).

Listeria monocytogenes is psychrotrophic in nature. It can grow at temperatures ranging from 2°C to 45°C, but the optimum temperature range is 30-37°C (Low and Donachie, 1997; FDA, 2005). *Listeria* grown at 37°C show little or no motility but tumbling motility can be detected by the incubation of cultures at approximately 20°C with motile organisms having peritrichous flagellae (Low and Donachie, 1997). *Listeria* may swim with 1 to 5 flagella to invade human cells (CDC PulseNet, 2005). Its ability to survive and grow at low temperatures in food products and food contact surfaces provides an advantageous niche from other food contaminant species (Bayles and Wilkinson, 2000). Once present in the processing facility, *L. monocytogenes* has been hard to control. Food facilities and processing plants that operate under low temperatures

may be more susceptible to the presence of *L. monocytogenes*. Although refrigeration conditions and high osmotic strength environments are strategies commonly used to control bacteria in ready-to-eat foods, the conditions mentioned provide an advantage for survival and growth *Listeria monocytogenes* over other microflora.

L. monocytogenes can also grow at a relatively wide pH range, from 4.8 to 9.6 (FDA, 2005). It has been documented to survive for long durations of time at a pH of 5 in such products as cheddar cheese (Ryser and Marth 1987). In meats, it is reported that it grows poorly or not at all at a pH near or below 5.0 and well at pH 6.0 and above (Post, 2004).

Distribution

Listeria monocytogenes is ubiquitous in nature, and can be found in water samples, soil, feces, as well as plants (Farber and Peterkin, 1991). Moreover, it can be found on a wide spectrum of foods, with it having being associated with fresh produce, poultry, and dairy products (Ryser and Marth, 1987; Farber, *et al* 1996; Ray, 2004; FDA, 2005) and it has been isolated in various animals (Cliver, 1990; Low and Donachie 1996). It has been found in 37 species of mammals and 17 species of birds. *Listeriae* have been identified in fish, shellfish, and molluscans as well (Elliott and Kvenberg, 2000). Through the FSIS Nationwide Sampling Programs, prevalence of *Listeria monocytogenes* have been reported as 30.5% for raw ground turkey, 41.5% for raw ground chicken, 11.7% for raw ground beef, 5.9% for turkey carcasses, 15% for broiler chicken carcasses, 7.4% for market hog carcasses, and 11.3% for cow/bull carcasses (USDA-FSIS, 1999). Since 1989, USDA-FSIS has conducted finished product testing

for *L. monocytogenes* in several RTE foods, in their finding to date, 5.7% of deli meats such as sliced ham, 4.4% of frankfurters, 3.4% of salads and spread, and 3.1% of roast/corned/cooked beef tested positive for the presence of *L. monocytogenes* (USDA-FSIS, 1999). It has also been suggested that 1-10% of humans are intestinal carriers of the organism (FDA, 2005). Hof (2003) states that 90% of adults possess immune lymphocytes to pathogenic *Listeriae*.

Listeriosis

Listeriosis was initially recognized as a disease of animals following the initial isolation and description by J. Pirie in 1925 and Murray and colleagues in 1926, who were working independently of each other (McLauchlin, 1996; Post, 2004). In the 1980s, however, it was described as a food borne human disease that fueled research of *Listeria monocytogenes* as a food borne pathogen (McLauchlin, 1996; Low and Donachie, 1997; Elliott and Kvenberg, 2000).

Infection may present itself as meningitis, sepsis, or more rarely, encephalitis. Listeriosis is also of great concern due to the possible onset of abortion of the fetus (50% of cases) in pregnant women, as well as immunocompromised, and the very young and the elderly (McLauchlin, 1996; Low and Donachie, 1997; Hof, 2003; Rocourt, *et al.*, 2003; Post, 2004, CDC Pulsenet, 2005). Hof (2003) states, "Pregnant women have a 12-fold increased risk in comparison with the normal population to acquire listeriosis after consumption of contaminated foods." Moreover, the acquired immune deficiency syndrome (AIDS) provides the highest risk population for listeriosis in North America. AIDS patients have an estimated 100-500 fold increased risk over the normal population

(Schlech, 1996). Other patients that are immunocompromised, such as those who have had organ transplants, diabetes, chronic liver disease, renal disease, and cancer patients undergoing chemotherapy have an elevated risk of listeriosis than the normal population. Although foodborne listeriosis has a low incidence rate, it is of great concern due to the high case fatality rate – at the low end of the spectrum to be about 20% to the mortality rate being suggested to be as high as 50% (McLauchlin, 1996; Rocourt, 1996; Elliot and Kvenberg, 2000). The infective dose of *L. monocytogenes* is unknown, but is believed to vary with the strain type and overall susceptibility of the host. From cases contracted through raw or under pasteurized milk, it is presumable that fewer than 1000 total organisms may cause disease in susceptible populations (FDA, 2005). As reported for the U.S. population, listeriosis annually accounts for roughly 2,500 cases and 500 deaths (USDA-FSIS, 2003; CDC PulseNet, 2005). However, due to lack of reporting for typical foodborne illnesses, the incidence rate may be much higher. The onset of symptoms of listeriosis may be as short as one day to as long as 70 days for some patients (McLauchlin, 1996). The Center for Food Safety and Applied Nutrition of the FDA (2005) states, “The onset time to serious forms of listeriosis is unknown but may range from a few days to three weeks. The onset time to gastrointestinal symptoms is unknown but is probably greater than 12 hours.”

Host susceptibility plays a key role in listeriosis as well as the bacteria’s virulence. Abnormalities in T-cell immunity are clearly associated with an increased risk of listeriosis. Incidences as described above reflect this and the age of the potential host weigh in as a factor for susceptibility as well. The immunosuppression associated with

pregnancy and the multiple immune defects of even normal newborn for being among the highest at risk population reinforce this concept. (Rocourt, 1996; Schlech, 1996).

Listeria monocytogenes associated gastroenteritis

Non-invasive listeriosis caused by *Listeria monocytogenes* associated gastroenteritis has also been described. It affects healthy individuals and usually presents symptoms more than 12 hours after the ingestion of the contaminated food. Symptoms of *L. monocytogenes* associated gastroenteritis are primarily those associated with gastrointestinal illness: chills, diarrhea, headache, abdominal pain and cramps, nausea, vomiting, fatigue, and myalgia (FDA, 2005). A variety of foods have been implicated as the vehicle of infection (CDC PulseNet, 2005). A high potential for underreporting listerial gastroenteritis exist because symptoms are mild, and it is more likely that foodborne diseases in general often go unreported by the sufferer. Data is not currently available through foodborne surveillance mechanisms such as FoodNet to record the incidence of listerial gastroenteritis since routine stool cultures do not include evaluation for *Listeria monocytogenes*, nor do other parts of the world report gastroenteritis from exposure to the pathogen (FDA, 2005; Makino, *et al.* 2005). Gastrointestinal and other mild symptoms were reported in individuals with no known underlying predisposition, and in examining the product that caused the disease, there was evidence of very high levels of food contamination. In a study reporting of an outbreak of food-borne listeriosis due to cheese in Japan in 2001, in environmental samples as well as in samples of the tainted cheese, concentrations of the organism found on samples were reported ranging from < 30 to $4.6 \times 10^9 / 100$ g of *Listeria monocytogenes* serotype 1/2b. *Listeria*

monocytogenes was not initially suspected due to the patients' mild symptoms who consumed the contaminated food (Makino, *et al.* 2005). It is likely that in normal individuals, listerial gastroenteritis may be associated with exposure to high levels of *Listeria monocytogenes*. It is possible that this manifestation of *Listeria monocytogenes* infection is a different disease compared to invasive and more severe listeriosis. Makino, *et al.* (2005) also suggests that due to the irregularity of monitoring for *Listeria monocytogenes* for foodborne gastroenteritis, rates of infection with *Listeria* may have been grossly underestimated to date and that testing for *Listeria* should be performed routinely for cases of gastroenteritis.

Pathogenesis

An infective dose of *Listeria monocytogenes* most commonly enters the body through consumption of contaminated food. The bacteria are capable of entering the host cell, evading the host cell's defense mechanism, multiplying within the cytoplasm, and spreading between the cells (Low and Donachie, 1997; Hof 2003). Though the mechanisms of intracellular multiplication have been identified, the means for bacterial adherence to cells are still of great interest (Low and Donachie, 1997). It has been proposed that a cellular surface protein of *Listeria monocytogenes* (internalin) interacts with receptors in the intestinal lining and induces phagocytosis of the bacterium (Brooks *et al.*, 1998). The secretion of a hemolysin (which is commonly used in serotyping within the species), listeriolysin O (LLO) is a crucial virulence factor of the organism. The production of LLO has been linked to the growth environment, where production of LLO increased with the deterioration of optimal environmental sublethal factors such as

lowering of pH in the environment and variance in temperature. This enables the organism to escape intracellular destruction within macrophages by lysis of the phagosomal membrane and escape into the cytoplasm (Low and Donachie, 1997). Movement within the host cytoplasm follows envelopment of the bacterium by polymerizing actin filaments from the host cytoskeleton to propel them towards the host cell membrane which they push on and create elongated protrusions into neighboring cells (Brooks et al., 1998; Hof, 2003). Other bacteria such as *Shigella flexneri* and *Rickettsia rickettsiae* use similar methods of evasion of immune response phagocytic cells and infection of the host body (Brooks et al., 1998). Although there are six species of *Listeria*, only *L. monocytogenes* is highly regarded as a foodborne pathogen; and within the species, pathogenicity in man has been correlated to a few distinct serotypes (Rocourt, 1996; Hof, 2003).

Serotyping strains

Serotypes of *Listeria* strains are identified based on somatic or O antigens (1-4) and flagellar or H (a-e) antigens. There are sixteen serotypes known for the genus *Listeria* and thirteen serotypes known for *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Low and Donachie, 1997; Allerberger, 2003). For *L. monocytogenes*, two primary divisions have been delineated, with serovars 1/2b, 3b, and 4b falling into one division and serovars 1/2a, 1/2c, and 3a falling into the other based on multilocus enzyme electrophoresis (MEE) analysis, which defines and compares the genetic relationships among the electrophoretic types (Low and Donachie, 1997). The identification of serotypes serves as a useful tool for epidemiological purposes.

Epidemiological data suggests that serotype 1/2 causes approximately 60% of sporadic cases, and serotype 4b causes approximately 29% of cases (Schlech, 1996). Rocourt (1996), however, noted that other studies report serovar 4b being responsible for 45-70% of sporadic cases of listeriosis. Among *Listeria monocytogenes* strains, serotype 1/2a, 1/2b, and 4b have been implicated in 90% of outbreaks of listeriosis (Gasnov, *et al.* 2005). Results of serotyping *L. monocytogenes* strains have established that most strains accounting for human infection differ in certain respects from strains typically found in foods as contaminants (Rocourt, 1996, Gasnov *et al.* 2005).

Recent studies examining large numbers of serotypes of *Listeria monocytogenes* have noted significant differences in the ability of some isolates to adhere and adsorb to stainless steel (Lund, *et al.*, 2000; Kalmokoff, 2001). Kalmokoff (2001) reported variances among strains in terms of the number of cells adhering to the model surface. Serotype 1/2b demonstrated less variability when compared to 1/2a and 4b serogroups, but they stated that their finding may only reflect the limited numbers of strains tested within each serogroup. It was also noted that no apparent trends in terms of an enhanced level of adherence among either the food or clinical isolates – including those from foodborne outbreaks were observed. However, marked differences were observed in cell numbers between the short term adsorption experiments and in the number of adhered cells found on stainless steel chips processed for SEM observations. *Listeria monocytogenes* strain Scott A on the surface after processing for SEM was approximately 10X lower than that found on the short term adsorption assay. Other strains demonstrated 100X fewer cells when compared between SEM analysis and short term adsorption analysis. They concluded that the differences between the short term

adsorption assay and the biofilm assay could be attributed to differences between cells which are 'adsorbed' vs. cells that had 'adhered' to the surface, as with biofilm formation. In a study performed by Lund, *et al.* (2000), difference in short term adsorption of persistent vs. non-persistent strains of *Listeria monocytogenes* onto stainless steel was observed. Strains within the 1/2c serotype demonstrated the highest degree of adsorption.

Foodborne transmission

The general consensus in the scientific community is that the consumption of contaminated foods is the principle route of transmission for listeriosis, although rarely listeriosis can be transmitted through direct contact with infected animals or cross infection during the neonatal period (McLauchlin, 1996; Norwood and Gilmour, 1998). Most cases of listeriosis outbreaks are attributed to foods that were generally designed as read-to-eat (RTE) foods. RTE food products are routinely heat treated and receive a high degree of processing (McLauchlin, 1996). The presence of *Listeria monocytogenes* in RTE foods is widely believed to occur due to post process procedures rather than being due to survival during the processing itself (McLauchlin, 1987). Food items that have been linked to the outbreak of listeriosis include dairy, soft cheeses, coleslaw; meat products such as undercooked poultry, uncooked hotdogs and deli meats; seafood; and vegetable based products (CDC 1985; McLauchlin, 1996; Elliot and Kvenberg 2000; CDC 2002; CSFAN-FDA, 2003). The recovery of the strains from specific food manufacturing environments further solidify suggestions that contamination is highly likely to occur during the production of other foods in the processing plant and it may be a hygiene / cleaning protocol problem.

Presence in food and food processing environment

The landmark case of listeriosis occurred in 1985, where between January 1, and June 14, 1985, 86 cases of *Listeria monocytogenes* infections were identified in Los Angeles and Orange Counties, California. Of those cases, 58 of the cases were among mother-infant pairs. Twenty-nine deaths occurred with eight neonatal deaths, 13 stillbirths, and eight non-neonatal deaths. The consumption of Mexican style soft cheese was implicated in the illness, and it was confirmed when samples of Mexican-style cheeses from three different manufacturers purchased from markets in Los Angeles were cultured at CDC. Four packages of Jalisco cheese products grew *Listeria monocytogenes* serotype 4b. Resultant of the outbreak, the manufacturer instituted a voluntary recall of the implicated cheese products. Television, radio, and newspaper announcements were made warning the public against ingestion of cheeses manufactured in the Jalisco plant. (CDC, 1985).

In late 2002, a RTE deli turkey meat was attributable for another multi-state outbreak of listeriosis. The outbreak spanned across eight states in the northeastern region of the United States, with a total of 46 reported cases. Seven fatalities and three stillbirths were resultant from the outbreak. One food sample and 25 environmental samples from where the turkey meat was produced were positive for *Listeria monocytogenes*. Subsequently, 27.4 million pounds of product was recalled and the processing plant suspended operation (CDC, 2002). Moreover, the outbreak led to the application of the new USDA-FSIS directive of a “zero tolerance” policy on RTE food products (USDA-FSIS, 2003).

The occurrence of an outbreak poses severe health and economic impacts. As stated, although the incidence of listeriosis is low, the mortality rate is very high. The contaminated product can lead to recalls and suspended operations as well as serve to provide the food manufacturer an unfavorable connotation from the consumer once an outbreak has been linked to the food product produced by the manufacturer. Although through new regulations and governance, the occurrences of *Listeria monocytogenes* linked outbreaks have decreased, it is still an organism of concern due to its characteristics aforementioned. *L. monocytogenes* has proven that it can contaminate many food products and survive well in the food processing environment.

U.S. Governance on regulations concerning Listeria monocytogenes

In 1999, the Food Safety and Inspection Service of the United States Department of Agriculture (USDA-FSIS) issued updated guidelines for *Listeria* in the food industry, noting changes in regulatory affairs concerning the tolerance level of acceptability of the presence of *Listeria monocytogenes* in food industries as well as sampling methods recommended. Moreover, the report rated RTE foods such as sliced ham, luncheon meat and small diameter sausages as having the highest prevalence rate of *L. monocytogenes*. This species of *Listeria* was officially recognized as a pathogen of concern that must be addressed in food safety plans as well as in hazard analysis critical control point (HACCP) plans (USDA - FSIS, 1999).

In an updated directive resultant to the public health significance of the emergence of *Listeria monocytogenes*, a zero tolerance policy was established in RTE food products, similar to the regulations on *Escherichia coli* O157:H7 in ground beef

(USDA - FSIS, 2003). It further classified food processing plants manufacturing RTE foods by their risk of contamination. In this assessment of the presence of *Listeria monocytogenes* in food products, it lists *Listeria monocytogenes* in FSIS Nationwide Sampling Programs that provided food manufacturers a degree of standardization in sampling, analysis, and provisions in the occurrence of the presence of the pathogen. All of the sampling data gathered in the plant would be made available to the USDA – FSIS (USDA –FSIS, 2003). The directive provided potential plans as to how to alter their current safety and HACCP plans to accommodate for the presence pathogen as well as steps to reduce and eliminate the pathogen.

A valid HACCP and sanitation program is imperative in food processing facilities to ensure the steps outlined within it effectively reduce and eliminate food pathogens such as *Listeria monocytogenes*. Equipment and storage facilities must be cleaned on a regular basis with emphasis on hard to reach locations within the plant where pathogens are likely to survive and grow. Sanitizers must be effective in eliminating microflora as well as provide no detrimental effects on the equipment and especially the end food products. Separation of raw products and finished RTE products is essential in the processing plant to eliminate the chances of cross contamination.

Bacterial attachment to food contact surfaces

The presence of pathogenic bacteria on food contact surfaces may present great health risks, since as few as ten colony forming units (CFUs) of certain pathogens can lead to life-threatening infections. The initial aggregation and formation of surface contaminants such as dental plaques and biofilms is the attraction and adherence of the

bacteria onto a surface. Cellular surface components such as fimbriae, pili or outer membrane polysaccharide layers are used by the bacteria in attaching to the surface as well as to other cells. Compared to the bacterial cell membrane, the structures are relatively fragile (McInnes, *et al.* 1992). Attached bacteria on a surface produce extracellular polymeric substances (EPS), that plays an important role in initial attachment as well as facilitate the formation of biofilms by providing a firm anchorage to solid surfaces (Costerton, *et al.*, 1985; Marshall, K.C., 1992). Once formed, biofilms are highly resistant to disinfectants and other cleaning agents. Bacterial attachment and biofilm formation on stainless steel pipes, vessels, valves, tables, utensils or other equipment in contact with food products impose serious trepidations to the food processing industry (Arnold and Bailey, 2000).

Many factors influence the adhesion of a microorganism. Attachment may be affected by the structural and physiological characteristics of the cell as well as the nature and temperature of the suspension. The physical and chemical properties of contact surface, such as geometry, porosity, roughness, composition, and hydrophobicity dictate the strength of adhesion (Hood and Zottola, 1997; Ong, and Razatos, 1999; Seo and Frank, 1999; Arnold and Bailey, 2000; Sinde and Carballo, 2000). Attachment of microorganisms to surfaces is metabolically favorable due to the concentration of organic molecules being more concentrated at the interface (Sinde and Carballo, 2000). After attachment, the microorganism's exocellular polysaccharides provide means for it to transition to irreversible attachment. This transition occurs very rapidly on surfaces such as stainless steel, with irreversible attachment being reported to start occurring in less than a minute, and increasing with the elapse of time (Frank, 2001). The type of bacteria,

its adherence strength, and the bacterial concentration on the surface varies with the type of surface (Helke, *et al.* 1993). The type of surface is divided into two categories; one being high surface energy materials that are hydrophilic often carrying a negative charge such as glass, minerals, and metals; and the other type being low surface energy that shows hydrophobicity with low charge (either positive or negative) such as plastics and rubber surfaces (Helke, *et al.* 1993; Sinde and Carballo, 2000; Frank, 2001; Chae, *et al.* 2006). Smoot and Pierson (1998) noted that the growth conditions prior to exposure influenced the level of attachment occurred. They concluded that exposing cells of *L. monocytogenes* to sublethal levels of environmental stress, such as pH and temperature, and the hydrophobicity of the surface is a determinate of the microorganism's ability to attach to common food contact surfaces. It was also noted that *Listeria monocytogenes* Scott A was found to attach at higher levels on stainless steel than Buna-N-rubber for all temperature and pH parameters tested.

Arnold and Bailey (2000) correlated the increase in roughness parameters of surface finishes to bacterial attachment and early biofilm formation. Using scanning electron microscopy, they showed that at various times following exposure of the steel surface to bacteria, bacterial counts on all the treated surfaces were significantly less than that of untreated surfaces with mill finish. Bacterial affinity of attachment was dependent on the surface type variations and both physical and electrochemical treatments improved resistance of stainless steel to bacterial adhesion.

Listeria and attachment

Listeria monocytogenes can adhere to almost all food contact surfaces. It has demonstrated attachment to stainless steel surfaces with the involvement of a polysaccharide and protein matrix. *Listeria monocytogenes* has been shown to form biofilms on solid food contact surfaces (Herald and Zottola, 1988; Norwood and Gilmour, 2001). Biofilm production increases the resistance of *L. monocytogenes* against common sanitation procedures. These factors heighten the risk of *L. monocytogenes* contaminating food products in a food processing facility. Stanfield, *et al.* (1987) demonstrated that *Listeria monocytogenes* could be recovered from the surface of inoculated plastic and waxed cardboard milk containers after 14 days of storage.

Vatanyoopaisarn, *et al.* (2000) demonstrated that *Listeria monocytogenes* used flagellar filaments in surface binding, which is an initial step of biofilm formation. Kalmokoff, *et al.* (2001) noted *Listeria monocytogenes* as well as *Listeria innocua* strains adsorbed to stainless steel at much lower levels than some other Gram positive and Gram negative isolates. They stated that no direct correlations between serotypes or source and the levels of adsorbed cells from stainless steel surfaces were observed, nor were correlations made in the rate of adsorption from foodborne outbreak strains from other test isolates. However, marked differences were noted among strains, including the density of attached cells, the presence / absence of extracellular fibrils and the ability of strains to form a biofilm in the study. All but one strain tested (*L. monocytogenes* CLIP 23485) did not form a biofilm under their testing conditions, but they did adhere to the surface as isolated cells.

In a study conducted by Herald and Zottola (1988), *Listeria monocytogenes* was observed under SEM and EPS were present at 21°C, but not at 10°C nor was it present at 35°C. The presence of EPS represents an inclination for attachment to solid surfaces. However, other studies suggest that while flagella facilitated initial attachment, their rotary actions may prevent long-term attachment (McSweegan and Walker, 1986; Herald and Zottola, 1989).

Even without the presence of EPS, *Listeria monocytogenes* has shown to attach to solid surfaces at lower and higher temperature ranges than when the organism flagellates. Norwood and Gilmour (2001) observed substantial adherence of *Listeria monocytogenes* Scott A and FM876 strains at 30°C and at 4°C (lowest of the three tested temperatures) - although they did note that optimal adherence occurred at 18°C. Moreover, it has been suggested that stress adaptation may increase virulence of *L. monocytogenes*, and therefore, more likely to cause illness due to it being able to survive digestion in the stomach (Bolton and Frank, 1999).

Bacterial recovery from food contact surfaces

In microbiological studies evaluating sterilization and sanitization of food industries, clean room operations, hospitals, as well as pharmaceutical industries and medical devices, removal and enumeration of viable microorganisms from material surfaces is a critical point for environmental control. The data obtained pertaining to survival and enumeration of microorganisms would affect sterilization dose settings, cycle development, and process validation (Jeng, *et al.* 1990; Frank, 2001). In risk based food safety management systems such as HACCP, traditionally the focus is on end-

product analysis when using microbiological testing. Although methods utilizing sponges and swabs for quantitative analysis are often recommended due to their ease of use in sampling difficult to clean, irregular, and uneven surfaces, there are many factors influencing recovery of microflora from surfaces sampled using traditional swabbing and sponging (Frank, 2001; Moore and Griffith, 2002a). Variables affecting the accuracy of the detection and enumeration using swabbing technique initially include the ability of the swab to remove the microflora from the surface as well as its effectiveness to release removed microorganism from the swab bud and their subsequent recovery and cultivation (Moore and Griffith, 2002a,b). The proportion of attached microflora on surfaces that are trapped or tenaciously bound to the interwoven fibers of a swab head are unknown, and sampling techniques that preserve the underlying surface as well as the viability of the detached microflora, will detach only a portion of the total population. Adherent bacteria on surfaces become increasingly difficult to remove by use of swabs and sponges, especially if they become associated with a biofilm (Frank, 2001; Moore and Griffith, 2002b). There lacks standardization of swabbing pattern and pressure applied to the swab during the sampling process, leading to technician-to-technician variability. Moreover, there exists an inherent problem in methods using the swabbing technique in that the buds of the swab retain a portion of the microflora removed from the sampled surface – resulting in reduced recovery (Salo, *et al.* 2000; Frank, 2001; Moore and Griffith, 2002a,b). Conventional recovery methods of bacteria from food contact surfaces yield less than optimal recovery of viable counts and possess high limiting factors, all of which attribute to a possibility of misleading results. Kusumaningrum, *et al.* (2003) reported that in evaluating the survival and recovery of *Bacillus cereus*,

Salmonella enteritidis, *Campylobacter jejuni*, and *Staphylococcus aureus* on stainless steel surfaces, the direct contact method using solidified agars done a single pass recovered 18% of *B. cereus*, 23% of *S. enteritidis*, 7% of *C. jejuni*, and 46% of *S. aureus* from the initial concentration applied to the surface. A validation and comparative study on recovery of microorganisms using swabs, Hygicult TPC dipslide, and contact agar plate yielded similar results and did not differ in precision, with recoveries ranging from 16-30% of the microbial load applied to the surface (Salo *et al.*, 2000).

Moore and Griffith (2002a) reported that variance in mechanical energy generated during swabbing significantly affected ($p < 0.05$) the number of bacteria removed from a surface, and that using swabs in tandem with a wetting agent (surfactant) to the swabbing solution such as triton-X100 and Tween 80 significantly improved bacterial release by lowering the surface tension of the swabbing solution, thereby increasing its ability to contact the entire surface area being sampled and aiding in the detachment of cells from the surface. In the release of bacteria from swab bud, the use of a 3% Tween solution yielded the highest percentage of bacteria released from the bud and was significantly higher than when any other swab-wetting agent was used. They also noted that along with its wetting effect, it prevents clumping of the cells as well as preventing the re-disposition of the detached microorganism back onto the surface, but, it should be noted that they also addressed the possible detrimental effects of using too high a concentration of wetting agents, in that the enhanced wetting effect may also reduce the mechanical energy generated by the swabbing action and thus reduce the removal of bacteria from the surface. Other studies concluded that wetting agents are dependent upon the charge

of the sampled surface as and may bring about no significant improvement of overall bacterial recovery (Raiden, *et al.* 2003).

Characteristics of Ultrasound

Coupland and McClements (2001) define ultrasound as “Mechanical vibrations similar to sound but at frequencies far beyond the range of human hearing.” Power ultrasound (US) is used at frequencies in the range of 20 kHz to 100 kHz and requires the presence of a liquid medium for power transmission (Seymour *et al.* 2002; Piyasena, *et al.*, 2003). Ultrasound fields consist of waves at high amplitude, which forms cavitation bubbles during the expansion phase of the wave. As these bubbles absorb energy, they grow in size until they can absorb energy no longer and violently implode (Seymour, *et al.* 2002). During implosion, there is a two sided effect. First, the collapse of the bubbles produces intense hydrodynamic shock waves, producing energy that can dislodge materials such as soil from a surface – providing a scrubbing effect (Scherba, *et al.*, 1991). Second, it may have a bactericidal effect in that it could momentarily generate pressures up to 50 mPa and generate intense heat (Raso, *et al.*, 1998). The phenomenon is known as cavitation, in which Earnshaw (1998) describes two types – stable and transient. In stable cavitation, the bubbles generated vibrate instead of growing and imploding as ultrasonic waves are passed through the liquid, which generates a vibrating current which spread throughout the fluid known as “microstreaming.” Microstreaming provides a substantial force that agitates the cell and causes disruption of microbial cell structures. In transient cavitation, ultrasonic energy passing through a liquid medium causes rapid expansion and implosion of bubbles. In the implosion of bubbles, as

described above for cavitation, high heat and pressures are generated to remove adhered microflora and other matter attached to a surface.

Ultrasonic cavitation is dependent on the frequency of the sonic energy, temperature, and pressure. Leadley and Williams (2002) states that lower frequencies in the range of 20 kHz produce larger bubbles that collapse and produce higher energies. Higher frequencies (≥ 2.5 MHz) may not produce cavitation in that at the higher frequencies, bubbles are more difficult to produce (Alliger, 1975). There lies an inverse proportion between the increase in temperature to a higher production of bubbles, but the intensity of implosion is reduced (therefore reducing cavitation) due to an increased vapor pressure. This phenomenon leads to a decrease in tensile strength (Piyasena, 2003). The effects of temperature can be offset if pressure is applied. A combination of 200 – 600 kPa and heat with ultrasound can increase the amplitude of the ultrasonic waves and increasing the effectiveness of cavitation. Gram positive bacteria have shown to be less sensitive and less liable to destruction due to the effects of ultrasonication and subsequent acoustic energy due to its thicker peptidoglycan layer versus Gram negative bacteria (Robrish, *et al.* 1976, Olsen and Socransky, 1981).

Ultrasonication enhanced removal and recovery of bacteria

The accuracy, precision, and repeatability of the recovery/enumeration methods in use such as swabbing, direct contact method, RODAC, and manual agitation are greatly affected by the manual skill of operators and their technique of recapture (Bloomfield, *et al.* 1994). By the use of automated mechanical treatments for recovery of bacteria, technician to technician variability is greatly decreased. Jeng *et al.* (1990) iterates that

the efficiency of surface microbial recovery is dependent on the techniques used to remove organisms from the surface and the ability to recover and enumerate them. Use of methods such as sonication and manosonication enables the investigator to more efficiently utilize time and reduces the labor of detaching bacteria from the surface manually, compared with swabbing, along with providing consistency to each sample treated. Moreover, ultrasonic removal of bacteria potentially has an advantage in that the cavitating bubbles can reach crevices that are not easily reached by conventional cleaning / sampling methods (Mason, *et al.* 1996).

Many studies have been performed using ultrasonication as a germicidal treatment (Lillard, 1993; Piyasena, *et al.* 2003). It has shown efficacy when used in tandem with other sanitizing methods such as chemical sanitizers, including sodium hypochlorite (Lillard, 1993). However, Puleo, *et al.* (1967a) determined that use of ultrasonication provided a faster means of removal than manual agitation and that removal of spores from porous surfaces yielded better results. He suggested that sonication could also be used as an efficient recovery technique to detach bacteria from surfaces (Puleo, *et al.* 1967a; Frank 2001). Due to the declumping of the bacterial clusters attached to surfaces and planktonic cells using ultrasonics, bacterial enumeration from surfaces may be truer to the original bacterial load applied (Puleo, *et al.* 1967a,b; Lillard 1993, Qian, *et al.* 1996; Joyce *et al.* 2003).

Adjustment of the duration of exposure to ultrasonication as well as the frequency to maximize recovery is imperative in using this method for recovery of viable microorganisms. Sinde and Carbello (2000) reported that the viability of *Salmonella* spp. and *Listeria monocytogenes* was not affected by sonication with 40 kHz for 20 seconds.

Puleo *et al.* (1967b) noted that suspensions of *Staphylococcus aureus*, *Pseudomonas alcaligenes*, *Escherichia coli*, and natural contaminants resulting from natural hand washing had no lethal effects on bacterial cells when they were insonated for up to 24 minutes at a frequency of 25 kc/sec held between a temperature range of 25°C - 32°C.

In the removal of *Proteus mirabilis* biofilm from water filled tubes using axially propagated ultrasound, Mott, *et al.* (1998) noted that the removal of biofilm from 7 cm tubes by two 30s pulses at 150 kHz yielded 54.8% recovery and 60.9% was recovered using 33 kHz sonication. In 50 cm tubes, three 30s pulses from the 20 kHz transducer removed 87.5% of the biofilm on the surface, at 150 kHz removal of 66.8% was observed, and exposure to 350 kHz removed 31.3% of biofilm formed.

In other studies, Oulahal-Lagsir, *et al.* (2000) compared the biofilm removal by sonication using 40 kHz and the swabbing method. They reported an 83% removal of fouling material using ultrasonics versus a 20% yield using the swabbing method. Qian, *et al.* (1996) reported that application of 10 mW cm⁻² ultrasound was reported as not changing the structure of the biofilm or the structural position of the cells. It was also noted that biofilm was appearing to grow during insonation at this level.

However effective or more accurate the use of ultrasonics in the removal and recovery of microflora attached to surfaces, as Frank (2001) iterates, the methods as they are presented in studies using this technology proves generally impractical for sampling and analyzing in the industry setting.

Use of sonic toothbrushes in removal of dental plaques (biofilms)

McInnes, *et al.* (1992) suggested in their study that since cell surface components such as fimbriae and pili that are used by bacteria to attach to a surface as well as other cells are relatively fragile in comparison to the bacterial cell wall, lower acoustic pressures and frequencies than ultrasonics could be used for cellular detachment and when acoustic energy is applied to fluid surrounding the surface, it may potentially prevent or hinder accumulation on the surface. They demonstrated that the ability of bacterial production of EPS was reduced when exposed to low-frequency (200 Hz) acoustic energy and detachment of bacterial cells on the surface was seen.

The use of a sonicating toothbrush generates fluid pressure and shear force (velocity) at the bristle tip via the high frequency vibration of the bristles to remove adherent colonies of cultured bacteria *in vitro* and *in vivo*. The resulting acoustic energy is transported through a fluid medium with the resulting dynamic fluid effects having been found to remove attached bacteria at distances up to 4 mm beyond the tip of the bristles (Wu-Yuan and Anderson, 1994; Stanford, 1997; Parini and Pitt, 2005). Studies have demonstrated that fluid dynamic effects generated from sonicating toothbrushes are capable of removing adherent oral bacteria from surfaces of dental relevance such as titanium and hydroxyapatite discs. Sonicating toothbrushes have shown to be significantly superior to manual tooth brushing in removing supragingival plaque *in vivo* (Ho and Niederman, 1997). In electron microscopic examination, MacNeill, *et al.* (1998) reported a decrease in aggregation tendency and loss of fimbriae in the sonic toothbrush group when compared to manual tooth brushing. They also found lack of morphological evidence that would indicate cell damage potentially due to aggressive mechanical action

or sonic energy from insonating toothbrushes. Increase in CFUs over that of the control group was apparent in that exposure to sonic toothbrushes did not affect cell viability and dispersed aggregates of *Actinomyces viscosus* into the fluid environment.

Parini and Pitt (2005), who reported in a previous study that a stream of bubbles striking an oral biofilm effectively removed bacteria from the surface, studied the effectiveness of biofilm removal by the collision of bubbles and sonic waves and also the effect of sonic waves at 260 Hz (vibration rate commonly used in sonicating toothbrushes such as the Sonicare series) and 520 Hz in the absence of any fluid or bubble flow. This was achieved by the use of a probe that generated sound waves at the specified levels. The authors mounted *Streptococcus mutans* biofilms in a chamber containing artificial saliva and exposed then to bubbles and sonic waves, where the waves were generated by an oscillator at frequencies and acoustic intensities of sonic toothbrushes. They also mounted biofilms at different angles to measure the effect of a bubble's impingement angle. In the study it was concluded that velocity, gas fraction, and mean bubble diameter are more important than the angle of bubble collision on the surface in the design of a powered toothbrush. They found no significant difference in the removal of adhered bacteria from a surface from bubbles impinging at more direct angles (45°) than those that impinged on biofilms at more glancing angles (5°). Leading them to conclude that the angle of the fluid forces generated by a sonicating toothbrush in relation to the surface did not affect in cell removal, moreover, they also concluded in the study that sound by itself is not an effective means of biofilm removal - at least not at the frequency of the sonicating toothbrush. They do suggest, however, from a cited study, that sound intensity at the ultrasonic level may be able to remove cells adhered to surfaces.

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Chapter II - Quantitative Evaluation of Recovery Methods for *Listeria monocytogenes* Applied to Stainless Steel

David Kang, Joseph D. Eifert, Robert C. Williams, Steven Pao

Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA

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ABSTRACT

The ability of *Listeria monocytogenes*, to attach to various food contact surfaces such as stainless steel, polypropylene, and rubber compounds is well documented. The retention of these or other pathogenic bacteria on food contact surfaces increases the risk of transmission to food products. The objective of this study was to compare several methods for quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces. A cocktail of four serotypes of *Listeria monocytogenes* (Scott A (4b)), 1/2b, 3b, and 4b) were mixed in equivalent concentrations and inoculated onto type 304 stainless steel coupons in a 2cm x 2cm area. After a one hour exposure, coupons were sampled by one of the following methods: 1) swabbing using a pre-moistened Dacron swab, 2) rinsing with phosphate buffered saline, 3) direct contact onto a tryptic soy agar containing 0.6% yeast extract (TSA+YE) plate surface for 10 seconds, 4) sonication in an ultrasonic water bath (40 kHz), 5) contact with the bristles of a sonicating brush head for 1 min, and 6) indirect contact (2-4 mm) with the bristles of a sonicating brush head for 1 min. Coupon rinses were plated onto TSA containing 0.6% yeast extract and incubated for 24 hours at 35°C. The three sonication methods yielded higher recovery than the other three methods ($p < 0.05$). Brushing the coupons with the sonicating brush head yielded a recovery level of 58% and indirect exposure to the sonicating brush head permitted a recovery level of 65% from the initial microbial load. The lowest cell recovery (~20%) was observed with the swab and direct agar contact methods.

INTRODUCTION

Quantifying microflora and pathogens on food contact surfaces provides valuable risk assessment data for modeling consumer exposure from cross contamination in food manufacturing, food transport, and in food service environments. A major purpose of food safety management systems such as HACCP is to understand and overcome the risks associated with microbiological contaminations that may lead to reduced shelf life of the product; or of greater concern, the presence of health risks associated with the possibility of the presence of pathogens in the environment such as on food contact surfaces- particularly those with low minimum infective doses (Moore and Griffith, 2002b; Lin, *et al.* 2006). As few as ten CFUs of certain pathogens can lead to life-threatening infections, especially in persons who are immunocompromised. The infective dose of *L. monocytogenes* is unknown, but it is believed to vary with the strain and susceptibility of the potential host. From cases contracted through raw or under pasteurized milk, it is presumable that fewer than 1000 total organisms may cause disease in susceptible populations (FDA, 2005). Pathogenic bacteria, including *Listeria monocytogenes* are known to be able to attach and adhere to food contact surfaces such as stainless steel, glass, as well as rubber compounds and are capable developing an extensive extracellular matrix commonly associated with biofilm formation (Helke, *et al.*, 1993). Short term adsorption and longer-term biofilm formation in a variety of *L. monocytogenes* strains have been investigated. Numerous studies have examined the effect of environmental factors and growth conditions on attraction and binding to these food-contact surfaces (Kalmokoff, *et al.* 2001).

In the 2003 draft of the “FSIS Risk Assessment for *Listeria* in RTE Meat and Poultry Products,” quantitative transfer to and from slicing machines, knives, and cutting boards in delicatessens was identified as a key informational gap (USDA-FSIS, 2003). Biofilm formation by *L. monocytogenes* may enhance bacterial presence in food processing environments, and consequently increase the chances of contributing to post processing contamination (Chae, *et al.* 2006). The extent of bacterial survival and cross contamination between hands and foods or various kitchen surfaces have been quantified in various studies (Zhao, *et al.*, 1998; Chen, *et al.*, 2001; Montville, *et al.*, 2001).

Listeria monocytogenes is especially of great concern in RTE foods and its facilities. There are 13 serovars of *L. monocytogenes*, but almost all cases of human listeriosis can be attributable to types 4b, 1/2a, and 1/2b, with serotype 4b appearing to be most often associated with large outbreaks of foodborne listeriosis (MacLauchlin, 1996; Rocourt, 1996; Schlech, 1996; Low and Donachie, 1997; Allerberger, 2003; Gasanov, *et al.*, 2005). A recent study of retail luncheon meats revealed contamination levels for 99.6% of samples of less one *L. monocytogenes* per gram. The bacteria has the ability to be transferred from a commercial slicer and equipment onto deli meats, and as Gombas, *et al.* (2003) states, the degree of transfer correlates with the number of *Listeriae* inoculated onto the slicer blade.

Listeria monocytogenes is of concern for food processors by its ability to survive and grow at low temperatures in food products and food contact surfaces. Along with its psychotropic nature, its ability to grow at a very broad range of pH, water activity, and salt concentration provides an advantageous niche from other food contaminant species (Bayles and Wilkinson, 2000).

The detection and enumeration of organisms is widely used in assessing the efficacy of sanitation programs. The quantification of cross-contamination risk associated with various steps in the food preparation processes would provide a scientific basis for risk management efforts both domestically and industrially (Kusumaningrum, *et al.*, 2003). Various methods for quantifying microbial surface contamination exist. The degrees in which these sampling methods are effective vary greatly and there is no consensus as to an accepted standard method (Bredholt, *et al.*, 1999; Moore and Griffith, 2002a,b). Due to a wide scattering of data and / or insensitivity of the enumeration method used in surface sampling, this can lead to misleading determinations of the initial cleanliness of the surface and / or the effectiveness of the cleaning procedure used in processing plant. Such risks play a major factor in cross contamination, which can ultimately lead to and maintain a cyclic procurement of premature spoilage of food products, and in certain cases, lead to foodborne diseases.

The make-up and porosity of the surface being sampled makes an impact in the sampling method's overall efficiency as well. In a study conducted with the recovery of *E. coli* cells inoculated onto cutting boards, Abrishami, *et al.* (1994) reported more than 90% of the inoculated *E. coli* which had been allowed to dry on the surface for two hours could not be recovered, despite 75% of the cells remaining viable. Moreover, the methodology and mechanics of a sampling method often dictate the procedure's success in recovery and consequent enumeration. Although conventional hygiene swabbing is widely used, this method has been reported to recover only a small proportion of the total bacteria population present on a surface, and technician-to-technician variability affects

the outcome of the enumeration results (Bredholt, *et al.*, 1999; Moore and Griffith, 2002a,b; Vorst, 2004).

Accurate detection and enumeration of microbial contaminants, either by use of conventional sampling techniques or novel methods employing sonication or ultrasonics, relies initially upon mechanical action to effectively dislodge and remove the contaminant from the surface. It is also imperative that the method employed also releases the microorganisms from potential devices used for subsequent recovery and cultivation. The mechanical action and ease of manipulation of the brushing technique using instruments such as typical toothbrushes are good for reaching and sampling hard to reach environments, where bacteria and filth may accumulate. The added effects of fluid dynamics of an insonating brush head in a liquid environment further enhance its sampling capabilities in the removal adherent bacteria. The bristles of a sonicating toothbrush such as the Sonicare[®] Elite series vibrate at a frequency of 260 Hz, creating mild cavitation and bubble activity about the brush head. Previous studies have shown that sonic vibrations in fluid similar to those generated by Sonicare[®] even at a distance of 4 mm away from the contaminated surface can alter cell surface structures of the oral bacterium *Actinomyces naeslundii* (formerly *Actinomyces viscosus* T14V) as well as remove these adherent bacteria from model dental surfaces in vitro (Wu-Yuan and Anderson, 1994).

In this study, we strived to contribute to the methodology in the field of quantitative microbiological sampling of food contact environments, using *Listeria monocytogenes* attached onto stainless steel. The study also considered the effectiveness of the experimental design, particularly monitoring for results that may not be as sensitive

in enumeration as other sampling techniques and also for results that may be invalidated due to the presence of excessive, uncontrolled variability and data scattering due to technician to technician variability.

MATERIALS AND METHODS

Cultures and culture maintenance

Listeria monocytogenes serotypes 1/2b, 3b, and 4b were provided through the courtesy of Dr. S. Kathariou (Dept. of Food Science, North Carolina State University, Raleigh, NC). *L. monocytogenes* strain Scott A (serotype 4b) was obtained from the culture collection of the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. Cultures were kept in an ultra low-temperature freezer (approx. -70°C) in a 25% glycerol solution until use. Cultures were revived in tryptic soy broth (TSB, Difco, Franklin Lakes, NJ) supplemented with 0.6% yeast extract (YE, Difco, Franklin Lakes, NJ) (TSB+YE) at 35±2°C for 24±2 hours and maintained on tryptic soy agar (TSA, Difco, Franklin Lakes, NJ) supplemented with 0.6% YE (TSA+YE) slants. The slants were kept at 4±2°C.

Preparation of inoculum

Each serotype was separately cultivated in 10 ml of TSB+YE for 24±2 hours at 35±2°C from stock slant. At 24±2 hour intervals, the culture was transferred by a single loop inoculation to 10 ml of TSB+YE. Prior to use in test media, it was subcultured in this manner at least twice, consecutively. A standard plate count method determined that the average concentration of each of the four serotypes of *Listeria monocytogenes* was ~1.0 x 10⁹ CFU/ml after 24 hours of incubation. Cultures used for experimentation were inoculated into 10 ml TSB+YE and incubated at 35±2°C for 24±2 hours prior to use. To simulate conditions faced by contaminants in a processing plant, where washing of

equipment and its maintenance are in the protocol of the plant's maintenance schedule, the inoculum used in the study underwent washings and drying. Each grown culture was centrifuged at 2000 x g for 20 minutes. The supernatant fluid was decanted and the cells were suspended in 15 ml of cooled ($4\pm 2^{\circ}\text{C}$) phosphate buffered saline (PBS, 7.4 pH). This washing procedure was repeated and the concentration for each serotype in the final suspension was determined. Spectrophotometric analysis was also done (Spec 20) at 600 nm for concentration yield. Each serotype was pooled into a *L. monocytogenes* cocktail in equal concentrations (based on spectrophotometric analysis and previous concentration determination per serotype) for use in the study. The cocktail was serially diluted in PBS for a concentration of approximately 10^6 CFU/ml. Culture purity and concentration (PBS used as diluent) were confirmed by standard plate counts and morphological examination of isolated colonies. Purity plates were incubated for 24 ± 2 hours at $35\pm 2^{\circ}\text{C}$, and the plates determining the concentration were incubated for 48 ± 2 hours at $35\pm 2^{\circ}\text{C}$. All colony morphologies observed were similar on all plates throughout the experiments. Isolated colonies were also identified with an API *Listeria* biochemical test kit (bioMerieux, Hazelwood, MO) on a regular basis.

Preparation of stainless steel coupons

Stainless steel was chosen as the test surface in that it is used extensively throughout the food processing and food transport industry. Stainless steel sheets, type 304, were provided courtesy of Mr. Paul Winniczuk (Citrus Research and Education Center, Institute of Food and Agriculture Sciences at the University of Florida, Lake

Alfred, FL). The sheets were from the lining of 3A commercial food grade tankers and cut into coupons measuring 2.5 cm x 8 cm.

Each coupon was soaked in acetone for 60 minutes, thoroughly rinsed with deionized water, and then placed in glass beakers to be treated in an ultrasonic water bath (Fisher Brand, 150-300 W, 40kHz) for 45 minutes. The beakers holding the coupons contained ultrasonic cleaning detergent (Fisher brand, Fisher Scientific, Pittsburgh, PA). After treatment, the coupons were thoroughly rinsed in tap water followed by rinsing three times in deionized water to remove debris as well as possible residual detergent.

Excess moisture was removed from each coupon processed with a lab wiper (Kimwipe[®]). On the cleaned coupons, a 2 cm by 2 cm area was scribed with permanent ink (Chae and Schraft, 2000). The designated area was scribed approximately 1.5 cm from the end of the coupon. Each scribed coupon was distributed into a wire test tube holder capable of holding a coupon into each opening with flame sterilized forceps dipped in 95% ethanol. The wire test tube holder was covered in aluminum foil. The coupons were steam sterilized by autoclave for 20 minutes at 121°C. Thereafter, the sterilized coupons were dried in a hot air drying oven at ~80°C until completely dry. Before use, the coupons were placed into individual, covered sterile Petri dishes using flame sterilized forceps, ensuring that the marked area was face up.

Enumeration of bacteria

An initial *Listeria monocytogenes* concentration was determined (time 0) per culture used per sets of studies performed. Inoculated coupons were sampled either after one hour of drying or 12 hours of drying, maintaining the testing temperature of 20±2°C

at relative humidity. Additional dilutions with PBS were required before plating. All plates were incubated at $35\pm 2^{\circ}\text{C}$ for 48 hours before being counted. Any plates that appeared to be contaminated were discarded and not used.

Application of inoculum on stainless steel

100 μL of the prepared inoculum ($\sim 10^5$ CFU / coupon) was placed on the center of the designated area per coupon tested and spread using a flame sterilized glass rod dipped in 95% ethanol, ensuring proper cooling of the rod before use. Coupons were covered and allowed to dry at $20\pm 2^{\circ}\text{C}$ at $\sim 50\text{-}60\%$ relative humidity for one, 12, or 24 hours. Five inoculated coupons were studied for each drying time and recovery method.

Preparation of sonicating brush heads

Due to the irregular lengths of the bristles on the head of the brush head attachments for the Sonicare[®] Elite 7300, all brush heads used in the study were cut to a uniform length of 7 mm at the ends to 8 mm at the middle of the brush head (Appendix A, Figure 1). All prepared brush heads were visually examined before use to ensure uniformity of the length and that the plane was level.

Because alterations had to be made to the bristles using non-sterile methods and the initial sterility of the brush heads was not guaranteed, preliminary studies were performed to ensure the methodology used for the initial sterilization as well as the subsequent sterilizations after its use would be satisfactory (Appendix D). For each used brush head, the base of the brush head was sprayed with 70% ethanol until saturated and the brush head and neck was submerged in 45 ml of 70% ethanol contained in a 50 ml

sterile centrifuge tube. Each brush head was turned on for 10 seconds in solution to knock off any potential bacteria that may have been attached to the bristles. It was held in this condition for at least 3 hours and submerged in 45 ml of sterile deionized water twice to rinse off the ethanol. The brush heads were stored in 45 ml of sterile deionized water in 50 ml centrifuge tubes until use.

Sampling / recovery procedures

Each of the following methods described subsequently were performed on five pieces of steel per drying time used. Serial ten-fold dilutions using PBS were plated from the solution(s) utilized in sampling using standard plating techniques. Duplicate plating was performed. Serial ten-fold dilutions were plated on the TSA+YE using standard plating techniques. Sampled coupons were washed gently with 20 ml of PBS and the coupons were placed into sterile Petri dishes, where they were overlaid with molten TSA+YE. All plates were incubated at $35\pm 2^{\circ}\text{C}$ for 48 hours.

Inoculum method

As a positive control and to serve as the initial concentration of inoculum applied in comparing the recovery level of the sampling methods tested, initial inoculum counts were made. Sets of five were performed per session. 0.1 ml of the inoculum used to inoculate the stainless steel coupons was added to 100 mL of PBS containing 1% polysorbate 80 (PBS+), mixed by swirling, and sampled within 1 minute of adding the inoculum.

Swab method

Per coupon tested, a Dacron tipped swab dipped in PBS+ was used to sample the contaminated area rigorously both in the horizontal and vertical direction for 1 minute. The used swab was then be submerged in 10 mL of PBS containing 0.1% polysorbate 80, breaking off the tip of the handled portion of the swab and agitated in a vortex type mixer for 1 minute.

Rinse method

Inoculated coupons were aseptically submerged into 100 ml of PBS+ using flame sterilized forceps dipped in 95% ethanol, ensuring that the inoculated area not touched and that the inoculated area was fully submerged. The lid of each container was capped and shaken in a 90 degree arc approximately 25 times in one minute.

Direct agar contact

After drying, each coupon was picked up using flame sterilized forceps dipped in 95% ethanol, ensuring that the inoculated area was not disturbed, and placed flat onto the surface of a TSA+YE plate, with the inoculated area making an impression onto the agar. The coupon was pressed once over the entire contaminated area of the stainless steel for 10 seconds with 200g of pressure per coupon ensuring that the impression plate did not break (Whyte, 1989; Kusumaningrum, 2003). To extrapolate countable numbers from the plate impressions, the impressed coupon was removed from the plate and the agar of the contact plate was dislodged from the plate using a flame sterilized “spatula” dipped in

95% ethanol. The agar was suspended in 100 mL of sterile PBS+ in a filtered stomacher bag and subsequently homogenized in a stomacher for 60 seconds.

Ultrasonic bath

Per coupon tested, the inoculated stainless steel was aseptically submerged into a suitable container holding 100 ml of PBS+ and exposed to ultrasonication in a cooled ($20\pm 2^{\circ}\text{C}$) ultrasonic waterbath (40 kHz with a power rating of 300W), (VWR Scientific, Model 250D). The containers holding the coupons were suspended in the ultrasonic water bath on top of a wire test tube holder inside the water bath. The containers were held in place with weights to ensure the containers stayed static. Water level in the bath was held constant and the containers were adjusted so coupons were below this level. The temperature of the water bath was maintained at $20\pm 2^{\circ}\text{C}$ using ice packs. The coupons were exposed to ultrasonication for 5 minutes.

Sonicating brush contact

A sonic toothbrush (Sonicare[®] Elite 7300, Phillips Oral Healthcare Inc., Snoqualmie, WA) was used in the recovery of *Listeria monocytogenes* dried onto stainless steel, with the bristles making direct contact on the inoculated area of the coupon. The coupons were aseptically submerged in 100 ml of PBS+ at an angle with flame sterilized forceps dipped in 95% ethanol. After placement, the inoculated area of the coupon was exposed to the bristles of the oscillating brush head, making direct contact on the contaminated surface for 1 minute while submerged in solution, where the entire contaminated area of the surface was ensured to be exposed to agitation from the

bristles by insonation (Appendix A, Figure 3). The brush head oscillates at 31,000 brush strokes min^{-1} , which translated to a fluid velocity of 260 Hz (Wu-Yuan and Anderson, 1994; Parini and Pitt, 2005). Although the pressure applied to the bristles was not measured, contact with the stainless steel did not visibly bend the bristles. The used brush head was pulsed for 10 seconds in a tube containing 10 ml of PBS+ to collect possible cells attached to the bristles. This was performed twice per brush head (data not shown).

Sonicating brush indirect contact

Testing was performed to look at the rate of recovery when there is no direct mechanical scrubbing action by the brush head and recovery is dependent on the cavitation and bubble actions of the solution generated by the brush head. Wu-Yuan and Anderson (1994) and Stanford *et al.* (1997) demonstrated that a sonic toothbrush can effectively remove bacterial colonies adhered to dental surfaces *in vitro* when the bristles were held 3-4 mm from plaque bacteria.

A fully charged base unit of the Sonicare[®] Elite 7300 was stationed in such a manner so that the height and angle of the bristles of the brush head when fastened to the base unit would be parallel to the surface area of the inoculated coupon submerged in 100 ml of PBS+. The container holding the solution and the coupon was adjusted to ensure the spacing between the inoculated area of the stainless steel coupon was 2-4 mm away from the bristle tip (Appendix A, Figure 4). The coupon was exposed to fluid velocity and generation of cavitation of the sonicating brush head for 1 minute, where the entire contaminated area of the surface was exposed to insonation. Following insonation, the

container holding the coupon was removed from the apparatus and the solution was serially diluted and plated using standard plating techniques. The used brush head was pulsed for 10 seconds in a tube containing 10 ml of PBS+ to collect possible cells attached to the bristles. This was performed twice per brush head (data not shown).

Data analysis

Each sampling procedure was replicated twice with quintuplicate analysis in each replicate, giving 10 observations for each value for the 12 hour drying time. For the 1 hour drying time, each sampling procedure was replicated three times with quintuplicate analysis in each replicate, giving 15 observations. The mean recovery values of duplicate plate counts of each analysis of the sampling method tested per drying time was determined. Numbers of CFU / coupon were transformed to \log_{10} for statistical analysis. The percent recovery for sampling methods was determined by transforming the average of the inoculum test represented in CFU / coupon into 100% and using this value to compare the percent recovery of the tested samples represented in CFU / coupon. The mean percent recovery values were averaged from the tested observations.

A one-way analysis of variance (ANOVA) was employed to determine significant differences between means for each treatment method and bacterial inoculation dry time at a statistical significance of $\alpha = 0.05$. When the ANOVA indicates a difference between means, Tukey's multiple range test was used to assess significant differences between means. All calculations were performed with JMP[®] Statistical Discovery Software, version 5.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

During the study, it was noted that use of PBS+ was not detrimental in the recovery of *Listeria monocytogenes*. The data obtained from the *inoculum* method was consistent with enumerations performed with PBS during the testing sessions conducted.

Recovery of *Listeria monocytogenes* on stainless steel dried for 1 hour

The mean \log_{10} CFU/ stainless steel coupon recovery of *L. monocytogenes* of the sampling methods tested were significantly different ($\alpha < 0.05$) from that of the inoculum method, which tried to capture as close as possible the initial concentration of the bacteria that was applied to the stainless steel coupon.

After drying the inoculum on stainless steel coupons for one hour at $20 \pm 2^\circ\text{C}$ with ~ 50-60% relative humidity, of the three conventional methods tested, the *Rinse* method yielded the highest mean recovery level when compared to that of the “Agar contact” method and the *Swab* method (Table 1). The *Swab* method was observed to have the lowest recovery level among sampling methods studied, followed closely by the *Agar contact* method, with the recovery levels being 20% and 21%, respectively (Figure 1). The mean recovery \log_{10} CFU/ coupon for these were significantly lower ($\alpha < 0.05$) from other sampling methods tested.

Among sampling methods that utilized fluid force dynamics and mechanical actions of the Sonicare[®] Elite 7300, the mean recoveries of *L. monocytogenes* were significantly higher than those that did not use a form of acoustic energy. The *Brush non-contact* method, where the bristles of the sonicating brush head were 2-4 mm away

from the surface, and the *Brush contact* method, where the bristles of the sonicating brush head made direct contact with the surface yielded mean \log_{10} CFU / coupon recoveries of 61% (5.10) and 59% (5.09) from that of the mean inoculum recoveries, respectively (Table 1; Figure 1).

The results from the sampling procedure using an ultrasonicating water bath (40 kHz) could almost be considered a “bridge” between methods using sonicating brushes and those that did not. The mean \log_{10} recovery CFU / coupon for the inoculated stainless steel coupons sampled with the use of an ultrasonic water bath, entitled, “Sonic bath” was 4.98, which was approximately 0.1 \log_{10} lower than the brush contact method and approximately 0.1 \log_{10} higher than the rinse method.

Recovery of *Listeria monocytogenes* on stainless steel dried for 12 hours

After drying the inoculum onto stainless steel coupons for 12 hours at $20 \pm 2^\circ\text{C}$ with ~ 50-60% relative humidity, there was a range of 0.77 to 1.74 \log_{10} loss from the mean recovery level of the *Inoculum* method for *L. monocytogenes* amongst the sampling methods tested ($\alpha < 0.05$). The *Swab* method yielded the lowest recovery of *Listeria monocytogenes*, producing less than a 2% recovery in comparing this method to the *Inoculum* (Figure 2). The highest recovery was seen using the *Brush contact method*, where a mean \log_{10} CFU / coupon recovery of 4.62, or a 17.3% recovery level when compared to the *Inoculum* counts (Table 2; Figure 2). It was significantly different ($\alpha < 0.05$) amongst all sampling methods, except for the *Brush non-contact* method, where the difference was 0.05 \log_{10} recovery. Recovery from swabs was significantly lower than recovery from the *Rinse*, *Sonic bath*, and the *Agar contact* methods.

Recovery of *Listeria monocytogenes* on stainless steel dried for 24 hours

The recovery levels were below the limit of detection when the inoculum of *Listeria monocytogenes* was dried onto stainless steel coupons for 24 hours at 20±2°C with ~ 50-60% relative humidity. In other words, recovery with each method was less than 5 CFU / coupon or less than 0.01 % when compared to the *Inoculum*. Since a high number of cells may have died off or been unrecoverable, a quantitative comparison of the recovery method efficiencies was not conducted.

Swab method

The *swab* method consistently yielded the lowest bacterial recovery of all the sampling methods tested. Previous studies (Moore, *et al.* 2001; Salo, *et al.* 2000; Frank, 2001; Moore and Griffith, 2002a,b; Foschino, *et al.* 2003; Vorst, *et al.* 2004) have noted problems associated with recovery of microorganisms, particularly from a dry surface, using the traditional swab hygiene method. Ineffective bacterial release from the swab head is one of the reasons noted for the comparatively poor performance of the *swab* method – in this study as well as others (Moore and Griffith, 2002a,b; Foschino, *et al.* 2003; Vorst, *et al.* 2004). However, in an auxiliary test that was performed where the swab used in sampling was exposed to ultrasonication in a water bath, recovery did increase, but the effect was only slight to moderate. In recovery following one hour of drying, the mean recovery of the swab heads exposed to ultrasonication was 5.6% increase over samples not exposed. In sampling *L. monocytogenes* dried for 12 hours, the mean recovery only went up to 0.63% for the sampled swabs exposed to ultrasonication as compared to 0.41% to those that did not in relation to *Inoculum* counts.

Another reason for its comparative inefficiency in recovery is the variability in the application of pressure and scrubbing action on the surface sampled. The degree of pressure applied is often difficult to measure in sampling and it has been suggested that the design of the swab allows only a limited amount of pressure applied in sampling and thus restricting the number of bacteria removed from the surface (Moore and Griffith, 2002b). Moreover, the accuracy and repeatability of measurement in use for methods not automated such as sampling in an ultrasonic water bath is greatly affected by the manual skill of technicians and the methods of recapture when using such methods as the *swab* methods and the *direct agar contact* method.

Effects of sonication by fluid flow on recovery

In surface sampling of *Listeria monocytogenes* attached to stainless steel, sampling methods using fluid flow acoustic energy were the most effective means of indicating the presence of the bacteria, and the difference between the most efficient methods (*sonic indirect contact* and *sonic direct contact*) to the least efficient methods (*swab* and *direct agar contact*) was approximately 40% when the inoculum was dried for one hour and nearly 20% when *L. monocytogenes* was dried onto the coupon surface for 12 hours (Figures 1 and 2).

In the study conducted by MacNeill, *et al.* (1998), examination of bacteria from samples exposed to sonic brushes with electron microscopy revealed that, regardless of time of exposure, the mechanical actions did not seem to have an effect on microbial integrity, i.e. no lysis of the cell membrane, or the overall viability of the bacteria. They indicated that sonic toothbrushes dispersed aggregates of *A. viscosus* in a fluid

environment. The bacterial cells showed distinct differences both with respect with decreased aggregation tendencies and a lack of demonstrable fimbriae – either severely shortened or removed them from the bacterial cell wall, especially at exposures greater than 30 seconds. This phenomenon was also noted by McInnes, *et al.* (1993), where damaged or missing fimbriae were observed on *A. viscosus* cells exposed to sonic energy. Shear forces in the liquid media resulting from cavitation and fluid flow were concluded to be responsible for the observed alterations.

Recovery of viable bacteria may increase with exposure to sonic energy. McInnes, *et al.* (1993) reported a two-fold increase in the number of recoverable *A. viscosus* colonies following a 30 second exposure to sonic energy. Although as MacNeil, *et al.* (1998) poignantly discusses that the dislodging and consequent dispersal of the plaque into the fluid media may not be the best scenario for the elimination of dental plaques and that it may in fact, have detrimental effects on the overall health of the gums and teeth of ones who are suffering from advanced stages of periodontal disease – potentially increasing the incidences of bacteremias. The dislodgement, disaggregation, and dispersal of bacteria within a fluid medium without loss of viability are ideal for sampling and enumeration processes.

Stanford, *et al.* (1998) reported through the use of observances in scanning microscopy that use of the Sonicare[®] toothbrush in indirect contact 2-mm with an enamel surface exhibited a virtually clean surface, with only residual bacteria being observed only in deep pits or in surface defects. At a distance of 3 mm between bristles and enamel, exposure to sonic energy removed 56-78% of plaque relative to non-treated controls. They also concluded that exposure to Sonicare fluid dynamic effects removed

highly significant numbers of bacteria ($p < 0.0001$). Correlation was also given to distance of the bristles to the surface and exposure time in reduction values. In comparing the percentage of reduction of *A. naeslundii* in relation to distance and exposure time, the data seen in the study conducted by Wu-Yuan and Anderson (1994) supports this. Exposed to the oscillating Sonicare brush head for 5 seconds, 83% of bacteria was removed, at 2 mm, 74% was reported dislodged; at 3 mm, 54%; and at 4 mm, 13% of attached bacteria were removed. The percentages recovered relative to the distance of insonation did increased with exposure time, however, the data presented were not as drastic. In comparison of recovery between exposures to 15 seconds vs. 30 seconds, there was little or no change in detachment of *A. naeslundii*. The distance of the sonicating brush head relative to the contaminated surfaced seems to have a greater impact than the duration of the insonation. This was in agreement with the data gathered on observations testing for duration of exposure length in an auxiliary test performed with this study (Appendix C).

Brushing possesses the desirable characteristics similar to that of swabs in that they are good for reaching hard to reach places. The additional effects of fluid dynamics to remove adherent bacteria even when the bristles are not in contact with the surface further enhances the sonicating brush's ability as a potential mechanism for surface / environmental sampling. Moreover, Seymour et al (2002) states the cleaning action of cavitation from fresh produce exposed to ultrasonication appears to remove cells attached to the surface of fresh produce, rendering the pathogens more susceptible to sanitizers. This may be due to the susceptibility of planktonic cells versus attached cells to chemical

biocides (Frank, 2001). Biofilms have shown much greater resistance to antibiotics and chemical sanitizers than their planktonic counterpart.

MacNeil, *et al* (1998) states that due to the sonic toothbrush operating at a high frequency with low power output, frictional contact between the bristles of the sonic toothbrush and oral tissues may completely or partially attenuate the production of an acoustic energy effect. For the present study, fluid forces generated by the sonic toothbrush seem to be effective in removal of adsorbed bacteria on stainless steel surfaces, at least at distances of 2-4 mm away from the surface.

Although the application of acoustic energy through fluid flow and cavitating actions have proven to be relatively effective in the removal, recovery, and enumeration of bacteria attached to various surfaces, an inherent problem lies in that a fluid medium is necessary for these methods to work. Frank (2001) states the methods as they are presented in studies using ultrasonics proves generally impractical for sampling and analyzing in the industry setting. In environmental sampling, where simplicity of design as well as efficient use of time is of the utmost importance, it is not feasible to use an ultrasonic bath in the sampling of machines and surfaces / objects that are either too bulky or otherwise unwieldy to be submerged into the waterbath. This problem also applies to commercially available ultrasonic probes as well. Sonicating brushes would conceivably remove attached bacteria on surfaces through the direct mechanical actions of the bristles scrubbing the surface of the area sampled, but the oscillating head at the present frequency would disperse and possibly aerosolize that could be pathogens. During the study, it was noticed that the entire brush head had to be fully submerged in solution, otherwise, the vibration of the brush head and bristles would spray the solution

onto the surrounding environment. However, with the relatively less expensive powered toothbrushes available to the general public sharing some of the principles of sonic toothbrushes, it is very conceivable that this technology could be used in a microbiological setting for environmental sampling using reduced power in a cost effective manner. In comparison to the cost of an ultrasonic water bath or an ultrasonic probe, the sonicating brush provides the similar principles of removal and recovery at a fraction of the cost.

Non-accounted-for cell recovery

In observing sampled coupons that were overlaid with molten TSA+YE, it was apparent that the sonic toothbrush in direct contact removed nearly all attached bacteria, while viable colonies grew on other sampled coupons (data not shown). However, when comparing the recovery of viable cells between sonicating brush with direct contact and indirect contact, there was no significant difference in recovery between the two.

Although viable cells attached to the stainless steel coupons were recovered, they did not significantly increase the total rate of recovery, since the addition of those viable cells to the total number of cell recovered would generally yield for less than a 1-2% increase.

Moreover, the data gathered in observance of recovery of entrapped cells in between the bristles of the sonicating bristles also did not significantly add to the total recovery of the sampled coupon. The contribution in adding the number of viable cells recovered would have yielded an increase on the order of less than one percent to the total number of recovered cells for the sample.

Effects of drying to recovery levels

For all sampling methods used, there was a noticeable reduction in the enumeration of *Listeria monocytogenes* as drying time for the inoculated coupons increased. While after one hour of drying, the highest mean recovery method was 0.22 \log_{10} lower than that observed of the *Inoculum*, after 12 hours drying, there was a 0.77 \log_{10} reduction when comparing the two (Tables 1 and 2). Moreover, after 24 hours drying, the recovery levels were below the limit of detection.

The reduction of minimum detection limits by the loss in microbial viability during drying over time has also been supported by studies conducted by Davidson, *et al.* (1999) and Moore and Griffith (2002b). In observation of the condition of the inoculum drying on the coupon, there seems to be a correlation between moisture content and survivability. At one hour drying time, the inoculum remained mostly beaded; however, at 24 hours drying (as well as in observation for 12 hour drying with lowered relative humidity), there was a disappearance of visible liquid on the surface of the coupon. Moreover, the presence / absence of organic and inorganic soils and nutrients in the liquid media would affect survivability of organisms in desiccation. Maxcy (1975) states that washed cells are extremely sensitive to drying, and in his study, he observed that when drying was extended even a few hours beyond the disappearance of visible liquid, vegetative cells did not survive. In recovery of cells washed three times by centrifugation, he reported that 48% of viable cells were recovered using water after one hour of drying.

Potential continuations of current study

Many studies can be performed to branch out and further the initial investigations. Research can be performed for potential differences in quantitative recovery using additional soiling such as organic soils; using materials such as serum and fats as well as inorganic soils such as the addition of minerals to the inoculum for greater surface attachment by the organism. Zottola and Sasahara (1994) stated that on food contact surfaces, it is likely that high levels of organic material would be present in a food processing setting, which could result in the formation of conditioning films which microorganisms could attach themselves and create biofilms.

There remain many questions in terms of comparative methods, whether one sampling method is paired in comparison with another, or whether differences in protocol within the sampling method would enhance the recovery of the sampled bacteria. Such investigations might include comparative studies using scouring methods, either in swab or pad form to those that are readily available such as cotton, Dacron, membrane filters, sponges, and laboratory wipes. Although recovery from swabbed samples is low, the swab method is chosen by many investigators due to its simplicity of use, familiarity, as well as its ability to be used in a broad range of settings. Investigations into optimizing methods in dislodging trapped microorganisms from the swab tip through the aid of surfactants or mechanical applications such as sonication, ultrasonication, and through the use of the likes of glass beads and agitation through the use of a vortex-type mixer would be prudent. Moreover, selection of recovery / dilution media may be a variable that should be further investigated to elucidate effective recovery of viable cells.

A possibility in making the application of sonication practical in sampling methodology could be the use of swab heads attached to a sonic generator to effectively remove the contaminants from the surface as well as use insonation to knock off trapped cells within the interwoven fibers of the swab head. Moreover, an apparatus such as a domed container covering the sonicating brush head could be manufactured with a water-tight seal on the bottom and with a flip top to introduce and extract liquid sampling media into and out of the apparatus using a pipette is conceivable and potentially cost effective when compared to the prices of other sampling apparatuses in the market. With the introduction of very low-cost vibrating toothbrushes in the market today, it is very likely that sonicating technology will be seen more readily in sampling and enumeration of contaminants.

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Level	N	Mean Recovery per Coupon ^a (log ₁₀)	Mean Std. Dev.
Inoculum	15	5.32 ^A	0.14
Brush non-contact	15	5.10 ^B	0.12
Brush contact	15	5.09 ^B	0.15
Sonic Bath	15	4.98 ^{BC}	0.17
Rinse	15	4.90 ^C	0.13
Agar Contact	15	4.64 ^D	0.16
Swab	15	4.62 ^D	0.14

^a Levels not connected by same letter are significantly different

Table 1. Mean recovery per coupon observed of *Listeria monocytogenes* dried for 1 hour at 20°C at ~50 - 60% RH using various sampling methods

Level	N	Mean Recovery per Coupon ^a (log ₁₀)	Mean Std. Dev.
Inoculum	10	5.39 ^A	0.12
Brush contact	10	4.62 ^B	0.17
Brush non-contact	10	4.57 ^{BC}	0.23
Rinse	10	4.31 ^{CD}	0.27
Sonic Bath	10	4.21 ^D	0.26
Agar Contact	10	4.02 ^D	0.15
Swab	10	3.65 ^E	0.27

^a Levels not connected by same letter are significantly different

Table 2. Mean recovery per coupon observed of *Listeria monocytogenes* dried for 12 hour at 20°C at ~50 - 60% RH using various sampling methods

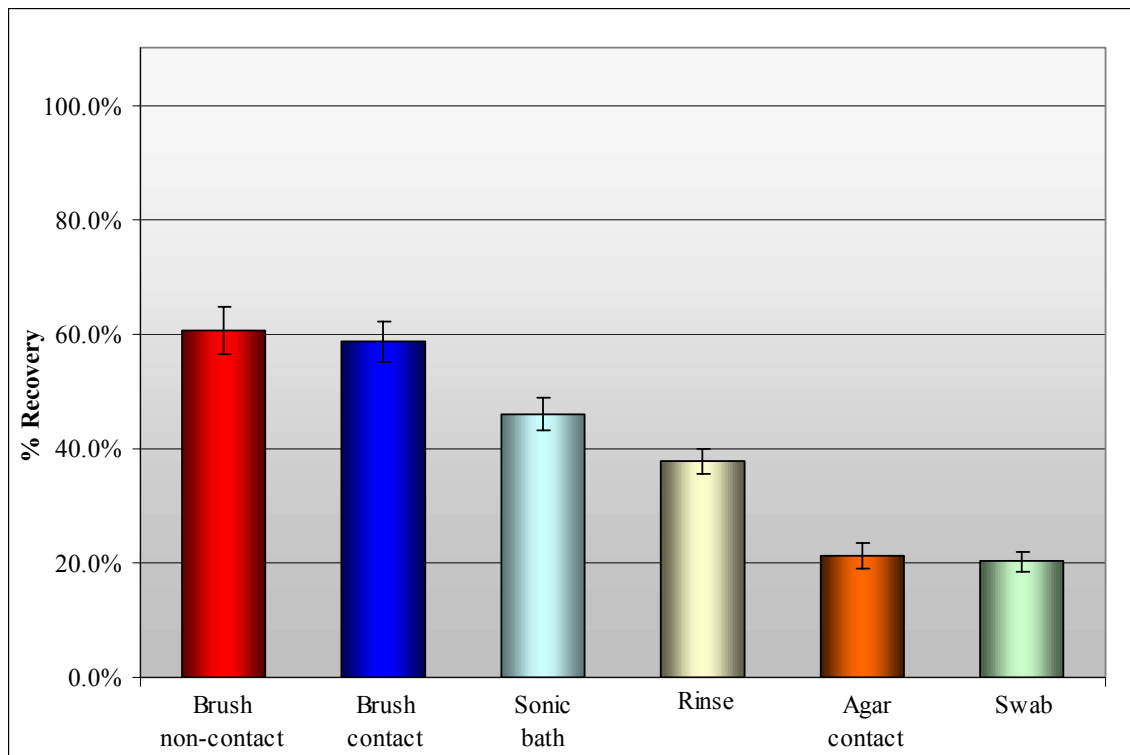


Figure 1. Mean percentage recovery of various sampling methods dried for 1 hour at 20°C with ~ 50-60% relative humidity as compared to the inoculum method

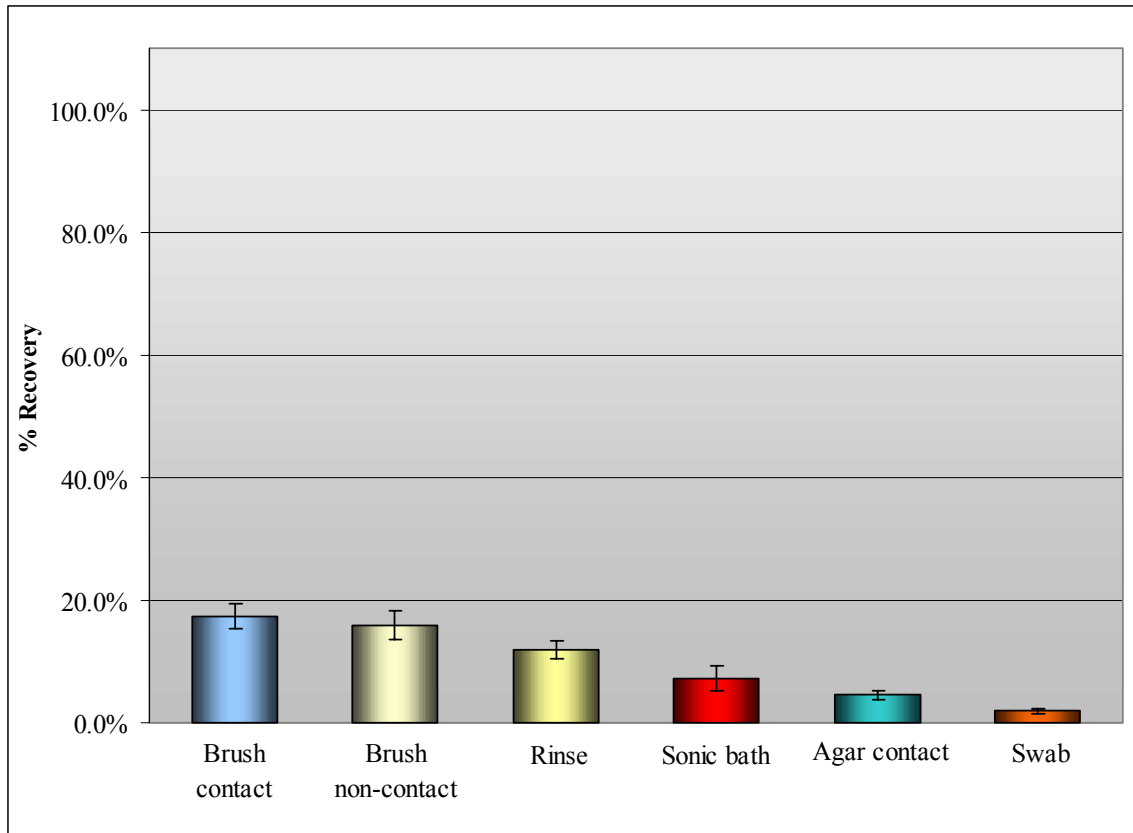
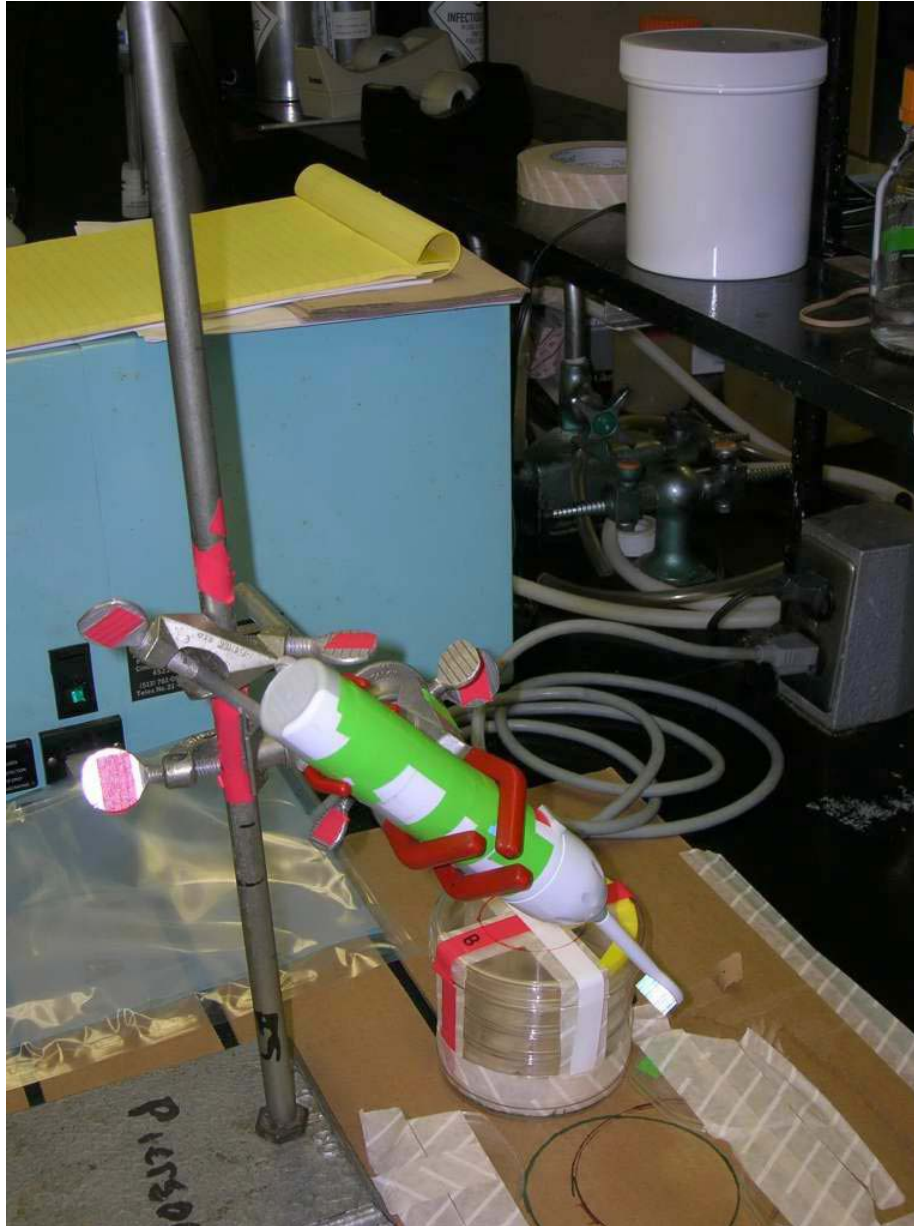


Figure 2. Mean percentage recovery of various sampling methods dried for 12 hour at 20°C with ~ 50-60% relative humidity as compared to the inoculum method



Appendix A, Figure 1: Bristles of the Sonicare® Elite 7300 brush head altered for uniform length for use in the study.



Appendix A, Figure 2: Apparatus setup for use of sonicating brush head for the “sonicating brush indirect contact” test.



Appendix A, Figure 3: Sonicating brush head in relation to the inoculated area of the stainless steel coupon submerged in 100 mL of PBS containing 1% polysorbate 80



Appendix A, Figure 4. Position of sonicating brush head relative to the angle and inoculated area of the stainless steel coupon in 100 mL of PBS containing 1% polysorbate 80 during brush non-contact method.

Appendix B. Description of media used

- PBS – PBS: Phosphate Buffered Saline 10X Ready concentrate, Biotech grade FisherBiotech. DNase, RNase, and Protease Free. 98.8g / 1L
 - Diluted 1:10 prior to use with sterilized deionized water

- Polyoxyethylenesorbitanmonooleate (Tween 80; Poly 80; Polysorbate), Sigma Chemical Co. St. Louis, Mo.

- Ultrasonic cleaning solution, Fisher Scientific Pittsburgh, PA.
 - Dodecylbenzene sulfonic acid sodium salt CAS# 25155-30-0
 - Dodecylbenzene sulfate CAS# 15-21-3
 - Sodium Bicarbonate CAS# 144-55-8
 - Sodium Triphosphate CAS# 7758-29-4
 - Water CAS# 7732-18-5

Appendix C. Effect of exposure time vs. cell recovery using the *sonicating brush* method and the effect of ultrasonication* on cell recovery from sampled swab heads. Percentage recovery based on comparison between Inoculum method.

* Exposed to ultrasonication (40 kHz) for 5 minutes

1 Hour Drying Time

Level	Avg. Log ₁₀ Recovery / Coupon	Std dev (log)	Avg. % Recovery	Std Dev (% Rec)
Inoculum	4.35	0.006	100.0%	1.46%
Brush (30 sec)	4.18	0.023	67.0%	3.86%
Brush (01 Min)	4.18	0.052	67.0%	3.51%
Brush (05 Min)	4.15	0.062	63.5%	6.88%
Swab-ultrasonics*	3.92	0.047	37.3%	9.50%
Swab	3.85	0.019	31.7%	1.32%

12 Hour Drying Time

Level	Avg. Log ₁₀ Recovery / Coupon	Std dev (log)	Avg. % Recovery	Std. dev (% Rec)
Inoculum	4.35	0.01	100.0%	1.46%
Brush (30 sec)	2.77	0.27	2.63%	0.15%
Brush (01 Min)	2.61	0.20	1.83%	1.06%
Brush (05 Min)	2.43	0.19	1.21%	0.73%
Swab-ultrasonics*	2.15	0.09	0.63%	0.15%
Swab	1.96	0.08	0.41%	0.07%

Appendix D. Validation of sonicating brush head sterility

Three prepared Sonicare® Elite Series 7300 brush heads, along with one prepared Sonicare® Elite Series 7300 brush head that was used as a viability control, were used per validation session. All environmental conditions used for testing were 20±2 °C with ~50-60% relative humidity.

Validation session one: exposure to 70% ethanol for one hour

- The bristles of four prepared Sonicare® Elite Series 7300 brush heads were submerged into 50 ml centrifuge tubes, each containing 30 ml of a cocktailed *Listeria monocytogenes* culture at a concentration of 3.7 x 10⁸ CFU / ml. The brush heads remained submerged for 20 minutes. Afterwards, the brush heads were transferred into empty sterile 50 ml centrifuge tubes, where it was dried for one hour.
- After drying, three brush heads were submerged into 50 ml centrifuge tubes containing 45 ml of 70% ethanol each; and the remaining brush head was submerged in 45 ml of sterile deionized water in a 50 ml centrifuge tube. The base for the Sonicare® Elite Series 7300 was attached to the submerged brush head and turned on for 30 seconds, sequentially. After insonation, the brush heads remained submerged and undisturbed in ethanol (or deionized water) for one hour.
- Following immersion, each brush head was “rinsed” by submerging the brush head into 10 ml of sterile deionized water in 15 ml centrifuge tubes, attaching the base, knocking off potential surviving cells by insonating for 10 seconds. This was repeated per brush head, using fresh sterile deionized water each time.
- The rinsed brush head was transferred into 40 ml of TSB+YE in 50 ml centrifuge tubes, then insonated for 10 seconds to remove entrapped air between the bristles.
- Per tube containing rinsed water, 8 ml was removed and replaced by 10 ml of TSB+YE. All tubes containing TSB+YE were placed in a 35±2°C incubator. After 24 and 48 hours post test, the tubes were observed for growth / turbidity (+) or no growth / clarity (0).

	Brush head 1		Brush head 2		Brush head 3		Viability	
<i>Incubation (hr)</i>	24	48	24	48	24	48	24	48
<i>Rinse 01</i>	+/-	0	0	+	0	+	+	+
<i>Rinse 02</i>	0	+	0	0	+/-	+	+	+
<i>Brush head</i>	+/-	+	0	+/-	0	+	+	+

Validation session two: exposure to 70% ethanol for three hours

- All procedures used in the second validation session was identical to the first session, with exceptions being the concentration of *Listeria monocytogenes* cultures used measured 4.2×10^8 CFU / ml and exposure to 70% ethanol was increased to three hours.

	Brush head 1		Brush head 2		Brush head 3		Viability	
<i>Incubation (hr)</i>	24	48	24	48	24	48	24	48
<i>Rinse 01</i>	0	0	0	0	0	0	+	+
<i>Rinse 02</i>	0	0	0	0	0	0	+	+
<i>Brush head</i>	0	0	0	0	0	0	+	+

- To ensure of its sterility and its maintenance of sterility, two brush heads tested were retained in TSB+YE for 21 days thereafter. The media remained clear during that time.

VITA

Suk-Kee Kang was born in Seoul, Korea but was raised in Vienna, Virginia where he attended George C. Marshall High School in 1996. After graduating high school, he attended Virginia Tech as a biology major. In 2000, the author graduated Virginia Tech with a Bachelor of Science in biology with a concentration in immunology and microbiology and a minor in chemistry. Soon thereafter, he attained a position at MICROBIOTEST, Inc. in Sterling, Virginia as a research microbiologist in the Division of Clinical and Applied Microbiology where he was promoted to Research Associate, then Study Coordinator, then Study Director. He moved divisions within the company to work primarily in the Division of Biofilm, Protozoology, and Virology, all the while retaining his title as Study Director in the previous division. He also performed extensive R&D research and held key roles in directing the development of the biofilms program. In 2003, Mr. Kang entered the Department of Food Science and Technology at Virginia Polytechnic Institute and State University where he is currently a Masters Candidate. While in graduate school, he was a member of the Institute of Food Technologists, the International Association of Food Protection, and Phi Sigma National Biological Honor Society.