SURFACE MATERIAL, TEMPERATURE, AND SOIL EFFECTS ON THE SURVIVAL OF SELECTED FOODBORNE PATHOGENS IN CONDENSATE

by

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(Under the Direction of Jeffrey L. Kornacki)

ABSTRACT

We evaluated four surfaces [stainless steel, Delrin® (DuPont) acetal resin, fiberglass reinforced plastic wall paneling (FRP), and mortar] to determine the effects that surface-type, soil (porcine serum), and temperature (4ºC and 10ºC) have on the survival of *Listeria monocytogenes*, *Salmonella* spp., and *Yersinia enterocolitica*, in the presence of condensate. Mortar had the most lethality among the four surfaces studied with *Listeria* and *Salmonella* surviving better than *Yersinia*. Temperature had little effect on survival of all three organisms across all surfaces. However, *Yersinia* displayed growth on FRP at 10ºC, but death at 4ºC. Serum had a protective effect on *L. monocytogenes* on all four surfaces, with populations sustained at significantly ($P \leq 0.05$) higher numbers over time than unsoiled coupons. Serum did not have an effect on the survival of *Salmonella* or *Yersinia* on stainless steel, acetal resin, or FRP, yet showed a protective effect on mortar.

INDEX WORDS: *Listeria monocytogenes*, *Salmonella*, *Yersinia enterocolitica*, biofilm, condensate, contamination

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DEDICATION

I would like to dedicate this thesis to my family and friends who have supported me over the years, with special thanks to my parents. Without their love, support, advice, and encouragement I would not be where I am today.

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CHAPTER 1

INTRODUCTION

Food product contamination from the post-processing environment is the most frequent cause of product contamination-based recalls, food poisoning outbreaks, and shelf-life problems in North America (51). It is impractical to create and maintain a sterile food processing environment. Therefore, manufacturers must try and maintain, as much as practically possible, strict control over the microbiological environment. Factory conditions that promote growth of microbes increase the risk of post-processing product contamination. Many factors affect the growth of microorganisms including moisture, nutrients, pH, oxidation-reduction potential, temperature, presence or absence of inhibitors, interactions between microorganisms in a population, and time. Conditions exist for the growth of microorganisms in most food processing factories. Moisture is the most critical of these as it is absolutely required for microbial growth (26). Air, water, tools, workers, traffic, and other means transfer microbes in the non-sterile factory environment into niches that are inaccessible for cleaning and sanitation. Within these niches many bacteria can attach themselves to underlying surfaces using cell wall-bound structures (e.g. proteins, polysaccharides, glycoproteins, etc.), given enough time. Bacteria that attach and are allowed to form biofilms can be protected from cleaners and sanitizers (21, 22). Attached cells have also been correlated with increased heat resistance (22).

Equipment and maintenance/repair practices that entrap moisture can result in microbial growth niche development (26). The same is true for operating conditions that release moisture into the environment. The water activity in niches also impacts the type of microflora that

develops therein (26). Disruption of these niches can ultimately result in contamination of the product stream (26). The probability of product contamination is affected by a number of variables including but not limited to 1) proximity of microbial growth niches to the product stream, 2) number of niches, 3) spatial relationship of niches to product stream, 4) microbial populations in niches, 5) extent of niche disruption, and 6) exposure of the product stream to the environment (26).

Entrapment of condensate in surface microenvironments (e.g. microscopic pores and irregularities) could result in niches with high microbial populations. Cold, moist niches favor the growth of cold-tolerating microbes over that of others. Cold-tolerating microbes of public health significance include *Yersinia enterocolitica*, *Listeria monocytogenes*, and selected strains of *Salmonella* spp. (50, 62). The chemical and physical nature of these microenvironments and the degree to which they can bind or entrap food residue is likely to play an important role in the growth and/or survival of selected microorganisms. The four surfaces studied in these experiments were as follows: 1) stainless steel which is used widely for food contact surfaces in food processing environments, 2) Delrin® by DuPont, an acetal resin used to make various machine parts (e.g. slicer parts, conveyor belt sprockets and rollers) which require durable, low friction surfaces, 3) fiberglass reinforced plastic wall paneling (FRP) which is often used in coolers and other rooms where the walls undergo frequent cleaning and need to be water impermeable and easy to clean, and 4) mortar, as used in concrete flooring and walls. [Mortar is primarily the mixture of sand and cement. The sand functions as aggregate and cement is the "glue" that binds the sand together. Concrete is mortar with a larger aggregate (e.g. rocks and pebbles) added for additional strength.] The working hypothesis was that growth of *Yersinia enterocolitica*, *Listeria monocytogenes,* and *Salmonella*, if present, would occur on these

surfaces provided that a source of nutrients (porcine serum), water (condensate), and sufficient time and temperature were present.

Research Objectives

1) To determine the influence selected food contact and non-food contact materials, which may be used in the ready-to-eat meat production environment, have on the survival of selected attached bacterial foodborne pathogens in the presence of condensate.

2) To determine the impact temperature has on the survival of selected surface-attached bacterial foodborne pathogens in the presence of condensate.

3) To determine the effect soiling selected surfaces has on the survival of selected attached foodborne pathogens in the presence of condensate.

CHAPTER 2

LITERATURE REVIEW

Foodborne Diseases Resulting from Meat Products

In the United States, foodborne organisms are estimated to cause 76 million cases of illness every year, including 5,194deaths (58). A large number of these cases are due to consumption of contaminated meat products. The Centers for Disease Control and Prevention (CDC) reported that from 1993 to 1997, the number of cases of illness due to the consumption of beef, pork, chicken, turkey, and other or unknown meats were 3205, 931, 1113, 758 and 645, respectively (13).

Meat from uninfected animals (absence of systemic bacterial, viral or parasitic infection) is generally regarded as being sterile before the animal is slaughtered. However, during and after slaughter, meat can be contaminated in many different manners such as knife sticks, cross contamination from animal hides, gastrointestinal contents, lymph nodes, hands of handlers, containers and other environmental surfaces. Contamination is not a great risk for products that are to be sold raw to the consumer, assuming that the meat is then handled and cooked properly. Unfortunately, as is often the case in many instances of foodborne illness, meats are not always properly handled or cooked in food service establishments or at home. Processed products that are ready-to-eat (RTE) can sometimes become cross-contaminated with pathogens from contact with contaminated processing environment surfaces after the kill step. These organisms are sometimes able to survive on the food and can potentially multiply to higher numbers and/or produce toxin, depending on the type of organism, food, storage and handling conditions.

Microbial Ecology of Food Processing Environments

There are numerous spoilage and pathogenic microorganisms that can contaminate food, including bacteria, fungi, parasites, and viruses. These organisms can originate from many locations in and around the processing facility (personnel, air, water, or non-food materials and equipment brought into the plant) or from the food ingredients entering the plant that were previously contaminated at another location (e.g. farm, slaughterhouse, or other processing facility). The focus of the research described in this thesis is on selected pathogenic bacteria. It has been well documented that *Yersinia enterocolitica*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria* spp. and *Aeromonas* spp. can be found in the slaughterhouse environment on floors and walls, hand washing sinks, splitting saws, chopping blocks, floors and drains (3, 27, 35, 67), all of which provide excellent opportunity for cross-contamination. Once organisms enter an environment, they can contaminate surfaces, attach, and grow given appropriate conditions. These growth niches may be located between sandwiched machine parts, in cracks and crevices in walls, floors and ceilings, and many other areas that may not routinely be cleaned and sanitized thoroughly. Microorganisms can become attached to or held within these niches by cell/substratum interaction or via retention by capillary action—such as within small, narrow cracks wherein cells are suspended in a liquid medium—or surface defects that provide protection from flow or other shear forces.

Attachment of Bacteria and Biofilm Development on Surfaces

Attachment. The attachment of bacterial cells to a surface is influenced by the properties of the surface itself, the cell's surface, and the medium, if any, surrounding the two. Attachment occurs in two stages: reversible followed by irreversible adhesion. Reversible attachment is an immediate, weak interaction involving Van der Waals and electrostatic forces

and weak hydrophobic interactions (60). Reversibly attached bacteria are easily removed by mild shear force. Irreversible attachment occurs when cells become anchored to the surface by extracellular polymeric substances (EPS) and/or appendages such as pili, flagella, or adhesin proteins (63, 77). This bonding usually occurs within hours of initial contact (44). Removal of irreversibly attached cells requires either strong shear force or disruption of the bonds through the use of detergents, surfactants, sanitizers, enzymes and/or heat (7, 34, 61, 64, 72).

Biofilm formation. Attached bacteria have the potential to form a biofilm, depending on the temperature, amount of moisture, nutrient supply, surface type, and frequency and efficacy of cleaning and sanitation of the surface. Biofilms are formed by cells that have attached to a surface and through the production of EPS, which cover and protect cells as well as provide firm attachment, create an immobilized microenvironment wherein cells can entrap and scavenge nutrients and potentially grow. EPS provide protection to cells within the biofilm from desiccation, prevent penetration of biocides, and sequester minerals and other nutrients (12). The biofilm formation process is generally regarded to consist of the following steps: 1) organic or organic/mineral material (food material, soil, etc.) from the environment coat the surface forming a conditioning film; 2) bacteria arrive at the conditioned surface; 3) bacterial surface structures (polysaccharides, proteins, glycoproteins, etc.) become attached to the film largely through hydrophobic bonds and electrostatic forces (23, 65); 4) as a result of arrival at the surface, production of EPS is initiated, possibly through induced genetic regulation of synthesis (8), or if these polymers were already present prior to attachment, they can assist in attachment. If conditions are favorable (especially in flowing, aqueous environments) the biofilm can grow and develop into a multi-structural community (10).

Influence of surface on attachment and biofilm formation. The topography of a surface can affect both soil retention and microbial attachment. Surface defects allow for increased soil/nutrient retention (30) and provide protection from shear forces which allow bacteria to remain on the surface (49). The properties of the conditioning film that forms when a surface comes into contact with an organic soil are influenced by the underlying substratum (6). The degree of hydrophobicity and free energy of a surface influence the type of organic molecules that can bind as well as their orientation—if they are polar. Therefore, a particular soil may have differing abilities to bind to microorganisms depending on the underlying surface. The concentration of a conditioning soil may also affect attachment. Flint, et al. (29) showed that a dilute milk soil enhanced bacterial adhesion more than undiluted milk soil. However, since accumulation of soil and microorganisms is also affected by surface topography, the molecular effects discussed above may have little overall influence on bacterial attachment. In one study, various floor surfaces were exposed to routine processing conditions, including cleaning and sanitation, in a plant for a 10 week period. The materials tended to have similar surface free energies at the end of the study which indicated that initial surface properties are not maintained over time in the environment and therefore do not affect later surface fouling (59).

Implications of Attached Bacteria and Biofilms in Food Processing

Because microorganisms are inherent in any raw food, the chances of contamination of a surface within a processing environment, and subsequent attachment, are great. Austin and Bergeron (2) found microbial attachment on rubber and Teflon gaskets in a dairy processing line, even though proper cleaning and sanitization procedures were performed. There is a greater chance of attachment as gaskets age. Czechowski (17) found that most organisms will attach to the inner edge of a gasket where it is in contact with the product. Stone and Zottola (73) showed

that during dairy processing *Pseudomonas* attaches to stainless steel equipment as soon as milk is introduced and can remain attached during processing. As pathogenic and/or spoilage bacteria accumulate on such surfaces there is a greater chance that they will form a biofilm.

Over time, attached cells and biofilm will detach from a surface, as part of the normal life cycle of microorganisms in natural systems, and may migrate to a new growth site (4). Depending on the population size and dynamics of the biofilm, this situation can lead to sporadic, low-level contamination of product, especially on surfaces in direct product contact. Low levels of *Salmonella* associated with cheese implicated in a 1989 salmonellosis outbreak (38) and with ice cream in 1994 (41) appear to be two examples of such a situation. In neither case was *Salmonella* isolated from the questionable surfaces, which had been suspected due to improper cleaning procedures. Possibly, evidence of contamination had disappeared due to the transient nature of attached cells and biofilms, an externally applied force which removed the evidence, and/or competition from other microbes.

Beyond increasing risk to the product or consumers, biofilms can cause damage to and the malfunction of machinery. Biofilms can cause mechanical blockage within fluid handling systems, the impedance of heat transfer (68), and corrosion of metal surfaces (9).

Role of Condensate in Bacterial Survival and Dispersal

Condensation of water occurs when the air above a surface is warmer than the surface and the humidity of the air is at a critical percentage. Given the large amount of steam generation that occurs in certain food processing environments, it is almost assured that condensate will form. Condensate provides bacteria and fungi a source of water for survival and potentially growth, if there are ample nutrients and time. Furthermore, as condensate builds up, the potential arises for carriage of any organism present within that niche, to other locations by

way of drips from ledges or overhead cracks, splashes, aerosols, or seepage through porous surfaces.

Control of Microorganisms in Food Processing Environments

Controlling the attachment of microorganisms to a surface is very difficult, practically speaking. In a food or beverage processing environment, the physicochemical properties of a surface, even one that is particularly resistant to attachment, changes once a conditioning film has formed. This film can vary in composition over time and so does the degree in which organisms can attach to it. Therefore, in order to effectively control attachment, one must focus on equipment design, proper cleaning and sanitization and other environmental controls.

Equipment and factory design can significantly affect the potential for biofilm formation. High shear forces can prevent attachment by limiting contact time to a surface and can help remove attached cells. Reducing the number of joints, valves, and other protected sites can also help minimize attachment by reducing both the build-up of product and the number of sites where cells would be shielded from shear forces. In addition, choosing construction materials for machine parts, floors, and walls that are less likely to hold water, become scratched, and are easy to clean, is important.

Wet cleaning procedures generally include the following basic steps: 1) removal of gross soil using warm or cold water; 2) application of cleaning agent; 3) hand or mechanical scrubbing using a brush or turbulent flow; 4) rinsing suspended soil and cleaning solution from surfaces; 5) application of disinfectant solution; and 6) rinsing of disinfectant (47). This cleaning process is designed to remove soil, but not necessarily biofilms. Research has shown that typical cleaning procedures do not remove all biofilm microorganisms or associated glycocalyx (42, 73, 78). Over time, if cleaning does not effectively remove all food and biofilm residue, then there may

be a gradual accumulation of these residues with each cleaning/processing cycle leading to a decrease in product quality or safety (24). Alkaline agents have been found to be more effective than acidic ones for removing biofilm and chlorinated alkaline cleaners may be the most effective (18). Effective removal requires the proper concentration, time, and temperature (25, 73, 78). Longer exposure times and higher concentrations than are normally used may be needed to remove all biofilm residues (78).

Sanitization procedures are aimed at killing microorganisms that are remaining and have been exposed following the cleaning process. These remaining organisms are often located in protected niches and/or contained within the protective glycocalyx of a biofilm. Commonly used disinfectants include chlorine (hypochlorous acid-containing compounds), quaternary ammonium compounds, anionic acid compounds and peroxyacetic acid.

The effect of sanitizers on different surfaces and organisms has received much attention. For instance, polymeric surfaces and aluminum require greater concentrations of sanitizer to inactivate adhering microorganisms than does stainless steel (33, 52, 57). Copper and carbon steel are less easily disinfected than stainless steel (15).

Resistance to sanitizers is of concern especially with regards to attached cells and those within biofilms. Frank and Koffi (31) showed that *L. monocytogenes* which has been allowed to attach for 4hours can be completely inactivated by a quaternary ammonium compound and anionic acid disinfectant after 16 minutes. However, a 14day old biofilm can survive a 20 minute exposure. Biofilms that were 8 days old were over 100 times more resistant to 200 mg/L hypochlorite than were 4 hour attached cells (54). Wirtanen and Mattila-Sandholm (79) observed that it takes at least 48 hours for adherent microorganisms to develop sanitizer resistance which they linked to the production of glycocalyx material. Essentially, the longer a

population of cells remains on a surface, the more likely it is to become resistant to sanitizers. Therefore, the more often that cleaning and sanitization can be performed, the better.

Some examples of other environmental controls that can be implemented to reduce the incidence of growth niches include steps that can reduce moisture, i.e. increasing airflow, exhausting steam to the exterior of the building, eliminating standing water, both on floors and in drains, thorough drying of factory before start up, and appropriate use of dehumidifiers. Lowering the temperature is another controllable factor that can slow the growth of microorganisms.

Pathogens Evaluated in the Present Study

Listeria monocytogenes. *Listeria* spp. are small (0.5 µm x 1-2 µm), gram positive bacilli, asporogenous, catalase positive, and facultative anaerobes. Listeriae produce flagella and are motile at 20-25°C but not at 37°C. *Listeria monocytogenes* is a non-fastidious organism and can tolerate many growth conditions. It grows at 10% NaCl and survives 25.5% NaCl at 4°C (43, 55). *L. monocytogenes* is a psychrotroph with a reported minimum growth temperature of 1.1 ± 0.3°C (55).

L. monocytogenes primarily causes clinical disease with high morbidity and mortality in the immunocompromised, elderly, and in fetuses and newborn infants. In healthy individuals there may be an absence of symptoms or a mild flu-like illness (66). The organism can cross the placenta and cause stillbirths, spontaneous abortion, fetal death, severe neonatal septicemia, and meningitis. In nonperinatal patients with predisposing factors, bacteremia is more frequent with central nervous system infections being the predominant syndrome. The overall case-fatality rate for systemic or invasive listeriosis is usually about 20 to 30% for both epidemic and sporadic cases (11, 32).

L. monocytogenes forms biofilms on stainless steel, plastic and polycarbonate surfaces and numerous other food contact surfaces (31, 39, 44, 56). It has been isolated from various environmental surfaces including conveyor belts, flood drains, condensate, storage tanks, hand trucks, cleaning aids (mops, sponges, hoses and brooms), peelers, slicers, and wash areas (37). In a recent survey of 31,705 samples taken from eight categories of ready-to-eat foods over a 14-23 month period, the overall prevalence of *L. monocytogenes* was 1.82% (36). These figures illustrate the need to understand how and where *L. monocytogenes* can survive and to be able to effectively control its prevalence in the food processing environment.

***Salmonella* spp.** Salmonellae are facultatively anaerobic, gram-negative bacilli belonging to the family *Enterobacteriaceae*. They are oxidase negative, catalase positive, generally produce hydrogen sulfide, can grow on citrate as a sole carbon source, decarboxylate lysine and ornithine, and do not hydrolyze urea. Salmonellae are generally flagellated, but a few non-flagellated and dysfunctional (non-motile) flagellated variants do exist which can hinder identification of the serotype. There are over 1,400 serotypes of *Salmonella enterica* subsp. *enterica* which encompasses the majority of the serotypes that cause foodborne illness. *Salmonella* spp. actively grow within a wide temperature range up to 54°C with a few strains having been found to grow in foods stored at 2-4°C (20). Certain isolates have been shown to grow at a pH as low as 3.99 at 22°C (1).

Salmonella infections in humans can lead to several clinical conditions, including enteric (typhoid) fever, which is caused by the Typhi and Paratyphi serotypes, enterocolitis, and systemic infections. Enteric fever is a serious disease characterized by diarrhea, prolonged and spiking fever, abdominal pain, and prostration. Nontyphoidal enterocolitis is generally a self-limiting disease consisting of diarrhea, fever, and abdominal pain which usually subside within 5

days of onset. CDC estimates that there are approximately 1.3 million cases of illness resulting in 553 deaths due to foodborne nontyphoidal salmonellosis in the U.S. each year (58).

Salmonella has been isolated in a wide range of foods that have been implicated in outbreaks. In 1996, *Salmonella* serotype Enteriditis was the cause of an estimated 224,000 cases that were linked to consumption of ice cream which had been carried in a tanker truck which had previously carried raw eggs but had not been appropriately sanitized (41). In another outbreak, *Salmonella* serotype Agona contamination of a dry, ready-to-eat breakfast cereal lead to over 200 reported cases and nearly 50 hospitalizations across 11 states in the U.S. (14). The poultry processing environment is by far the largest reservoir for *Salmonella* among all food categories (19). Several studies have shown that *Salmonella* can attach and form biofilms on surfaces commonly found in food processing plants including plastic, stainless steel, and cement (40, 48).

Yersinia enterocolitica. *Yersinia enterocolitica* is an organism which is frequently isolated from the soil, water, animals, and a variety of foods. It is a member of the family *Enterobacteriaceae*, along with *Salmonella*. *Yersinia* is a gram negative rod. It is oxidase negative, urease positive, and esculin negative and is a psychrotrophic organism being able to grow between 0 and 44°C (71). *Yersinia enterocolitica* can survive in frozen foods for long periods even after repeated freezing and thawing (76). They can grow over a pH range from approximately 4 to 10, with an optimum pH of around 7.6 (69).

The serogroups of *Yersinia enterocolitica* which are most prevalent in human disease include: O:3, O:8, O:9, and O:5,27. Infections with *Yersinia enterocolitica* can result in a wide range of clinical presentations and outcomes. Most symptomatic infections result in a self-limiting diarrhea, however, yersiniosis may also give rise to a number of syndromes including, but not limited to suppurative lymphadenitis, bacteremia, cellulitis, septic arthritis, and urinary

tract infection (16). Enteritis usually occurs in children who present with diarrhea, low-grade fever, headache, and abdominal pain being predominant (45). Sore throat is also common and may be the dominant symptom in older patients (74). Acute yersiniosis may also present as a pseudoappendicular syndrome which often leads to misdiagnosis and the patient undergoing an unnecessary appendectomy (16). Outbreaks that have occurred in which a source was traced have most often been attributed to consumption of contaminated milk, although cattle do not appear to be an important reservoir of these bacteria. In these cases, the milk could have been contaminated from pig (probably the most important reservoir) or human feces during or after processing (70).

Yersinia enterocolitica has been isolated on several surfaces during surveys of pig abattoirs, including worktables, slaughtering floors, dehairing machines, and other processing equipment where detritus had built up (35, 67). However, very little, if any, research has been done to study the potential for attachment and survival on any of these or other surfaces.

CHAPTER 3

MATERIALS AND METHODS

Culture Preparation

The isolates/strains used and their origin, if known, where as follows: *Listeria monocytogenes* serotypes 4b (101M; beef), 4b (processed meat associated outbreak), 1/2a (Silliker Laboratories, South Holland, IL; SRL #34; meat processing environment), 3a (Silliker Laboratories, South Holland, IL; SRL #81; meat), and 1/2b (Silliker Laboratories, South Holland, IL; SRL #498; pork sausage); *Salmonella* serotypes Agona (Silliker Laboratories, South Holland, IL; SRL #141; cereal), Typhimurium DT104(clinical), Heidelberg (swine), Enteriditis, and Anatum (Silliker Laboratories, South Holland, IL; SRL #377; cattle); and *Yersinia enterocolitica* isolate 7YP (undetermined serotype) and isolates from Centers for Disease Control and Prevention of serotypes 0:8 (Pasteur Institute Collection), 0:9 (Pasteur Institute Collection), 0:3 (environmental-swine farm), and 0:5,27 (clinical). Cultures were stored at -80ºC on Cryobeads™ (TSC, Ltd., Lancashire, UK).

Each culture was prepared from an isolated colony obtained from a plate of Tryptic Soy Agar with 0.6 % Yeast Extract (TSA-YE) (Beckton, Dickinson and Company, Sparks, MD) previously streaked from a frozen culture. Isolates were sequentially transferred three times after 24 hours incubation at 37ºC in Trypticase Soy Broth with 0.6 % Yeast Extract (TSB-YE) (Beckton, Dickinson and Company, Sparks, MD) before use in the experiment to ensure an early stationary phase culture. On the third and final transfer, 100 µL of each culture in TSB-YE was inoculated into 100 mL (30 mL for mortar experiments) of TSB-YE and incubated (24 h; 37ºC).

Cells where centrifuged (10 min. @ 5520 x g), after the final transfer, using a Beckman J2-MI centrifuge (Beckman Coulter, Inc., Fullerton, CA), washed three times and resuspended to 100 mL (30 mL for mortar experiments) with sterile Butterfield's phosphate buffer (pH 7.2) (BPB) .

Preparation of Surface Coupons

Coupons of stainless steel (type 304; #4b finish), fiberglass reinforced plastic wall paneling (FRP), and Delrin® by DuPont (Wilmington, DE), an acetal resin, were cut measuring 8 x 3.3 cm. Surfaces were wiped with acetone to remove any oil residue, hand-washed in hot water with Terg-A-Zyme (Alconox, Inc., White Plains, NY), rinsed in deionized water, sonicated at 42 kHz ± 6% (FS30H Ultrasonic Cleaner, Fisher Scientific, Pittsburgh, PA) in an alkaline solution of 2% Microsoap (International Products Corporation, Burlington, NJ) at 60ºC for 5 minutes, and then rinsed again in deionized water. Coupons were autoclaved for 15 minutes at 121ºC before use.

Mortar samples were formulated, mixed, poured, cured, and cut (2.5 x 2.5 x 1.3 cm) under defined conditions at the direction of Dr. Kimberly Kurtis, School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta. Mortar was selected for use in this experiment due to the smaller aggregate size, as compared to concrete, that enabled easier cutting of coupons to the desired dimensions. The mortar formulation was based on mixes used in industrial flooring applications. See Table 1 for the formulation. The mortar was prepared by first mixing ASTM (American Society for Testing and Materials) type I cement, Class C fly ash and natural siliceous sand, obtained from Brown Brothers Quarry in Junction City, Georgia, USA, in a Hobart mixer (Hobart Corp., Troy, OH) for 3 minutes at 60 rpm. Separately, superplasticizer (ADVA Flow superplasticizer, Grace Construction Products, Cambridge, MA; 3.1 μL per gram of cement) was added via a pipette to deionized/distilled

water. Subsequently to the 3 minutes of dry ingredient mixing, the water and superplasticizer were slowly added to the mixer, to improve the workability of the mortar, and mixed for another 5 minutes, from the first addition of water, at 120 rpm. From this mix, 2.5 x 28.8 x 1.3 cm beams were cast in brass molds. These molds were placed on a vibrating table for at least 5 minutes to aid compaction. After compaction, samples were allowed to set for 5 minutes before the addition of the surface hardener (Maximent HD, ChemRex, Shakopee, MN). The surface hardener was sprinkled, at the manufacturer's recommended rate of 4.9 kg/m^2, on the exposed surface of the mold and a trowel was used to finish the surface. Samples were demolded after 24 hours and placed in a limewater curing tank for an additional 27 days at 20 ± 2ºC. Furthermore, the samples were cut with a diamond blade masonry saw to the desired 2.5 x 2.5 x 1.3 cm dimensions. Coupons were brushed by hand to remove excess mortar dust and autoclaved for 15 minutes at 121ºC before use.

Soiling of Coupons

All coupons to be soiled where dipped into sterile pork serum (Atlanta Biologicals, Atlanta, GA) and allowed to dry for 45-60 minutes before inoculation.

Inoculation of Coupons

Coupons were inoculated by immersion, standing on edge, into cell suspensions of ca. 10^7 CFU/mL in sterile BPB prepared from a five-strain cocktail made of equal volumes of the washed cell suspensions (as prepared above) of each organism-type evaluated (either *Listeria*, *Salmonella*, or *Yersinia*). Coupons were incubated at 25ºC for 2 h to allow attachment of cells to the surfaces. Afterwards, the coupons were rinsed by dipping into consecutive buckets of sterile BPB [two for stainless steel, FRP, and acetal resin (8 L in each) and five for mortar (12 L in each)] to remove unattached and loosely attached cells. The unfinished side of FRP was wiped

thoroughly with 70% ethanol to remove and kill attached cells (due to the fact that in a processing environment only the finished side is exposed). [Note: CFU counts for the finished side (26.4 cm^2) were doubled to yield a count for a surface area totaling 52.8 cm^2. Counts on mortar were doubled as well, since the coupons had approximately 25 cm^2 of surface area. These calculations were done to enable comparisons of data obtained from all surface types each with ca. 52.8 cm^2 surface area.]

Incubation of Coupons

After rinsing in BPB, coupons were placed into sterile Petri dishes (150 mm diameter x 10mm height; 100 x 15 mm for mortar). Each individual Petri dish contained 2-3 coupons of one surface-type, either soiled or non-soiled, inoculated with one organism cocktail to be held at a specific temperature. Each Petri dish contained a sterile filter paper disk (12.5 cm diameter; 80 mm for mortar; Fisher Scientific, Pittsburgh, PA) on the bottom along with sterile wooden or plastic supports to keep the coupons elevated above the filter paper (Figure 1). The filter paper was moistened with ca. 3 mL sterile water. The Petri dishes were then sealed using Parafilm M (Pechiney Plastic Packaging, Chicago, IL) to produce a 100% relative humidity environment and incubated at either 4ºC or 10ºC. [Note: *Salmonella* was only incubated at 10ºC on stainless steel, acetal resin, and FRP for two reasons: 1) most strains of *Salmonella*, including those used in this study, unlike *Listeria monocytogenes* and *Yersinia enterocolitica*, are not psychrotrophs and thus do not have growth potential at 4ºC and 2) the results of this study show that temperature did not have a significant effect on *Salmonella* survival on mortar, a very harsh environment (see Figure 3 in Chapter 5: Results), therefore the decision was made that it was not necessary to evaluate the survival at 4ºC on the other surfaces.]

Sampling of Coupons

Sampling of stainless steel, FRP, and acetal resin took place immediately after attachment and rinsing (day 0) and then on days 3, 6, 9, 12, and 15. Three coupons of each surface type per soil and temperature condition were removed from incubation at each sampling time. Mortar coupons were sampled at 9-10 time periods over a total of 120 h.

Sampling for bacteria on stainless steel, FRP, and acetal resin involved removing coupons from the sealed Petri dishes and placing each coupon, on edge, into its own jar containing 100 mL BPB plus 3 g glass beads (425-600 µm diameter; Sigma, Saint Louis, MO). Each jar was shaken on an orbital shaker (New Brunswick Scientific, Edison, NJ) at 350 rpm for 2 minutes to remove attached cells. An aliquot of the buffer was then plated onto TSA-YE and incubated 24 h at 37ºC, after which colonies were enumerated.

Bacteria on mortar coupons were sampled by placing them into jars containing 100 mL sterile BPB and sonicating them for 30 seconds, without heat, and then plating an aliquot of the buffer onto TSA-YE. For sampling periods ≥ 24 h after inoculation, the remaining buffer in each jar was enriched by adding 15 mL 5X TSB-YE and incubation for 48 h. In instances where the TSA-YE plates had no colonies but the enrichments were visibly turbid, a loopful (10µL) of the enriched BPB was streaked for isolation to selective media (Modified Oxford Medium [Becton, Dickinson and Company] for *Listeria*, Xylose Lysine Tergitol™ agar [XLT4; Becton, Dickinson and Company] for *Salmonella*, and *Yersinia* Selective Agar [Oxoid, Basingstoke, England] for *Yersinia*). The presence of typical colonies was considered as positive confirmation and results were recorded as either positive or negative.

Experimental Design and Statistical Analysis

A split-plot design was used with temperature, soil, and surface-type as independent variables. Two to three coupons were sampled for every condition at each sampling time and entire experiments were replicated three times. Data were pooled from the three experimental replications and analyzed using the general linear model in SAS (Statistical Analysis Software, SAS Institute, Cary, NC). Mean separations were performed using Fisher's student *t*-test ($P = 0.05$).

Table 1. Mortar coupon mix ingredients per liter

Type I portland cement	573.2 g
Class C fly ash	172.0 g
Sand (FM* = 1.80)	1117.7 g
Deionized/distilled water	334.9 g
Superplasticizer	1775 µL

* FM = fineness measurement

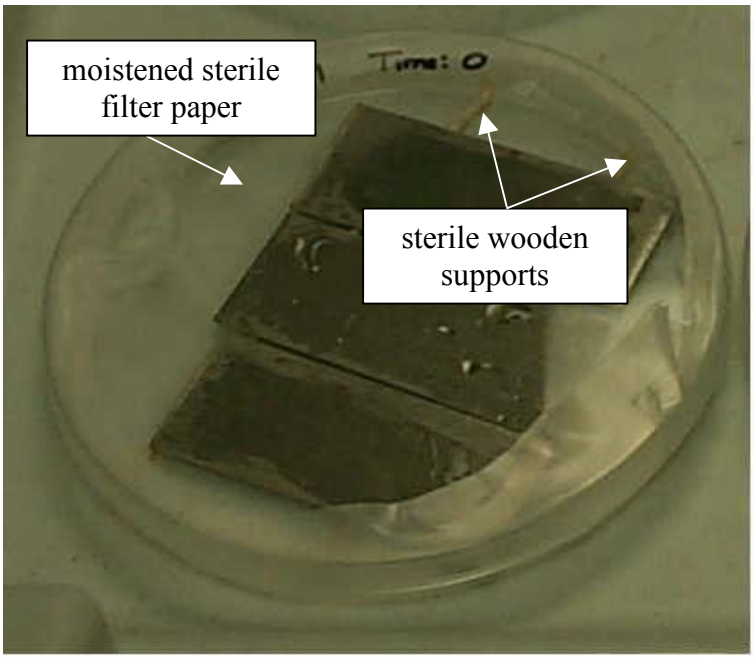

Figure 1. Parafilm-wrapped Petri dish with 100% humidity environment

CHAPTER 4

RESULTS

Objective I: To determine the influence selected food contact and non-food contact materials, which may be used in the ready-to-eat meat production environment, have on the survival of selected attached bacterial foodborne pathogens in the presence of condensate.

Mortar appeared to have the most lethality of any of the four surfaces studied. As shown in Figure 2, on mortar with serum present, *Yersinia* had more than a 5 log decrease in population in only a 24 h period and could only be detected by enrichments out to 120 h (Table 2). *Listeria* and *Salmonella* (Figures 3 and 4, respectively) had better survival on mortar, in the presence of serum, than *Yersinia* throughout the 120 h incubation period.

Stainless steel did not support the survival of *Listeria* as well as acetal resin or FRP when compared across all soil and temperature conditions (Table 3). However, for *Salmonella*, acetal resin and stainless steel were both less supportive than FRP, which sustained the highest populations (least reduction). There were no significant differences ($P > 0.05$) between population changes over 15 days for *Yersinia* on any of these three surfaces across all soil and temperature conditions.

Objective II: To determine the impact temperature has on the survival of selected surface-attached bacterial foodborne pathogens in the presence of condensate.

Temperature appears to have very little effect on the survival of *Listeria monocytogenes*, in general, on the surfaces tested. The only significant differences ($P \leq 0.05$) seen between 4ºC

and 10ºC on stainless steel were at day 6. The populations also declined more rapidly at 10ºC than at 4ºC after 24 h on mortar (Figure 3).

Salmonella was only evaluated at both 4ºC and 10ºC on mortar (Figure 4). The other *Salmonella*-inoculated surfaces were incubated at 10ºC. Differences in temperature did not effect the survival of *Salmonella*, with one exception. Only at the 48 h sampling time was there a significant difference ($P \leq 0.05$) with 10ºC having a 0.65 log higher average population (combined soiled and non-soiled data).

Unlike *Listeria* and *Salmonella*, temperature had an effect on the survival of *Yersinia enterocolitica* on stainless steel, acetal resin, and FRP, with incubation at 10ºC sustaining the populations (and even allowing growth on FRP) more so than at 4ºC. This effect is the most profound at day 15 for each of these surfaces (Figure 2). On mortar, however, there was no overall difference in survival of *Yersinia* noted between the two temperatures, with the exception of one sampling time (16 h) (Figure 2).

Objective III: To determine the effect soiling selected surfaces has on the survival of selected attached foodborne pathogens in the presence of condensate.

Serum had a protective effect on the survival of *L. monocytogenes* on all four surfaces tested, sustaining populations at significantly ($P \leq 0.05$) higher numbers over time than unsoiled coupons, as illustrated in Figure 3. The presence of serum did not have an overall significant effect on the survival of *Yersinia* or *Salmonella* on stainless steel, acetal resin, and FRP, yet showed a significant protective effect on mortar (Figures 2 & 4, respectively). However, the protective effect for *Salmonella* on mortar was much more pronounced than for *Yersinia*.

Additional Observations

There were no significant differences ($P > 0.05$) among the mean CFU/coupon of *L. monocytogenes*, *Y. enterocolitica* or *Salmonella* upon initial attachment onto any of the four surfaces (unsoiled), with one exception. Attachment of *L. monocytogenes* to stainless steel was significantly less than on FRP, as determined by Fisher's student *t*-test ($P \leq 0.05$).

Table 2. Recovery by enrichment of foodborne pathogens on mortar, with or without soil, after incubation at either 4°C or 10°C

Bacteria	Conditions		Time (h)					
			16	24	48	72	96	120
Listeria	soil	4 C	nt	6/6	6/6	6/6	6/6	3/3
		10 C	nt	6/6	6/6	4/6	2/6	0/3
	no soil	4 C	nt	6/6	5/6	4/6	1/6	1/3
		10 C	nt	5/6	1/6	2/6	0/6	0/3
Salmonella	soil	4 C	6/6*	6/6	6/6	6/6	6/6	5/6
		10 C	6/6	6/6	6/6	6/6	6/6	2/6
	no soil	4 C	2/6	3/6	1/6	1/6	1/6	0/6
		10 C	0/6	0/6	0/6	0/6	0/6	0/6
Yersinia	soil	4 C	nt	7/9	2/9	0/9	2/9	0/3
		10 C	nt	2/9	0/9	0/9	0/9	0/3
	no soil	4 C	nt	0/9	1/9	0/9	2/9	0/3
		10 C	nt	0/9	0/9	0/9	0/9	3/3

nt = not tested

* # of positive enrichments/# coupons sampled

Table 3. Decline in $\log_{10}$ CFU/coupon of foodborne pathogens over a 15 day incubation period on three surface types*

Surface	*Listeria monocytogenes*	*Salmonella*	*Yersinia enterocolitica*
Stainless steel	-3.39 B	-2.60 B	-0.91 A
Acetal resin	-1.85 A	-3.10B	-1.43 A
FRP	-1.79 A	-1.02 A	-0.28 A

* Values (given in $\log_{10}$cfu/coupon) are derived from the combined data for all temperature (4°C and 10°C) and soil (soiled and unsoiled) conditions. Significant differences ($P \leq 0.05$) between population changes among surfaces for each organism are designated by different letters following the numerical value.

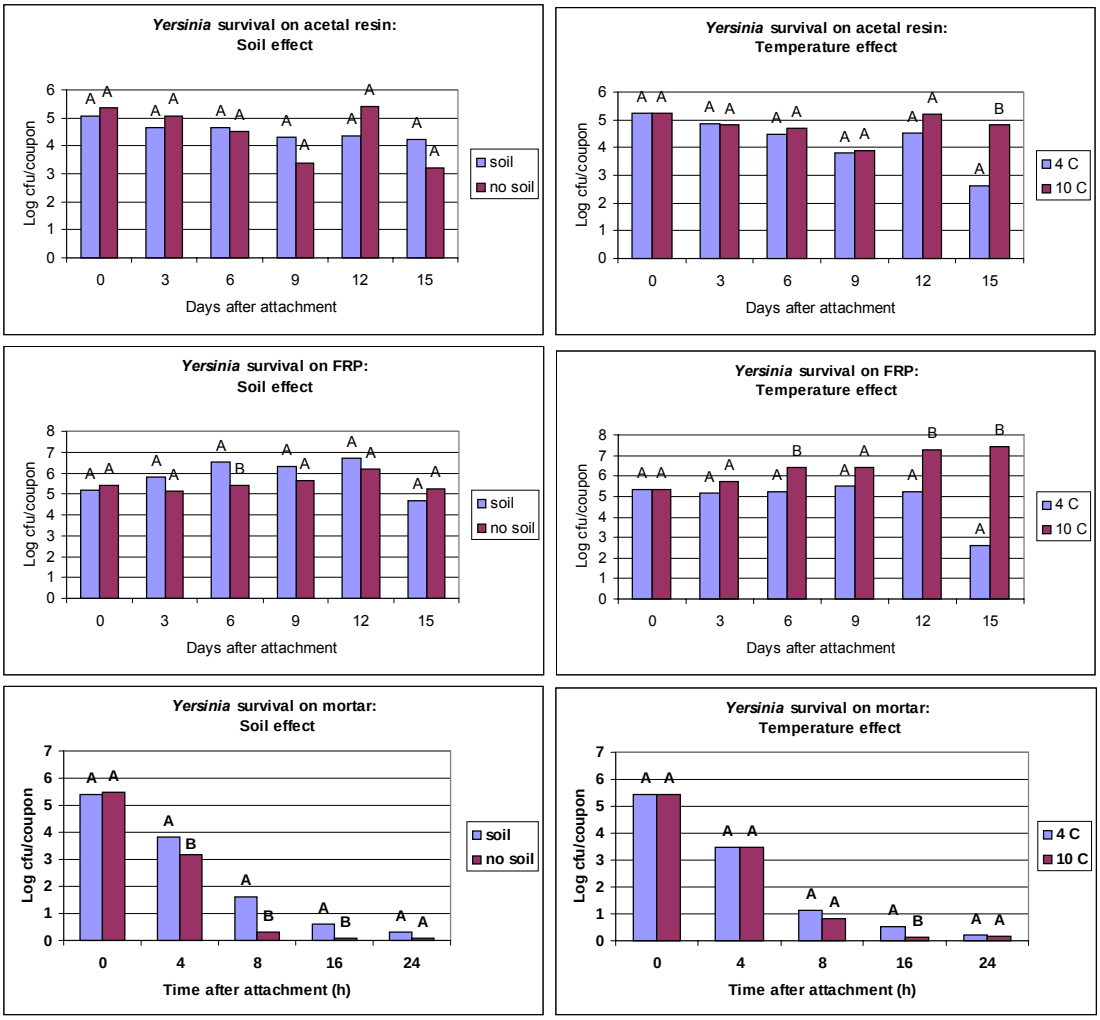

Figure 2. Influence of soil and temperature on the survival of *Yersinia enterocolitica* attached to four different surfaces. At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test.

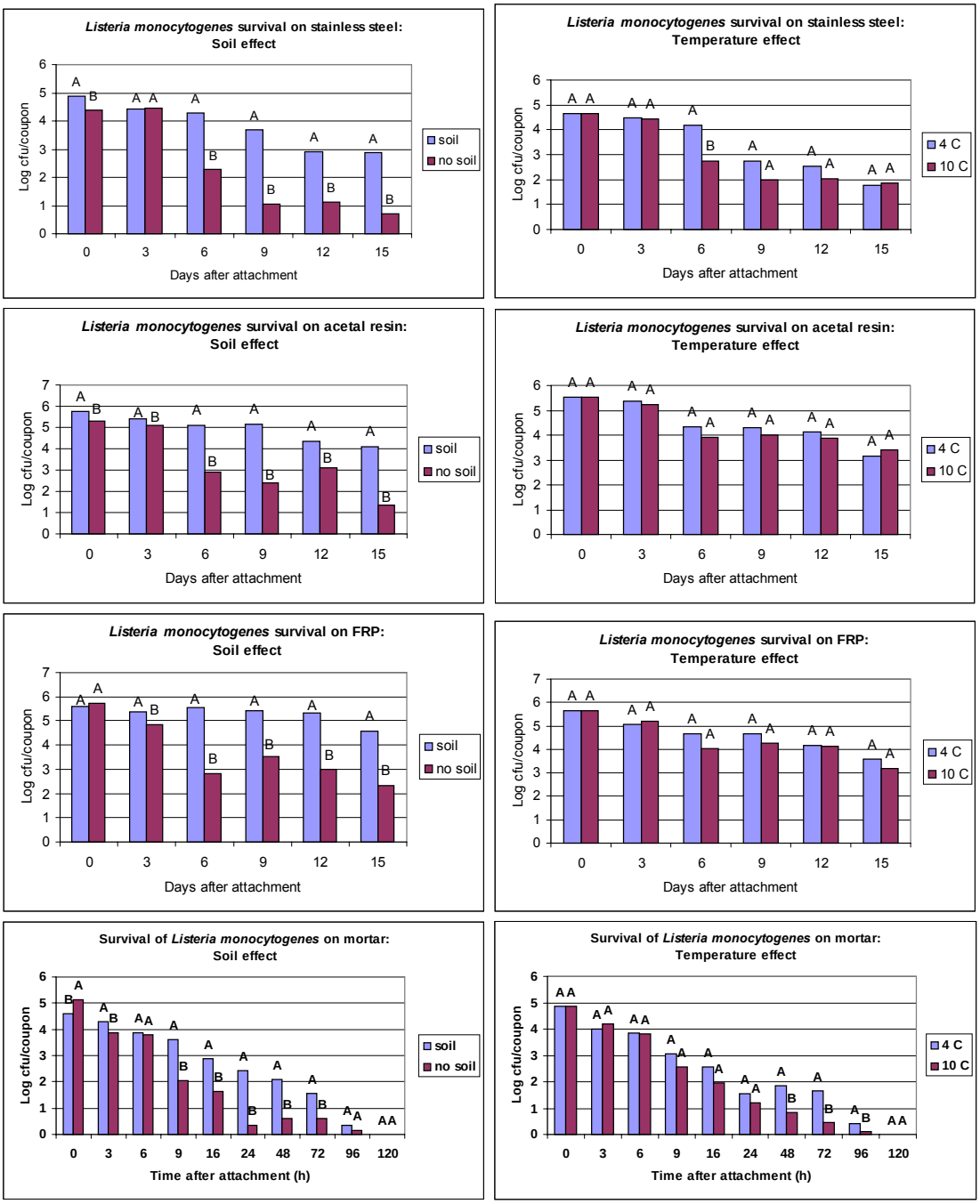

Figure 3. Influence of soil and temperature on the survival of *Listeria monocytogenes* attached to four different surfaces. At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test.

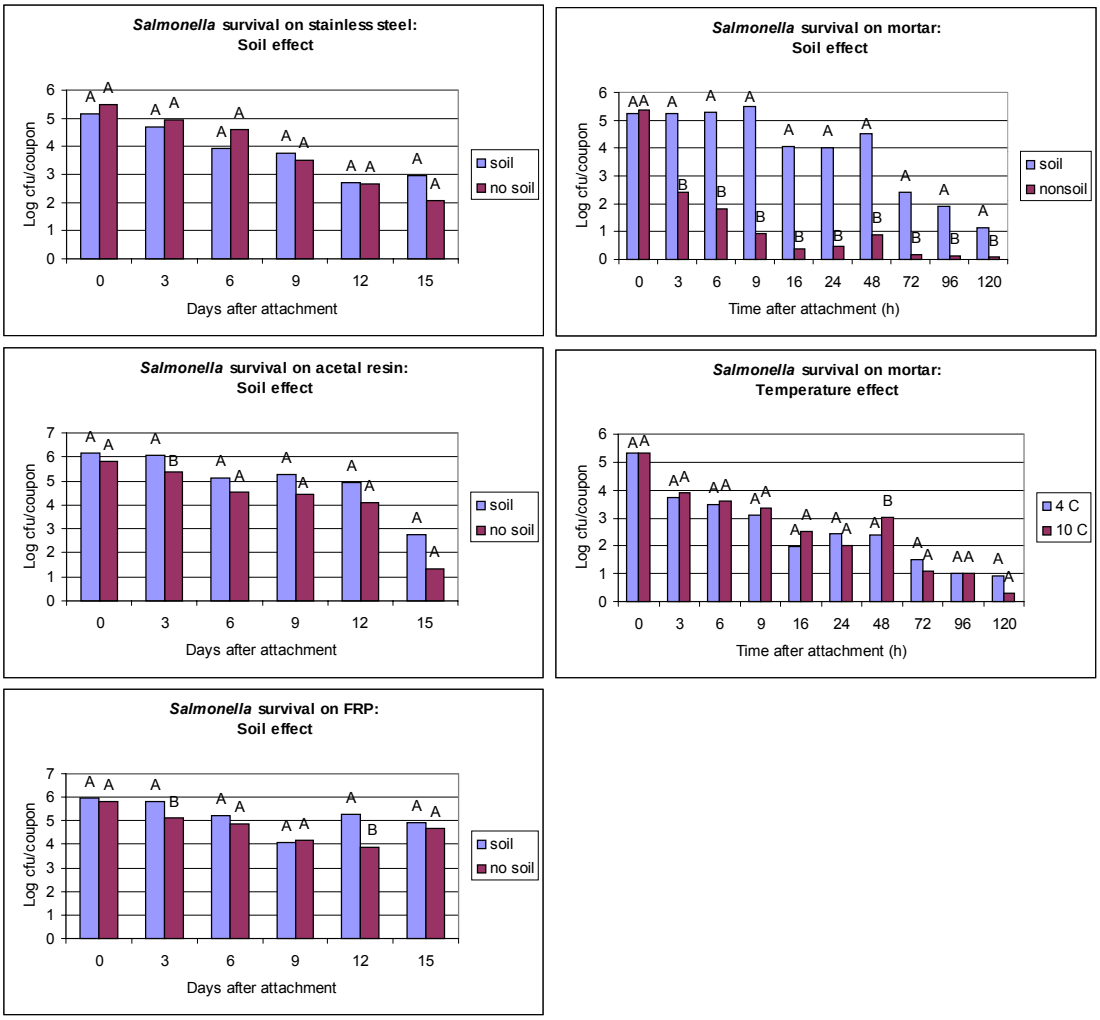

Figure 4. Influence of soil and temperature on the survival of *Salmonella* attached to four different surfaces. At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test

CHAPTER 5

DISCUSSION AND CONCLUSION

Bacterial survival while attached to a surface is dependant on many factors. Temperature, time, and the availability of nutrients and water to bacterial populations are all important. The attachment surface itself plays a role in how well an organism can survive. The physicochemical properties of a surface influence cellular as well as nutrient attachment. It has been previously reported that the chemical nature of a surface (surface charges, pH, hydrophobicity/hydrophilicity) can either inhibit or promote attachment and survival of different organisms. In most cases, bacteria attach more to hydrophilic (stainless steel and glass) than hydrophobic surfaces (Teflon, nylon, buna-N rubber and fluorinated polymers) (5, 46, 56).

Substratum preconditioning can have varying effects on how well a cell is able to attach to and survive on that surface. Fletcher et. al (28) showed that bovine serum albumin inhibited bacterial attachment to various surfaces. This effect, though, could not only be a property of the conditioning layer but also be due to the interaction of freely suspended albumin with cell surface structures involved in attachment that led to a reduced ability of the cells to bind to the substratum-bound albumin.

If a surface is able to chelate ions and/or prevent microbial access to vital organic nutrients then the chances of bacterial survival on that surface, without replenishment of these materials (such as in an aqueous environment wherein nutrients could readily flow over the surface), would be low. The initial hypothesis was that given a soiled surface and condensate there would not only be survival, but also growth, to some extent (as shown for *Y. enterocolitica*

on soiled FRP). But, our results show relatively stable populations in some instances (e.g. *L. monocytogenes* on FRP with serum and *Y. enterocolitica* on stainless steel with serum) and declines in others (e.g. *Salmonella* on stainless steel with serum). Cell death in the presence of serum could be due to either insufficient amounts of required nutrients in the serum to support growth or the binding of these nutrients to the surfaces thus preventing their uptake into the cells. Given that porcine serum was the only soil evaluated, it is possible that other porcine tissues (muscle, bone, organ, etc.), especially in combination, could provide for not only survival, but growth. However, the ability of these organisms to survive for at least fifteen days, even on unsoiled surfaces, demonstrates that effective cleaning and sanitization of such surfaces in a processing environment is critical to prevent the spread of organisms throughout the environment and ultimately into the product stream.

The presence of highly alkaline or acidic components can inhibit the survival of most bacteria. Mortar has a pH of about 12.5 due to incorporated CaOH (53). *Yersinia enterocolitica* populations declined much faster on soiled mortar than did *Listeria* and *Salmonella*. However, the death rate for *Yersinia* and *Salmonella* were similar on unsoiled coupons. *Listeria* survival was greater on unsoiled mortar coupons than both *Yersinia* and *Salmonella*, suggesting that *Listeria* is more tolerant to alkaline conditions. Taormina and Beuchat (75) demonstrated that *L. monocytogenes* can survive at least 144 hours (with a 4 log unit decrease) at pH 11. The protective effect of soil to the survival of all three organisms on mortar could be due to the buffering capacity of the serum which slowed the damage to the cells by the high pH. The observation that these pathogens (especially *L. monocytogenes* and *Salmonella*) are able to survive, with and without soil, on mortar for at least 120 hours, illustrates the need for proper cleaning and sanitization of walls and floors made from cement. In this study, mortar was used

without any surface coating such as a waterproofer, sealant, or paint as might commonly be used on concrete floors and walls in a food processing environment. The impact of these coatings on bacterial attachment and survival is unknown.

The bacterial ecology within a food processing plant is a dynamic system wherein organisms are constantly entering (either through raw ingredients, workers, air, water, etc.), attaching to surfaces and possibly establishing biofilms, then detaching and being transferred from one niche to another or into the product. Proper equipment and plant design, environmental control through effective cleaning and sanitization, and control over airflow and moisture, among other things, are the key elements needed to control this ecological system. The ideal "operating room" level of factory sterility is impractical and cost-prohibitive. Realistically, producers of non-sterile foods can only try to minimize the risk of product contamination to the most practical level. This study provides further evidence and insight into how select pathogenic organisms can survive on certain surfaces under defined conditions. This information may help in the design of cleaning and sanitization protocols as well as in the selection of materials used in food plant construction.

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