EFFECT OF CONCRETE SEALANT ON SURVIVAL OF FOODBORNE

BACTERIA IN PROCESSING ENVIRONMENTS

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EFFECT OF CONCRETE SEALANT ON SURVIVAL OF FOODBORNE BACTERIA IN PROCESSING ENVIRONMENTS

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IN PROCESSING ENVIRONMENTS

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VITA

Diego Moreira Paiva, son of Francisco e Fatima Paiva, was born March 25, 1983 in Belo Horizonte, Minas Gerais - Brazil. He attended Universidade Federal de Minas Gerais in Belo Horizonte, Brazil and graduated with a degree in Veterinary Medicine in July 2006. He then entered Graduate School at Auburn University in August 2007 in the Department of Poultry Science under the direction of Manpreet Singh, Ph.D.

THESIS ABSTRACT

EFFECT OF CONCRETE SEALANT ON SURVIVAL OF FOODBORNE BACTERIA IN PROCESSING ENVIRONMENTS

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Concrete sealants have been frequently used in the food industry to increase durability of concrete and decrease maintenance costs. In an attempt to associate these desirable properties of topical concrete sealants to a necessity in the food industry (reducing and controlling microbial levels), BioSealed for Concrete™ was created. This study was then designed to determine the efficacy of this product to prevent bacterial attachment, colonization, and antimicrobial effect against multiple microorganisms (*Salmonella*, *Listeria*, *Clostridium* and *Bacillus*) on concrete.

Cement blocks were divided into four different treatment groups: A) No Biosealed application, B) Biosealed applied before inoculation, C) Biosealed applied after inoculation, or D) Biosealed applied before and after inoculation. The cultures were prepared by inoculating microorganisms into individual brain and heart infusion broth

(BHI) and incubating them at their optimum growth temperature for 24 h. Cement blocks were inoculated by submerging in BHI broths containing one of the bacteria tested and incubated for 24 h (ca. 10^9 CFU/ ml). External surfaces of the inoculated blocks were swabbed using sterile swabs and placed in 10 mL peptone water (PW). The cement blocks were broken in half and interior surfaces were swabbed to determine bacterial levels. A completely randomized design was used to assign concrete blocks to the four treatment groups. Three replications of this experiment were performed and averages of the survival populations ($\log_{10}$ CFU/ sq. cm) of various bacteria were analyzed using analysis of variance (ANOVA) with SAS PROC GLM procedures (2002-03 SAS 9.1 Institute, Gary, NC). Statistical significance was reported at a *P*-value of less than or equal to 0.05 ($P \leq 0.05$).

BioSealed for Concrete™ proved to be a potent antimicrobial with immediate bactericidal effects that has potential to facilitate biofilm removal. Results from this study indicate that BioSealed for Concrete™ can be used as an alternative in food processing plants that have persistent and recurrent biofilm problems. Although antimicrobial capabilities of BioSealed for Concrete™ were shown in this study, its use should not be substituted for good manufacturing practices and/ or efficient cleaning and sanitizing procedures. The factors required for biofilm formation are still unknown, and although some progress has been made in this area, understanding the full mechanism of colonization to inert surfaces is a key factor on the process of attempting to prevent its occurrence.

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1 – INTRODUCTION

The food industry is under constant transformation and adaptation as a result of market demand and regulatory agencies determinations. The number of emerging pathogenic microorganisms, some of which are resistant to antibiotics or to traditional preservation methods, and which sometimes cause illness with low infectious doses, has increased (Sofos, 2002). The major goal of the food industry is to offer a safe, wholesome and palatable food to consumers while providing sufficient profit for the company to remain in business.

In addition to microorganism-associated concerns, societal changes including changing consumer food preferences, lack of adequate food handling education, increases in the human population at-risk for foodborne illness, complex food distribution patterns, increased international trade, and better methods for microbial detection also contributes to the changes that the food industry constantly undergoes (Sofos, 2002).

In the microbial world, it is more advantageous to microorganisms to live in surface-associated communities than in floating isolation. Most microorganisms have shown the ability to form biofilms. Regardless of the microorganism species, increased resistance to antimicrobials and sanitation procedures are fundamental characteristics shared by these microenvironments. Therefore, pathogen-control procedures are needed to assure food safety by preventing, reducing, inhibiting, inactivating, or eliminating pathogens. In addition to pathogens, it is of interest to the food

industry to address the issues of spoilage bacteria. The industry goal in this regard is to extend product shelf life, making the product more stable through reduction of spoilage and thereby reducing economic losses.

There are several indications of continuous adaptation and development of resistance by pathogenic microorganisms to antibiotics and to traditional food preservation barriers (pH, temperature, moisture and chemical preservatives) which lead us to think that the microbial ecology of our food supply is undergoing changes that parallel with the modernization taking place in our food-processing and distribution industries and the transformation of our society (Sofos, 2002). To reduce or eliminate pathogens and spoilage microorganisms from food, the food processing industry have relied primarily on physical and chemical methods. However, due to the development of microbial resistance to these preservation methods, the food industry has been trying to address these issues by developing alternative antimicrobials that could be used in their daily operations.

2 - LITERATURE REVIEW

2.1 – *Salmonella*

Salmonella spp. are facultative anaerobic, Gram-negative rod-shaped bacteria belonging to the Enterobacteriaceae family (Portillo, 2000; D'Aoust et al., 2001). These organisms are typical Gram-negative bacteria that are able to grow on a large number of culture media and produce visible colonies within 24 h at about 37 ˚C (Jay et al., 2005). *Salmonella* are chemoorganotrophic, with an ability to metabolize nutrients by both respiratory and fermentative pathways (Anderson and Ziprin, 2001; D'Aoust et. al, 2001). As with most Enterobacteriaceae, *Salmonella* organisms may harbor temperate bacteriophages and plasmids that may code for virulence factors, antibiotic resistance, bacteriocins, metabolic characteristics and antigenic molecules (Minor, 1992).

A typical *Salmonella* isolate produces acid and gas from glucose in triple sugar iron (TSI) agar medium and does not utilize lactose or sucrose in TSI or in differential platting media - such as brilliant green (BG), xylose lysine desoxycholate (XLD), and Hektoen enteric (HE) agars. Additionally, typical salmonellae readily produce an alkaline reaction from the decarboxylation of lysine to cadaverine in lysine iron agar (LIA), generate hydrogen sulfide gas in TSI, XLD and lysine iron media, and fail to hydrolyze urea (Anderson and Ziprin, 2001; D'Aoust et. al, 2001). Also, *Salmonella spp.* does not produce indole and are unable to deaminate phenylalanine or tryptophan (Anderson and

Ziprin, 2001). They are generally unable to ferment lactose, sucrose, or salicin, although glucose and certain other monosaccharides are fermented, with the production of gas (Portillo, 2000; Anderson and Ziprin, 2001; Jay et al., 2005). The bacteria grow optimally at 37 ˚C and catabolize D-glucose and other carbohydrates with the production of acid and gas. *Salmonella* are oxidase negative and catalase negative, grow on citrate as a sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolyze urea (Anderson and Ziprin, 2001; D'Aoust et. al, 2001).

The pH, salt concentration and temperature of the microenvironment can influence the growth kinetics of salmonellae. The *Salmonella* group consists of microorganisms with a great adaptability to extreme environmental conditions due to their ability to proliferate at pH values ranging from 4.5 to 9.5, with an optimum pH for growth between 6.5 and 7.5. The bactericidal effects of acid conditions can be reduced due to acid adaptation by salmonellae (D'Aoust et. al, 2001; Jay et al., 2005).

The bacteriostatic effect of high salt concentrations, which can also cause cell death, results from a dramatic decrease in water activity (a_w) and from bacterial plasmolysis commensurate with the hypertonicity of the suspending medium (D'Aoust et. al, 2001). Studies have revealed that foods with a_w values of ≤ 0.93 and neutral pH do not support the growth of *Salmonella* (Portillo, 2000; Jay et al., 2005). Although *Salmonella spp.* are generally inhibited in the presence of 3 to 4% NaCl, bacterial salt tolerance increases with increasing temperature in the range of 10 to 30 ˚C. The magnitude of this adaptive response is food- and serovar-specific (D'Aoust et. al, 2001). Nitrite is bactericidal, with the effect being greatest at lower pH values – the bactericidal effect of this compound is related to the undissociated form of this compound (Jay et al., 2005).

Nomenclature of the *Salmonella* group has progressed through a succession of taxonomical schemes based on biochemical and serological characteristics and on principles of numerical taxonomy and DNA homology. Although food microbiologists, scientists, and epidemiologists treat the 2,463 serovars as though each was a species, all salmonellae have been placed in two species, *S. enterica* and *S. bongori* (Jay et al., 2005; Shaw, 2007). According to the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*, *S. enterica* and *S. bongori* currently include 2,443 and 20 serovars respectively – most researchers and clinicians are now using this nomenclature (Anderson and Ziprin, 2001; D'Aoust et. al, 2001).

As like all Enterobacteriaceae, the genus *Salmonella* has three kinds of major antigens with diagnostics or identifying applications: somatic, surface and flagellar (Minor, 1992). Biochemical identification of foodborne and clinical *Salmonella* isolates is generally coupled to serological confirmation, a complex and labor-intensive technique involving the agglutination of bacterial surface antigens with *Salmonella*-specific antibodies. These include somatic (O) lypopolysaccharides on the external surface of the bacterial outer membrane, flagellar (H) antigens associated with the peritrichous flagella, and the capsular (Vi) antigen, which occurs only in *Salmonella* serovars Typhi, Paratyphi C, and Dublin (D'Aoust et. al, 2001).

Somatic antigens are heat stable and alcohol resistant. Cross-absorption studies individualize a large number of antigenic factors; 67 of which are or have been used for serological identification. O factors labeled with the same number are closely related, although not always antigenically identical. Antigenic specificity is carried by the lipopolysaccharide which may be divided into three parts: lipid, core (common to all

Salmonella serovars) and specific polysaccharide chains. Surface or envelope antigens, commonly observed in other genera of enteric bacteria, may be found in some *Salmonella* serovars. These surface antigens may mask O antigens and the bacteria will not be agglutinated with O antisera (Minor, 1992).

A great majority of bacteria from this genus are motile by peritrichous flagella. However, nonflagellated variants, such as *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum, and nonmotile strains resulting from dysfunctional flagella do occur (Anderson and Ziprin, 2001; D'Aoust et. al, 2001; Shaw, 2007). Flagellar (H) antigens are heat-labile proteins and antiflagellar antibodies can immobilize bacteria with corresponding H antigens (Minor, 1992).

Portillo (2000) divides salmonellae disease in humans into two major groups: (1) gastroenteritis or "non-typhoid salmonellosis" – a localized, self limiting bacterial infection of the intestinal epithelium and (2) "typhoid-salmonellosis" or "enteric-fever" – systemic infection. While the first one is characterized by abdominal pain, diarrhea, fever and vomiting, the second one is characterized by malaise, headache, non- productive cough, abdominal pain and constipation. Newborns, infants, the elderly and immunocompromised individuals are most susceptible to *Salmonella* infections (Portillo, 2000).

Salmonella strains may produce a thermolabile enterotoxin that bears a limited relatedness to cholera toxins both structurally and antigenically. Additionally, a cytotoxin that inhibits protein synthesis and is immunologically distinct from *Shiga*-toxin produced by *Escherichia coli* has been demonstrated. Both of these toxins play a role in the diarrheal symptoms of Salmonellosis (Minor, 1992).

2.1.1 – *Salmonella* Infections in Humans

Human *Salmonella* infections can lead to several clinical conditions which include enteric typhoid fever, uncomplicated enterocolitis, and systemic infections by non-typhoid microorganisms. Typhoid fever is a serious human disease associated with the typhoid and paratyphoid strains, which are particularly well adapted for invasion and survival within host tissues (D'Aoust et. al, 2001; Ziprin and Hume, 2001). The primary source of infection is a human carrier, symptomatic or asymptomatic, that contaminates the food. The infection is systemic (bacteremia) with a low mortality rate when properly diagnosed and treated (Ziprin and Hume, 2001). Clinical manifestations of typhoid fever appear after a period of incubation ranging from 7 to 28 days and may include diarrhea, prolonged and spiking fever, abdominal pain, headache, and prostration (D'Aoust et. al, 2001).

Human infections with non-typhoid *Salmonella spp.* commonly result in enterocolitis, which usually develop in 12-14 hours after contact with the pathogen; shorter and longer times have been reported (Jay et al., 2005). The time between the ingestion of contaminated food and the development of symptom is dose dependant (Ziprin and Hume, 2001). The clinical condition is usually self-limiting - and remission of the characteristic non-bloody diarrheal stools and abdominal pain usually occurs within 5 days of the onset of symptoms (D'Aoust et. al, 2001). The pathogenic action of *Salmonella* varies with the serovars, strain, infectious dose, nature of the contaminated food and the host status. Strains of same serovars may also differ in pathogenicity (Minor, 1992) and it is well established that newborns, infants, the elderly and immunocompromised individuals are more susceptible to *Salmonella* infections than

healthy adults (Minor, 1992; Portillo, 2000; D'Aoust et. al, 2001). The incompletely developed immune system in newborns and infants, frequently weak and/or delayed immunological responses in elderly and debilitated people, and the generally low gastric acid production in infants and seniors facilitates the intestinal colonization and systemic spread of salmonellae in these segments of the population (D'Aoust et. al, 2001). An additional factor of importance on the infectious dose of *Salmonella* is that foods high in fat content (i.e. meat, chocolate, milk and dairy products) significantly decrease the number of cells necessary to cause disease. Foods implicated in outbreaks with low infection doses usually have a high fat content as the entrapment of salmonellae within lipid micelles may afford protection to gastric acidic pH (Portillo, 2000; D'Aoust et. al, 2001).

2.1.2 – *Salmonella* in Food Systems and Related Outbreaks

The principal habitat for *Salmonella* is the intestinal tract of humans and animals (Minor, 1992; Jay et al., 2005).However, this bacterium has often been isolated from soil, water and animal feed (Anderson and Ziprin, 2001). The widespread occurrence of *Salmonella spp*. in the natural environment, coupled with the intensive husbandry practices used in the animal industry and the recycling of offal and inedible raw materials into animal feeds, have favored the continued prominence of this bacterial pathogen in the global food chain (Minor, 1992; Portillo, 2000; D'Aoust et al., 2001).

Salmonella can enter poultry houses carried in the air, food, water as well as by wild birds. Once the microorganism has entered the production environment, its presence is maintained in the soil and litter material (Minor, 1992). Among the many different sectors of the meat industry, poultry and eggs remain a predominant reservoir of

Salmonella spp. in many countries (Portillo, 2000; D'Aoust et al., 2001). Salmonella has been isolated from the skin, feathers and in the feces of broiler chickens at the time of slaughter, and conditions during slaughter tend to allow the spread of these bacteria among the carcasses (Nisbet and Ziprin; 2001). Eggs and egg-containing products are of particular concern since there is transovarian transmission of the pathogen into the interior of egg before shell deposition (Portillo, 2000). Contamination of meat may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing and transportation of carcasses (Minor, 1992).

Every year, approximately 40,000 cases of salmonellosis are reported in the United States according to the Centers for Disease Control and Prevention (CDC) (CDC, 2008). Salmonellosis is an important public health problem in the United States with an estimated number of non-typhoidal *Salmonella* infections ranging from 800,000 to 4,000,000 annually (Voetsch et al., 2004). Although most outbreaks cause mild to moderate self-limited illness, serious disease resulting in death does occur particularly in elderly and immunocompromised populations. Because many milder cases are not diagnosed or reported, the actual number of infections may be higher than the reported numbers. The CDC estimates that *Salmonella* infection causes approximately 1.4 million foodborne illnesses annually (Lynch et al., 2006). Accounting for medical costs and lost productivity, the estimated yearly costs associated with salmonellosis is approximately $2.3 billion (Frenzen et al., 1999). The CDC (2008) states that bacterial agents are the most common microorganisms associated with foodborne illnesses, accounting for 55% of laboratory diagnosed foodborne illnesses and outbreaks. Among bacterial pathogens,

Salmonella Enteritidis accounted for the largest overall number of outbreaks and outbreak-related illnesses. Major outbreaks of foodborne salmonellosis in the last few decades are of particular interest because they underline the multiplicity of foods and *Salmonella* serovars that have been implicated in human illness (D'Aoust et. al, 2001). Several foods, including cereal, peanut butter, tomatoes, cantaloupe, beef, pork and poultry, have been implicated in *Salmonella*-related human illnesses (Minor, 1992, USDA-FSIS 2008). Recent *Salmonella* outbreaks were reported by the U.S. Department of Agriculture's Food Safety and Inspection Services (USDA – FSIS) involving fresh poultry and further processed poultry products such as chicken pot pies and raw frozen breaded and pre-browned stuffed chicken entrees. However, it is not possible to obtain an accurate estimate of the proportion of human salmonellosis cases attributed to poultry. A given case could be attributed to any of the many possible sources in the home, including pets, but the patient may only associate the illness with food recently consumed (Nisbet and Ziprin, 2001).

Salmonella spp. have developed several strategies to survive in the environment and their ability to adhere to surfaces and form biofilms are among the most important ones. Extracellular structures contributing to bacterial adherence include curli fimbriae, cellulose, capsular polysaccharide and other polysaccharides such as lipopolysaccharides (LPS) (Malcova et al., 2008). However, mechanisms involving the adhesion of *Salmonella* spp. to inert surfaces are still unclear; different studies have shown that the bacterial attachment partially depends on bacterial characteristics and partially on surface properties (Austin et al., 1998; Sinde and Carballo, 2000; Joseph et al. 2001).

Regulatory agencies all over the world are working intensely in an attempt to make food safer and reduce the risk of *Salmonella* outbreaks. *Salmonella* regulations all over the world vary greatly according to the agency in charge. USDA – FSIS present compliance guidelines for *Salmonella* in poultry products and states the performance standard for *Salmonella* in broilers shall be less than 20% over a sample set of 51 samples (USDA, 2006). Lately, USDA – FSIS is moving towards a reduction of the performance standard to less than 10% of positive samples. In addition, poultry processing plants will have to maintain a culture library of the isolates. These two measures are an attempt by USDA – FSIS to reduce the presence of this pathogen in foods and to better understand the colonization pattern of *Salmonella*.

2.2 – Listeria

Listeria spp. are facultative intracellular Gram-positive, non-spore forming, motile, catalase positive, facultatively anaerobic and non-acid-fast rods. Their nutritional requirements are typical of many other Gram-positive bacteria (Jones and Seelinger, 1992; Kathariou, 2000; Jay et al., 2005) and they grow best in the pH range 6-8 and an optimum temperature between 30 – 37 ˚C. However, they will grow in a wide pH range (4.1 – 9.6) and over a temperature range of 0 – 45 ˚C (Kathariou, 2000; Swaminathan, 2001; Jay et al., 2005). Growth at low pH is highly influenced by the temperature and the acid type. At pH $\leq$ 4.3, the bacteria can survive but not multiply (Swaminathan, 2001). *Listeria* is capable of modifying its membrane composition in order to maintain membrane fluidity which allows growth at refrigeration temperatures (Beresford et al., 2001; Lado and Yousef, 2007). This modification is related to the proportion of different

fatty acids that are part of the cellular membrane. At lower temperatures, there is a reduction in the proportion of long aliphatic chains and an increase in asymmetric branching which reduce van der Waals interactions among membrane constituents. Tight packing of membrane phospholipids at low temperatures is reduced, therefore increasing membrane fluidity (Lado and Yousef, 2007). Temperatures below 0 ˚C preserve or moderately inactivate the bacterium; hence, survival and injury during frozen storage depend on the substrate and the rate of freezing (Swaminathan, 2001).

Listeriae grow optimally at $a_w \geq 0.97$. For most strains, the minimum a_w is 0.93, but some strains can grow at a_w values as low as 0.90. Furthermore, the bacterium may survive for long periods at a_w values as low as 0.83 (Donnelly, 2001; Swaminathan, 2001; Jay, 2005). *L. monocytogenes* is able to grow in the presence of 10 to 12% sodium chloride; it grows to high populations in moderate salt concentrations (6.5%). The bacterium survives for long periods in high salt concentrations; and its survival in high salt environments is significantly increased by lowering the temperature (Swaminathan, 2001; Lado and Yousef, 2007).

The genus *Listeria* consists of six species: *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri*, and *L. grayi*. Within this genus, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic. *L. monocytogenes* is a human pathogen of high public health concern; *L. ivanovii* is primarily an animal pathogen (Swaminathan, 2001).

Listeria monocytogenes is represented by 13 serovars, some of which are shared by *Listeria innocua*. According to Jay et al. (2005), although *L. innocua* is represented by only 2 serovars, it is sometimes regarded as the nonpathogenic variant of *L.*

monocytogenes. *Listeria* serotypes are designated by assigning letters, which refer to flagellar antigens, whereas a number refers to somatic antigens (Kathariou, 2000). *L. monocytogenes* isolates are often characterized below the species level for the purpose of public health surveillance and to assist in outbreak investigations. There are 13 serotypes of *L. monocytogenes* which can cause disease (Swaminathan, 2001). *L. innocua* and *L. monocytogenes* are very close related genetically, making *L. innocua* of special interest to food microbiologists, since it is frequently used as a surrogate to *L. monocytogenes* (Donnelly, 2001).

2.2.1 – *Listeria monocytogenes*: Occurrence in Food Processing Environments

Listeria is widely distributed in the environment, where its primary habitat is the soil and decaying vegetation (Jones and Seelinger, 1992; Kathariou, 2000; Swaminathan, 2001). There are several opportunities for this organism to spread and colonize the industrial environment following its entrance in a processing and further processing poultry facility. Entry of *L. monocytogenes* into food processing plants occurs through soil on worker's shoes and clothing and on transport equipment, animals which excrete the bacterium or have contaminated surfaces, hides or paws, raw plant tissue, raw foods of animal origin, and possibly healthy human carriers (Swaminathan, 2001). Floors and drains have been implicated as the primary source of this microorganism in poultry processing plants, but this microorganism has been frequently isolated from high humid areas such as condensed and stagnant water, floors, processing residues, and processing equipment (Chae and Schraft, 2000; Donnelly, 2001; Swaminathan, 2001). The persistence of this microorganism in hostile processing environments is enhanced by their ability to attach to surfaces and form biofilms. This unique survival property makes the

presence of *Listeria* difficult to control and remove from processing environments (Lawrence and Gilmour, 1995; Swaminathan, 2001). Most of the preceding research on the attachment of *Listeria* to inert surfaces has been done on classic few materials such as stainless steels and rubber (Beresford et al., 2001). But research has shown that several other materials present in poultry processing facilities such as door handles, concrete floors, squeegees, gloves and drains can harbor *Listeria* (Lawrence and Gilmour, 1994; Lado and Yousef, 2007).

2.2.2 – *Listeria monocytogenes*: Implications and Outbreaks

It is well known that a great variety of fresh food products of animal and plant origin may be a source of *L. monocytogenes*(Beresford et al., 2001; Jay et al., 2005). This organism has been isolated from many different foods, such as milk and dairy products, fresh and frozen meat – including beef, pork, poultry and seafood, fruits and vegetables (Jones and Seelinger, 1992; Ryser, 2007).

L. monocytogenes has been isolated from a great variety of poultry meat and poultry products, raw or cooked, such as chicken patties, chicken salad, diced poultry, poultry salad, poultry spread, poultry frankfurters, poultry bologna and turkey sausage (Berrang et al., 2002; Ryser, 2007). Poultry supports the growth of *L. monocytogenes* better than other meats due to its pH, and the type and cell populations of the competitive flora (Franco et al., 1995; Swaminathan, 2001). This high incidence of *L. monocytogenes* in raw chicken is a problem because of cross contamination to other foods in the home and the possibility of the microorganism surviving in processed chicken (Franco et al., 1995). In addition, raw chicken must be considered one of the many sources of contamination to further processing facilities. The presence of *Listeria* on incoming raw

meat ingredients can serve as a continuous source of contamination for further processing plants (Franco et al., 1995; Gamble and Muriana, 2007).

Poultry products have been implicated in several contamination episodes with *L. monocytogenes* and the USDA – FSIS has dealt with numerous recalls and outbreaks that have resulted from *L. monocytogenes* contamination. Over a 21-month period in the United States, 19.3% of 3,700 raw broiler carcasses were positive to *L. monocytogenes* (Jay et al., 2005). *L. monocytogenes* multiplies readily in meat products, including vacuum-packaged meats at pH 6, whereas there is little or no multiplication at pH 5. Cooked, ready-to-eat (RTE) meat and poultry products have been implicated as the source of sporadic and epidemic listeriosis on several occasions in the USA and Europe. When contamination of poultry occurs after cooking, there is a high probability that this product will carry the pathogen to the market place, ultimately reaching the consumers. Consumption of inadequately reheated frankfurters and undercooked chicken has been identified as a risk factor for sporadic listeriosis (Swaminathan, 2001).

Recent studies have demonstrated that the processing environment is a major source of *Listeria* contamination of raw and cooked poultry products. Major risk situations for contamination of poultry meat are during normal processing activities such as de-feathering, evisceration and further processing (Ryser, 2007). Contamination of animal muscle tissue can occur either from symptomatic or asymptomatic carriage of *L. monocytogenes* by the food animal before slaughter, but it can also occur after slaughter. Regardless of the route of contamination, *L. monocytogenes* attaches strongly to the surface of raw meat and is difficult to remove or inactivate (Swaminathan, 2001). Research has shown that the species obtained from the environments of establishments

producing *Listeria*-positive foods remained constant. This observation is critically important because it is a strong suggestion that once it is established in a food processing environment, *L. monocytogenes* may persist in that location for several years even if the products produced at that location are *Listeria*-free for several months (Lawrence and Gilmour, 1995; Chae and Schraft, 2000; Swaminathan, 2001).

RTE foods are usually preserved by refrigeration and offer an appropriate environment for the multiplication of *L. monocytogenes* during manufacture, aging, transportation and storage. The foods in this category include unpasteurized milk and products prepared from unpasteurized milk, soft cheeses, frankfurters, delicatessen meats and poultry products (Swaminathan, 2001). The United States government classifies the presence of *L. monocytogenes* in foods as an "adulterant". This means that any RTE food that contains this organism will be considered adulterated and, thus be subjected to recall and/or seizure (Jay et al., 2005). In 2008, approximately 50 recalls were announced by USDA - FSIS involving RTE poultry and poultry products. The products recalled included chicken sausage, hot dogs, turkey wraps and sandwiches, chicken sandwich, eggs and cheese burritos, chicken teriyaki , Thai style chicken, chicken salads, chicken burrito, chicken fajita , chicken parmigiana, chicken strips, grilled chicken breast and breaded and fried chicken. Several states were implicated in these recalls announcements such as AL, CA, CO, MO, IL, MA, MI, NY, OH and WA (USDA-FSIS, 2009).

2.2.3 – *Listeria monocytogenes*: Public Health Concerns

L. monocytogenes infection is commonly transmitted by means of contaminated food, and individuals at risk include pregnant women, infants, the elderly and immunocompromised individuals (Jones and Seelinger, 1992; Kathariou, 2000; Beresford

et al., 2001). Listeriosis is an atypical foodborne illness of major concern to public health and the food industry because of the ability of this microorganism to grow at refrigeration temperatures, the severity of the disease (meningitis, septicemia and abortion), a high case fatality (approximately 20-30% of cases), a long incubation time, and a predilection for individuals who have an underlying condition which leads to impairment of T-cell mediated immunity (Kathariou, 2000; Beresford et al., 2001; Swaminathan, 2001). Every year, approximately 2,500 cases of listeriosis are reported in the United States according to the CDC. While morbidity is low, this microorganism can cause life-threatening illness, with a mortality rate which is much higher than that of salmonellosis or campylobacteriosis (Berrang et al., 2002).

Complex interactions between various factors reflecting changes in social patterns resulted in the emergence of listeriosis as a foodborne illness. These factors include improvements during the past years in medicine, public health, sanitation, and nutrition that have resulted in increased life expectancy. The widespread use of immunosuppressive medications for the treatment of several clinical conditions expanded the immunocompromised population increasing the risk for listeriosis (Donnelly, 2001; Swaminathan, 2001). Changes in food production practices and increased use of refrigeration as a primary means of preservation of foods is another factor contributing to the increased incidence of listeriosis. These changes in food production practices result from consumer's demand for convenience foods that have a fresh-cooked taste, can be purchased RTE, refrigerated or frozen. These changes in food habits largely affected the way food is prepared and handled before consumption (rapidly and little cooking required before consumption). All these changes in social behavior contributed to the emergence

of listeriosis as a major foodborne disease during the past two decades (Swaminathan, 2001).

In non-pregnant adults, *L. monocytogenes* primarily causes septicemia, meningitis, and meningoencephalitis with a mortality rate of 20 to 25% (Jones and Seelinger, 1992; Swaminathan, 2001). Other infrequent manifestations of listeriosis include endocarditis in people with underlying cardiac lesions and various types of co-infections (septic arthritis, osteomyelitis) (Donnelly, 2001; Swaminathan, 2001, Painter and Slutsker, 2007). Although pregnant women, particularly in the third trimester of pregnancy, may experience only mild flu like symptoms as a result of *L. monocytogenes* infection, the infection can result in fetal loss, stillbirth, premature delivery, or neonatal infection (Painter and Slutsker, 2007). In neonates who are less than seven days old, sepsis and pneumonia are predominant symptoms, whereas in neonates older than seven days, the infections manifest as meningitis and sepsis (Swaminathan, 2001).

Public health concerns regarding *Listeria* contaminated products led USDA-FSIS to create monitoring / verification programs for cooked and RTE meat and poultry products. The collection of samples is based on Hazard Analysis and Critical Control Points (HACCP) under processing categories as defined in the Code of Federal Regulations (CFR) for RTE products (Ryser, 2007). USDA - FSIS regulations for cooked and RTE meat and poultry products state a "zero tolerance" policy for the presence of *L. monocytogenes*. USDA-FSIS request under this program that the food industry must issue a Class I recall for all lots of cooked and RTE meat and poultry products in which *L. monocytogenes* was detected in monitoring samples taken from intact packages of a product. However, *Listeria*-positive lots under direct control of manufacturer could be

recalled internally (avoiding adverse publicity) and if the pathogen was identified in samples from unpackaged products USDA – FSIS does not require these food processing plants to recall the sample lot (Ryser, 2007).

2.3 – Bacterial Spores

Bacterial spores are both metabolically dormant, catalyzing no detectable metabolism of endogenous or exogenous compounds and having no high energy compounds, and extremely resistant to a variety of harsh treatments, including heat, radiation, and chemicals. The major cause of the spore's metabolic dormancy is undoubtedly the low water content of the core which may preclude enzyme action (Setlow and Johnson, 2001).

The initiating signal for sporulation is not known, but its initiation is associated with a decrease in the intracellular GTP (guanine triphosphate) pool (Slepecky and Hemphill, 1992). Sporulation results from an unequal cell division (Slepecky and Hemphill, 1992; Setlow and Johnson, 2001) that creates the smaller nascent spore or forespore compartment and the larger mother cell compartment. As sporulation proceeds, the mother cell engulfs the forespore, resulting in a cell (the forespore) within a cell (the mother cell), each with a complete genome. Since the spore is formed within the mother cell, it is termed an endospore (Setlow and Johnson, 2001).

Sporulation is generally divided into seven stages based primarily on the morphological characteristics of cells throughout their developmental process. Growing cells are in stage 0, although sporulation is initiated only following completion of chromosome replication (Slepecky and Hemphill, 1992). Stage I is defined by the

presence of two nucleoids in an axial filament which can be observed in electron micrographs. Stage one is a discrete stage in sporulation and its significance remain obscure. The first morphological feature of sporulation is the formation of an asymmetric septum (Setlow and Johnson, 2001) which divides the sporulating cell (now stage II) into the larger mother cell and smaller forespore compartment. A biochemical marker for stage II, specifically late stage II, is the synthesis of high levels of alkaline phosphatase (Slepecky and Hemphill, 1992; Setlow and Johnson, 2001). Following septum formation, the mother cell membrane grows around and eventually engulfs the forespore which characterizes stage III. Thus, the forespore is surrounded by two complete membranes termed the inner and outer forespore membranes which have opposite polarities. In the transition from stage III to stage IV, a large peptideoglycan structure termed the cortex is laid down between the inner and outer forespore membrane. The cortex has a structure similar to that of the cell wall peptideoglycan but with a number of differences (Setlow and Johnson, 2001), and studies have shown that the cortex plays a fundamental role in the dehydration of spores which will be described further in this section. The cortex is also needed for accumulation of DPA (diaminopimelic acid) and for refractivity of the spore (when observed under phase-contrast microscope) (Slepecky and Hemphill, 1992). The spore's nascent germ cell wall is also made at about the same time as the cortex but appears to have the same structure as the cell wall peptideoglycan and is formed between the cortex and the inner forespore membrane. During the transition of stage III to stage IV, the spore also synthesizes two biochemical markers, glucose dehydrogenase and SASP (small acid soluble spore proteins). Although the function of glucose dehydrogenase in spores is unknown, a number of the SASP are involved in spore

resistance (Setlow and Johnson, 2001). The developing spore acquires full UV resistance and some chemical resistance at this time (Slepecky and Hemphill, 1992; Setlow and Johnson, 2001). Late in stage IV, the forespore pH falls by 1 - 1.13 units and dehydration begins. In the transition from stage IV to stage V a series of proteinaceous layers are formed outside the outer forespore membrane. Forespore resistance to radiation and chemicals increase in this stage, and dehydration continues. While transitioning from the stage V to stage VI the spore core's depot of DPA is accumulated following DPA synthesis in the mother cell. DPA uptake is paralleled by uptake of enormous amounts of divalent cations (predominantly Ca^{2+}). The great majority of these cations are also in the spore core, presumably associated with the DPA. During this period the spore core undergoes its final process of dehydration (Setlow and Johnson, 2001) and the spore becomes metabolically dormant acquiring increased resistance to radiation and chemicals. Finally, lytic enzymes lyse the sporangial or mother cell liberating the free spore (Slepecky and Hemphill, 1992).

The spore that is released from the mother at the end of sporulation is biochemically and physiologically different from the vegetative cell. The spore structure is also extremely different from that of a cell as many spore structures, including the exosporium and coats, have no counterparts in the vegetative cell (Setlow and Johnson, 2001). Sporeformers causing foodborne illness and spoilage are particularly important in low-acid foods (equilibrium pH > 4.6) packaged in cans, bottles, pouches, or other hermetically sealed containers (canned foods) which are processed by heat (Setlow and Johnson, 2001).

Spores are much more resistant than vegetative cells to a variety of chemical compounds and treatments, including cross-linking agents such as glutaraldehyde, oxidizing agents, phenols, formaldehyde, chloroform, octanol, alkylating agents including ethylene oxide, iodine, and detergents as well as to pH extremes and lytic enzymes such as lysozyme (Slepecky and Hemphill, 1992; Setlow and Johnson, 2001). The degree of resistance has been shown to depend not only on the species but also on the physiological environment in which the spores were formed and the temperature at which it was grown (Slepecky and Hemphill, 1992). The spore metabolic dormancy is undoubtedly one factor in its ability to survive extremely long periods in the absence of nutrients and hostile environments. Despite the spores' extreme dormancy, if given the appropriate stimulus spores can rapidly return to life via spore germination (Setlow and Johnson, 2001).

2.4 – *Bacillus subtilis*

Bacillus subtilis is neither an important pathogen nor an important agent of poultry spoilage. However, its natural transformability, as well as an abundance of molecular biological and genetic information, has made *B. subtilis* the organism of choice for mechanistic studies on sporulation, spore germination and spore resistance (Setlow and Johnson, 2001). In addition, several researchers have been using *B. subtilis* as a surrogate to study the physiopathology, behavior, sporulation and resistance of *B. anthracis* due to the low pathogenic profile of *B. subtilis*. *B. subtilis* is a Gram-positive aerobic sporeforming rod and it is actively motile by peritrichous flagella (Merchant and Packer, 1961). This microorganism is able to ferment glucose, sucrose and maltose with

the production of acid but no gas. It also has the ability to hydrolyze starch as an energy source (Slepecky and Hemphill, 1992) but is unable to grow on acetate as a sole carbon source. This is probably due to the absence of a glyoxylate shunt or a similar pathway that salvages carbon atoms of acetate from being completely oxidized to CO_2 and thereby provides carbon for anabolism (Hederstedt, 1993). Indol is not formed and nitrates are reduced by this bacterium. The organism is catalase positive, Methyl Red (M.R.) negative and Voges-Proskauer (V.P.) positive. *B. subtilis* is easily cultured on any nutrient medium and is extremely resistant to heat by virtue of the spores (Merchant and Packer, 1961; Slepecky and Hemphill, 1992). *B. subtilis* is able to grow at 50°C and on media containing 7% NaCl (Slepecky and Hemphill, 1992).

B. subtilis is found in the soil and decaying vegetation where they are most active in organic substances decomposition (Merchant and Packer, 1961; Slepecky and Hemphill, 1992). It is spread by water, wind and normal traffic (Merchant and Packer, 1961; Priest, 1993).

Although *B. subtilis* is not recognized as an important pathogen, there are reports of this bacterium causing iridocyclitis and panophthalmitis, and it has been known to produce fatal septicemia under ideal conditions (Merchant and Packer, 1961; Slepecky and Hemphill, 1992). In addition, there are several reports of *B. subtilis* being an important spoilage microorganism to the baking industry in rope-inducing in bread resulting in significant economic losses (Lindsay et al.,2005; 2006).

The industry takes advantage of several attributes of *B. subtilis*, such as good secretion of proteins and metabolites, simple to cultivate and easy genetic manipulation, for production of fermentation products. Antibiotics, insecticides, nucleotides and

nucleosides (for food flavor enhancement), aminoacids, and over half of the total enzymes sales are products in commerce today that are produced by *Bacillus* fermentations (Arbige et al., 1993). In addition, *B. subtilis* has been used to control several types of fungal disease associated with fruits, vegetables, field crops and flower crops, largely through the secretion of antifungal antibiotics (Priest, 1993).

2.5 – Clostridium perfringens

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic encapsulated bacterium that causes a broad spectrum of human and veterinary diseases (Smith, 1992; McClane, 2001; Wrigley 2001). This bacterium produces spores that are oval and subterminal (Wrigley, 2001). *C. perfringens* differs from many other clostridia in being nonmotile, reducing nitrate, and carrying out a stormy fermentation of lactose in milk (Setlow and Johnson, 2001; Wrigley, 2001). *C. perfringens* also ferments glucose, fructose, galactose, inositol, maltose, mannose, starch and sucrose. The fermentation products include acetic and butyric acids with or without butanol (Smith 1992).

The virulence of *C. perfringens* largely results from its prolific toxin-producing ability, including at least two toxins, *C. perfringens* enterotoxin (CPE) and beta-toxin, that are active on the human gastrointestinal (GI) tract (McClane, 2001). The classification scheme for *C. perfringens* assigns isolates to one of the five types (A-E) depending upon their ability to express one or all four "typing" toxins(alpha, beta, epsilon and iota) (Smith, 1992; McClane, 2000; 2001; Wrigley, 2001). The principal habitats of type A are the soil and the intestines of humans and animals. The habitat of types B, C, D and E appears to be the intestine of animals (Smith, 1992).

The lowest a_w supporting vegetative growth of *C. perfringens* is 0.93 to 0.97, depending upon the solute used to control a_w of the medium (McClane, 2001; Wrigley, 2001). *C. perfringens* will grow over a wide pH range varying from 5.5 to 8.5, while optimum growth of this bacterium occurs at pH 6 to 7 (McClane, 2001; Setlow and Johnson, 2001). The optimal temperature for growth of types A, D and E is 45 ˚C, whereas for types B and C optimal temperature varies from 37 – 40 ˚C (Smith, 1992; Wrigley, 2001). Minimum temperatures for growth range from 14 to 18.5 ˚C and incubation of cells between 0 and 10 °C results in rapid cell death (Wrigley, 2001). The optimum temperatures referenced above depend greatly on the pH and growth medium.

The widespread natural distribution of *C. perfringens* in both soil and the gastrointestinal tract of humans and animals provide this bacterium with ample opportunities to contaminate food (McClane, 2001; Setlow and Johnson, 2001). *C. perfringens* has two main characteristics that contribute to its ability to cause foodborne disease. First, its low generation time (reportedly <10 minutes for vegetative cells) allows *C. perfringens* to quickly multiply in food (McClane, 2001; Setlow and Johnson, 2001). Secondly, in addition to its ability to survive in undercooked foods due to relative heat tolerance of its vegetative cells, *C. perfringens* has the ability to form spores which are resistant to environmental stresses such as radiation, desiccation, and heat; the ability to form such resistant spores facilitates survival of *C. perfringens* in undercooked or inadequately warmed foods (McClane, 2001).

In addition to vegetables and fruits that acquire spores from soil, foods of animal origin are contaminated during slaughtering with spores in the environment or spores residing in the intestinal tract (Setlow and Johnson, 2001). It is estimated that

approximately 50% of raw and frozen meat contains some *C. perfringens* (McClane, 2001). Dried foods such as spices are a common source of *C. perfringens* to the food industry (Setlow and Johnson, 2001).

The exceptionally short generation time of *C. perfringens* (10 minutes) makes it relatively easy for this bacterium to contaminate foods at levels necessary for causing foodborne disease (10^6 – 10^7 vegetative cells/gram of food) (McClane, 2001). *C. perfringens* can grow extremely fast on high protein foods, such as meats that have been cooked which would eliminate competitors and are kept under inadequate conditions (Setlow and Johnson, 2001).

The two most common foodborne diseases caused by *C. perfringens* are each associated with a distinct *C. perfringens* type. The first type is called necrotic enteritis and is caused by type C isolates, with the beta-toxin produced by those isolates considered the primary virulence factor for that disease. The second one, as implied by its name, *C. perfringens* type A food poisoning is almost always associated with type A isolates, even though other *C. perfringens* types sometimes express an enterotoxin with similar physical, serologic, and biologic properties as *C. perfringens* enterotoxin (CPE) (McClane, 2001). Almost all *C. perfringens* foodborne illness in the U.S. and other industrialized countries involves *C. perfringens* type A food poisoning (McClane, 2000). Because most cases of *C. perfringens* type A food poisoning are not recognized or reported, the official CDC statistics significantly understate the true prevalence and impact of this foodborne disease. Economic costs associated with *C. perfringens* type A food poisoning are estimated to exceed U.S.$ 120 million (McClane, 2001).

C. perfringens type A intoxication rarely results from consumption of foods containing preformed CPE. The first step in acquiring *C. perfringens* type A food poisoning is consuming food, usually a meat product, that has become contaminated with large number of vegetative cells or spores of *C. perfringens* (McClane, 2000). Humans appear to be a common source for many *C. perfringens* outbreaks, as the contaminant is often introduced during food preparation (Wrigley, 2001). A great number of these ingested cells will be killed in the stomach, though some of these bacteria may survive exposure to gastric acid and escape into the small intestine. The survival of these bacteria is due to their ability to sporulate once exposed to gastric acid or bile salts in the intestines (McClane, 2001). It is during sporulation of the cells that the enterotoxin is produced (McClane, 2000; Wrigley, 2001). No significant release of CPE into the intestinal lumen occurs until sporulation is completed. The newly synthesized CPE accumulates inside the cytoplasm of the mother cell until it is released into the intestinal lumen when the mother cell lyses to free its now mature endospore (McClane, 2001). Once present in the lumen, CPE quickly binds to receptors on the intestinal epithelium and induces epithelial desquamation. CPE is a membrane active toxin inducing alterations in the normal permeability properties of exposed intestinal mammalian cells (McClane, 2000; 2001).

C. perfringens type A food poisoning is clinically characterized by diarrhea and abdominal cramps that develop about 8 – 16 hours after ingestion of contaminated food (McClane, 2000). In most affected people symptoms of *C. perfringens type A* continue for 12-24 hours before self resolving. Fatalities are relatively rare but may occur specially

in some elderly and immunocompromised individuals (Smith, 1992; McClane, 2000; 2001).

Identified outbreaks of *C. perfringens* type A foodborne illness are usually large and often occur in institutionalized settings (McClane, 2000; 2001). The large size of most recognized *C. perfringens* type A foodborne disease outbreaks is mostly attributable to two factors. First, large institutions often prepare food in advance and then hold that food for later serving. Institutions such as nursing homes, prisons, and hospitals represent favorable environments for *C. perfringens* type A food poisoning outbreaks since these establishments depend heavily on serving foods involving large meat items, such as roasts and turkeys, in order to feed many people at peak meal times (McClane, 2000). Large meat items are the most common food vehicles for *C. perfringens* type A food poisoning because they are fairly difficult to thoroughly cook. Food poisoning by *C. perfringens* nearly always involves temperature abuse of a cooked food, and the great majority of food poisonings caused by *C. perfringens* could be avoided if cooked foods were eaten immediately after cooking or rapidly chilled and reheated before consumption to inactivate vegetative cells (Setlow and Johnson, 2001).

Second, given the relatively mild and non-distinguishing symptoms of most cases of *C. perfringens* type A foodborne illness, public health officials usually only become involved in investigating and reporting such foodborne illnesses when large numbers of people become ill (McClane, 2000; 2001). CDC estimates that the most common vehicles for *C. perfringens* type A foodborne illness in the United States are beef and poultry, specially when prepared containing gravy and in the form of stews (McClane, 2001).

2.6 – Biofilms

Bacterial biofilms are defined as microbially-derived sessile communities characterized by cells, often of different species, that are attached to a substratum, to an interface, or to each other (Ofek et al., 2003; Costerton, 2004). At this point, however, it is not clear to what extent biofilms at these different interfaces share metabolic or physiological traits (Costerton, 2004). Biofilm cells are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription (Scher et al., 2005). Nutrient rich conditions are often found in food processing plants due to residues of proteins and fats being deposited on surfaces which allow colonization and maintenance of microbial populations within the food industry (Lindsay et al., 2006).

Planktonic and sessile are two microbiology jargons largely used to differentiate the environment in which bacteria is being accessed. The first one is defined by the Oxford English Dictionary as “drifting or floating organic life found at various depths in the ocean or fresh water”. This concept was extended by Marshall (1992) at the micrometer level of a planktonic habitat for prokaryotes can also include water films around soil particles, saliva, fluids in the intestinal lumen, blood and urine. Sessile on the other hand, according to the Oxford English dictionary means “immediately attached, without a foot-stalk”. This concept was also extended to the microbial world by Marshall (1992) as those prokaryotes directly adhering to surfaces, those embedded in biofilms developing as a result of extracellular polymer production by bacteria colonizing surfaces.

A bacterial biofilm is formed in a number of distinct steps: initial reversible adsorption of cells onto a solid surface, production of surface polysaccharides or capsular material followed by formation of an extracellular polymeric matrix resulting in irreversible attachment, early development of biofilm architecture, and maturation and dispersion of single cells from the biofilm (Kim and Wei, 2007).

Because bacterial adhesion is an indispensable component of biofilm formation, the factors involved in bacterium-substratum interactions are an important basis for understanding biofilm physiology (Ofek et al., 2003). Adhesion of one or more bacterial cells to a substratum is the first event in biofilm formation and the first stage of bacterial adhesion is called adsorption (Brisou, 1995; Ofek et al., 2003). This event occurs either on unconditioned substratum or on substratum covered with organic materials from the surrounding environment. It is not known whether this event requires a minimal number of bacterial cells or whether a single bacterium will suffice (Ofek et al., 2003).

All interfaces, whatever their nature, have properties of attracting substances dispersed or dissolved nearby (Brisou, 1995). Various workers have attempted to relate the extent of bacterial adhesion to the variation in surface free energy of the substratum with variable results. More extensive studies revealed that, in addition to the substratum-surface free energy, it was necessary to consider the bacterium-surface free energy and the surface tension of the liquid (Marshall, 1992; Hood and Zottola, 1995). When a clean sterile surface is immersed into a natural habitat, a molecular film rapidly forms on the surface as a result of adsorption of macromolecules and smaller hydrophobic molecules. This film serves to condition the surface causing alterations in surface charge and surface free energy (Marshall, 1992). The properties of the surface to which these organisms

adhere can profoundly impact the structure and composition of the community (Costerton, 2004).

It is well known that some bacteria adhere more avidly to surfaces than do other bacteria and even that the same bacteria may undergo time-dependent changes in avidity of adhesion (Ofek et al., 2003). Bacteria are carriers of negative electric charges and several researchers have accounted the initial adhesion of bacteria to solid surfaces as a result of the attraction of a negatively charged bacterium to a negatively charged substratum surface resulting from the interaction of Van der Waals attraction forces and electrical repulsion forces in the overlapping double layers of cations surrounding the negatively charged surfaces (Marshall, 1992; Brisou, 1995; Hood and Zottola, 1995). Regardless of the strength of adhesion, the bacterium must remain surface localized in order to become a community (Ofek et al., 2003).

Irreversible adhesion to surfaces in nature is generally considered to be nonspecific. That is, the bacteria adhere to a wide variety of different inanimate and animate surfaces with varying degrees of adhesive strength. Bridging polymers involved in most cases of nonspecific adhesion are either extracellular polysaccharides, proteins or glycoproteins (Marshall, 1992). Adhesins are one of the most important elements of adherence that can be encountered in both microorganisms and different surfaces. Some correspond to veritable anchorage devices, with precise structures, whereas others are reduced to gels, mucous, or soluble substances, but are nevertheless active (Brisou, 1995).

Once the initial adhesion is successful, accretion of bacteria can proceed with concomitant proliferation. Daughter cells may also become bound to the surface. The

composite population consisting of the initially adherent cells and their progeny may now secrete various polymers, especially acidic polysaccharides (Ofek et al., 2003). Extracellular matrix provides the environment which the cells are localized and also acts as a protective layer against potentially harmful agents as well as a carbon and energy source at times of nutrient deprivation (Purevdorj-Gage and Stoodley, 2004). The bacteria and the extracellular components produced or acquired may then serve as substratum for additional progeny or for the adhesion of additional bacterial species. Distinction between initial adhesion and subsequent accretion and eventual biofilm formation has not been well defined (Ofek et al., 2003).

The polymeric substance matrix has a very dynamic nature; cells will attempt to influence its surrounding by secreting specific biological macromolecules. However, this environment will also harbor components of abiotic origin and matrix nonspecific biological molecules derived from the lysis of cells (Starkey, 2004). Secreted polysaccharides represent the most extensively studied component of the extracellular polymeric matrix and are often assumed to be the most abundant extracellular component in biofilms. Depending on the species present in the biofilms and environmental conditions, the amount and types of exopolysaccharides produced can vary extensively (Starkey, 2004). The secreted polysaccharides of the biofilm matrix are capable of trapping ions and various organic materials in addition to their ability to trap additional bacteria (Ofek et al., 2003). There are three general forms of exopolysaccarides, based on their chemistry and structure: linear, branched and cyclic. The exopolysaccharides can also be classified on the basis of cell association – cell-associated exopolysaccharide is

frequently termed capsular (anchored to the cell membrane through a diacyl glycerol moiety or some other type of lipid) and unbound is called “slime” (Starkey, 2004).

The density of the developing bacterial community in the various stages of biofilm formation varies and depends on many factors, including the rate of multiplication. The metabolic activity of each of the individual species depends on the bacterial cell densities (Ofek et al., 2003). Biofilm formation is one of many bacterial activities controlled through cell-cell signaling. When cells live in biofilms, embedded in matrix material, the amount of a particular signal that turns on a specific gene in an individual cell may depend on the proximity of the signal-producing cell to the signal-receiving cells and on the diffusion path between them (Costerton, 2004). Cell density-dependent regulation of gene expression, a phenomenon known as quorum sensing, is used by many Gram-negative bacteria (Ofek et al., 2003). Recent studies have shown that “waves” of gene expression are triggered as certain signals diffuse through the matrix and reach critical local concentrations. Therefore, there is strong evidence that quorum-sensing signals are essential during biofilm formation, development and maintenance (Costerton, 2004).

Recent research strongly suggests that quorum sensing is a system involving acyl homoserine lactones. At a critical population density, the lactone compounds produced reach a concentration that affects the synthesis of a number of products, some of which may be the adhesions required for biofilm development and integrity (Ofek et al., 2003). Unlike liquid cultures, where the signal reaches a certain level in the bulk fluid and all cells up-regulate the gene, quorum signals may regulate gene expression in biofilms much more individually and subtly, in the same way that hormones regulate tissue and

organ development (Costerton, 2004). Another mechanism for density-related regulation of biofilm development is the ability to sense the proportion of dead bacteria in the community. The full mechanism of how dead cells can influence cell density is still unknown. There is strong evidence that lectins released by dying cells have an important role in this signaling process (Ofek et al., 2003).

Biofilm architecture can be influenced by many different external and internal parameters, both biotic and abiotic. Biofilms can have a rich repertoire of structures which can range anywhere from patchy monolayers, to thin or thick flat biomasses, to more organized mushrooms, ripples, and filamentous streamers (Purevdorj-Gage and Stoodley, 2004). One of the most obvious advantages of the sessile state is the increased probability of access to nutrients accumulating at surfaces, particularly in flowing, oligotrophic conditions (Marshall, 1992). These nutrients must diffuse through the mass transfer boundary layer (external mass transfer) and then through the biofilm matrix (internal mass transfer) to reach the bacterial cells. The thickness of the boundary layer is inversely correlated to the flow pattern over the biofilm surface, meaning that at higher shear conditions, the thickness of the boundary layer decreases and results in an increased rate of nutrient diffusion into the biofilms (Purevdorj-Gage and Stoodley, 2004). At the bottom of the biofilm, the oxygen tension is relatively low, creating a microaerophilic environment, although in an undefined manner. Oxygen levels at the periphery of the biofilm are equivalent to those in the medium. Channel formation in biofilms is an important aspect of biofilms functionality and architecture. Their main role appears to be an attempt to increase the surface area of the biofilm, making it possible to more efficiently trap nutrients from the bulk medium. In addition, channels have the important

function of water and waste transport. The process of channel formation is dictated by the genetics of the inhabiting bacteria (Ren et al., 2002; Ofek et al., 2003).

A biofilm is a dynamic microenvironment, such that the microorganisms divide or are released and shed into the medium and its organic constituents are turned over (Ofek et al., 2003). Biofilms are self-regulating, and as they age, individual cells or parts of the biofilm may release or detach from the primary microbial community. The detachment process is an important component of the biofilm life cycle and plays a fundamental role in dissemination and contamination and ultimately long term survival in either natural or artificial settings (Purevdorj-Gage and Stoodley, 2004). Although bacteria attached to particle surfaces may gain an advantage by utilization of adsorbed nutrients or by the dissolution of organic particles, such bacteria would sink to the sediments and would be unable to colonize new particle surfaces if mechanisms did not exist for their release or the release of daughter cells from the particle surfaces (Marshall, 1992).

The process of periodic detachment of relatively large particles of biomass from the biofilm is defined as sloughing. Individual cell release and sloughing allows the contamination and colonization of new substrates (Kumar and Anand, 1998; Gamble and Muriana, 2007;). Very little is known about the biological, chemical and physical mechanisms that takes part during the detachment process. Apart from recognized internal mechanisms like enzymatic dissolution of the matrix, physical forces such as hydrodynamics and shear are known to cause biofilm detachment via either erosion of single cells or sloughing of large aggregates of biomass (Purevdorj-Gage and Stoodley, 2004). The microbial load transferred from an inert surface to a food or food like surface depends on the properties of the biofilm: surface density of the microbial population, the

structure of the film, its capacity to produce exopolysaccharides, and the attachment strength of the microbial cells (Midelet and Carpentier, 2002). These mechanisms of bacterial colonization and detachment are extremely important since these cycles allow the microorganisms to be maintained in the food processing environment. Since all the sessile bacteria are derived from the planktonic state and, in addition to active growth and metabolism at surfaces, these sessile organisms have also evolved a variety of methods to ensure that representatives of the population can return to planktonic state and colonize other areas which are essential to maintaining their presence (Marshall, 1992).

Several authors have reported that bacterial cells are more resistant to environmental stresses such as nutritional deprivation and oxidative stress when in a biofilm environment. In addition, when in a biofilm, the cells are more resistant to antimicrobial agents and antibiotics than are free cells (Scher et al., 2005; Kim and Wei, 2007; Sandasi et al., 2008). So far, very few studies have reported specific reasons for this; however there are two hypotheses that are widely accepted by the scientific community. The first hypothesis refers to the slow or incomplete penetration of antimicrobial substances into the biofilm environment. This retarded penetration is probably due to the polysaccharides matrix that serves as a shield to the cells, preventing the antimicrobial substances from being in contact with the bacterial cells (Scher et al., 2005; Kim and Wei, 2007). In addition, reaction of these reagents with the outer layer of cells or with the extracellular polymer that makes up the matrix of the biofilm can reduce or inactivate these antimicrobials (Marshall, 1992). The second hypothesis is the occurrence of an altered microenvironment within the biofilm which modifies the properties of the bacterial cells or the antimicrobial itself (Scher et al., 2005; Kim and

Wei, 2007). In addition, it has been reported that biofilms have reduced metabolic activity mainly due to reduced nutrient and oxygen supply and it is this reduction in metabolic activity that has been correlated with resistance (Ofek, 2003; Gamble and Muriana, 2007; Sandasi et al., 2008).

Biofilms have developed into a significant issue for public health as they are less susceptible to antimicrobial agents and treatments (Scher et al., 2005). Inorganic interfaces are rapidly colonized by microorganisms, sometimes posing serious problems for the food industry and hygiene in general (Brisou, 1995). Biofilms formed in food processing environments are of significant importance because when viable cells are transferred to food, they could have a serious negative impact on the storage quality and safety of that food (Hood and Zottola, 1995; Stepanović et al., 2004). As a consequence, biofilms lead to serious hygienic problems (presence of foodborne pathogens leading to foodborne illness) and economic losses due to food spoilage (shelf life reduction) and potential recalls (Lindsay et al., 2006).

2.7 – Concrete

Concrete is present in food industry facilities especially in flooring, walls and ceilings. During processing, concrete receives a great amount of organic matter. The organic matter in the poultry industry is a result of usual processing steps such as bleeding, scalding, feather-picking and eviscerating. This organic matter has the potential to serve as an initial source of nutrients to microorganisms, allowing them to colonize on and / or in concrete. Concrete is a microporous, microstructure-sensitive construction material, and the pores in concrete are randomly sized, arranged, and connected (Yang et

al., 2004). These pores form capillary systems in concrete allowing water and other substances to traffic freely in concrete structures. When liquids flow freely on concrete, they may serve as carriers in the transport of microorganisms such as bacteria. To understand and interpret the behavior of a composite element such as concrete, knowledge of the characteristics of its components is necessary. Concrete is produced by the collective mechanical and chemical interaction of a large number of constituent materials. Disintegration of concrete due to cycles of wetting, freezing, thawing, drying, chemicals and the propagation of the resulting cracks is a matter of great importance for the food industry (Nawy, 1996). The disintegration of concrete will serve as great attachment sites for bacteria to form niches. These niches will serve as permanent sources of contamination for whatever food is being processed. Contamination of food may occur from direct contact of food to concrete surfaces or indirect contact (water splashes during sanitation, staff shoes and clothes).

2.8 – BioSealed for Concrete™

BioSealed for Concrete™ is manufactured and commercialized by GreenSealed Solutions, Inc. This product is a hydrosilicate catalyst in a colloidal liquid base with an extremely alkaline pH (12), is odorless appears white/opaque. BioSealed for Concrete™ is applied using a spray apparatus; no special ventilation conditions nor breathing equipment are required.

The main difference between BioSealed for Concrete™ from other typical topical sealants is that BioSealed for Concrete™ couples an antimicrobial with a gel barrier to eliminate pathogens. In addition, its deeper penetration into concrete (7 inches against 3

inches from typical topical sealants) closes down concrete capillary system sealing out water, moisture, pathogens and contaminants. BioSealed for Concrete™ permanently stops water migration and halts contaminants ingress from any direction with the benefit of preventing alkali-silicate reactions to thereby reduce concrete disintegration and abrasion.

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3 – ANTIMICROBIAL EFFICACY OF COMMERCIAL GRADE CONCRETE SEALANT AGAINST MULTIPLE STRAINS OF *SALMONELLA* SPECIES AND THEIR BIOFILMS ON CONCRETE SURFACES

(Submitted to Poultry Science for Publication, May 2009)

3.1 – Abstract

Salmonella is an important foodborne pathogen often associated with poultry and highly prevalent in poultry processing plants. The objective of this study was to determine the efficacy of a commercial grade concrete sealant (BioSealed for Concrete™) to prevent bacterial attachment, colonization, and antimicrobial effects against multiple strains of *Salmonella* (*S.* Enteritidis*, S.* Kentucky, *S.* Typhimurium, *S.* Seftenberg and *S.* Heidelberg) on concrete blocks. Individual strains of *Salmonella* spp. were inoculated onto the concrete blocks and divided into 4 different treatment groups: (A) Bricks which were not treated with BioSealed for Concrete™, (B) Bricks which were treated with BioSealed for Concrete™ before inoculation, (C) Bricks which were treated with BioSealed for Concrete™ after inoculation, and (D) Bricks which were treated with BioSealed for Concrete™ before and after inoculation. External and internal surfaces of the treated concrete blocks were swabbed, serially diluted and plated onto XLD agar. Reductions of survival counts were enumerated and recorded as $\log_{10}$ CFU/ sq. cm.

Significantly ($p<0.05$) lower viable counts were observed following treatments C and D as compared to treatments A and B. However, no significant differences ($p>0.05$) in the survival populations of *Salmonella* were observed between treatments A and B for all five strains tested and between treatments C and D for any of the strains tested. This indicates that BioSealed for Concrete™ proved to be a potent antimicrobial against multiple strains of *Salmonella* and can be used as an alternative method to control this pathogen in processing plant environments.

3.2 – Introduction

Salmonellae are small, Gram negative, non-spore forming rods. Every year, approximately 40,000 cases of salmonellosis are reported in the United States according to the Center for Disease Control and Prevention (CDC). Salmonellosis is an important public health problem in the United States with an estimated number of nontyphoidal *Salmonella* infections ranging from 800,000 to 4,000,000 annually (Voetsch et al., 2004). Although most outbreaks cause mild to moderate self limited illness, serious disease resulting in death does occur particularly in elderly and immunocompromised populations. Because many milder cases are not diagnosed or reported, the actual number of infections may be higher than the reported numbers. The centers for disease control and prevention (CDC) estimates that *Salmonella* infection causes approximately 1.4 million foodborne illnesses annually (Lynch et al., 2006). Accounting for medical costs and lost productivity the estimated costs associated with salmonellosis is approximately $2.3 billion (Frenzen et al., 1999). The CDC states that bacterial agents are the most common microorganisms associated with foodborne illnesses accounting for 55% of

laboratory diagnosed foodborne illnesses and outbreaks. Among bacterial pathogens, *Salmonella* Enteritidis accounted for the largest overall number of outbreaks and outbreak-related illnesses. Several foods including cereal, peanut butter, tomatoes, cantaloupe, beef, pork and poultry have been implicated in *Salmonella* related human illnesses. Recent *Salmonella* outbreaks were reported by the U.S. Department of Agricultures' Food Safety and Inspection Services (USDA – FSIS) involving fresh poultry and further processed poultry products such as chicken pot pies and raw frozen breaded and pre-browned stuffed chicken entrees.

Salmonella spp. has developed several strategies to survive in the environment and their ability to adhere to surfaces and form biofilms are among the most important ones. Extracellular structures contributing to bacterial adherence include curli fimbriae, cellulose, capsular polysaccharide and other polysaccharides such as lipopolisaccharides (LPS) (Malcova et al., 2008). However, the mechanisms involving the adhesion of *Salmonella* spp. to inert surfaces are still unclear; different studies have shown that the bacterial attachment partially depends on bacterial characteristics and partially on surface properties (Joseph et al. 2001; Sinde and Carballo, 2000; Austin et al., 1998). Bacterial biofilms are defined as microbially derived sessile communities characterized by cells that are attached to a substratum, to an interface, or to each other. Biofilm cells are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription (Scher et al., 2005). A bacterial biofilm is formed in a number of distinct steps: initial reversible adsorption of cells onto a solid surface, production of surface polysaccharides or capsular material followed by formation of an extracellular polymeric matrix resulting in

irreversible attachment, early development of biofilm architecture, and maturation and dispersion of single cells from the biofilm (Kim and Wei, 2007).

Biofilms have developed into a significant issue for public health as they are less susceptible to antimicrobial treatments (Scher et al., 2005; Dunowska et al., 2005; Cloete, 2003; Joseph et al. 2001). This is especially important considering that biofilms have increased resistance towards the most commonly used biocides in the food industry such as iodine, chlorine, peroxygens and quaternary ammonium compounds (Cloete, 2003). Biofilms formed in food processing environments are important as they have the potential to act as a chronic source of microbial contamination that can eventually lead to food spoilage or transmission of diseases (Stepanović et al., 2004). Biofilms lead to serious hygienic problems and economic losses due to food spoilage and potential recalls. The important aspects essential in controlling biofilm formation and minimizing the biotransfer potential in the food processing equipment and environments include proper cleaning and sanitation procedures. The control of biofilms represents one of the most persistent challenges within food and industrial environments where the microbial communities are problematic (Kumar and Anand, 1998). Regular sanitation programs typically consist of removal of gross debris, rinsing, presoaking in detergent, rinsing, disinfection and final rinsing. Since, removal of biofilms is a very difficult and demanding task, these sanitation programs are usually not sufficient to remove biofilms. The food industry has been looking for cost efficient cleaning and sanitation alternatives to facilitate biofilms removal and to prevent new biofilm formation on inert surfaces. These new strategies usually include physical and chemical methods which interfere on bacterial colonization and biofilm development.

It has been reported that bacterial cells are more resistant to environmental stresses such as nutritional deprivation and oxidative stress when in a biofilm environment. In addition, when in a biofilm, the cells are more resistant to antimicrobial agents and antibiotics than free cells (Kim and Wei, 2007; Dunowska et al., 2005; Scher et al., 2005; Cloete, 2003; Hood and Zottola, 1997; Leriche and Carpenter, 1995). So far, very few studies have reported specific reasons for the increased resistance of biofilms; however there are two hypotheses that are widely accepted by the scientific community. The first hypothesis refers to the slow or incomplete penetration of antimicrobial substances into the biofilm environment. This retarded penetration is probably due to the polysaccharides matrix that serves as a shield to the cells avoiding that the antimicrobial substances get in contact to the bacterial cells. The second hypothesis is the occurrence of an altered microenvironment within the biofilm which modifies the properties of the bacteria cells or the antimicrobial itself (Kim and Wei, 2007; Scher et al., 2005).

Concrete is widely used in the food industry especially in flooring, walls and ceilings. During processing, concrete receives a great amount of organic matter due to the usual processing steps such as bleeding, scalding, eviscerating and feather-picking. This organic matter has potential to serve as an initial source of nutrients to microorganisms allowing them to colonize on and / or in concrete. Concrete is a microporous, microstructure-sensitive construction material, and the pores in concrete are randomly sized, arranged, and connected (Yang et al., 2004). These pores form capillary systems in concrete allowing water and other substances to traffic freely in concrete structures. When liquids flow freely on concrete, they may serve as carriers in the transport of microorganisms such as bacteria. To understand and interpret the behavior of composite

element such as concrete, knowledge of the characteristics of its components is necessary. Concrete is produced by the collective mechanical and chemical interaction of a large number of constituent materials. Disintegration of concrete due to cycles of wetting, freezing, thawing, drying, chemicals and the propagation of the resulting cracks is a matter of great importance for the food industry (Nawy, 1996). The disintegration of concrete serves as a great attachment site for bacteria to form niches that have the potential to work as permanent sources of contamination in food processing plants. Contamination of food may occur from direct contact of food to concrete surfaces or indirect contact (water splashes during sanitation, staff shoes and clothes). Therefore, the food industry has placed a great deal of effort on the reduction of bacterial niches and avoiding the formation of biofilms. There is no single action which will reduce or eliminate biofilms from industrial environments. Several actions must be taken collectively to prevent the formation of biofilms and eliminate these chronic sources of contamination. This study focuses on the attachment of *Salmonella spp.* on concrete and its potential to form biofilms. The objective of this study was to determine the efficiency of BioSealed for Concrete^{TM}; a hydrosilicate catalyst in a colloidal liquid base (GreenSealed Solutions, Inc. – Georgia) to prevent *Salmonella* spp. attachment on concrete surfaces.

3.3 – Materials and Methods

3.3.1 – Bacterial Cultures

Salmonella strains used in this study were chosen based on their incidence in the poultry industry report published by the United Stated Department of Agriculture (USDA

– FSIS, 2008) and the Centers for Disease Control and Prevention (CDC). The five strains used in this study were *Salmonella* Typhimurium, *S.* Heidelberg, *S.* Enteritidis, *S.* Seftenberg, and *S.* Kentucky. These strains were all independently cultured in Brain Heart Infusion (BHI) broth (Acumedia Manufacturers Inc., Lansing, MI) and incubated at 39 °C for 24 h before being used to challenge the concrete bricks. The length of incubation and inoculation of the bacterial cultures were based on 24 h growth curves that were performed in the laboratory (data not shown). At the time of inoculation the average count of the inocula varied based on the strain of *Salmonella* (table 1).

3.3.2 – Bricks Preparation

Quikrete® (Quickrete, Georgia) powder mix was used to produce concrete bricks in commercial sized ice cube trays, as per manufacturer's directions. Miniature bricks were made in ice cube trays in order to simulate concrete blocks to conduct laboratory experiments.

3.3.3 – Sampling

Concrete bricks were divided into four groups. (A) Bricks which were not treated with BioSealed for Concrete™, (B) Bricks which were treated with BioSealed for Concrete™ before inoculation, (C) Bricks which were treated with BioSealed for Concrete™ after inoculation, and (D) Bricks which were treated with BioSealed for Concrete™ before and after inoculation. The inoculums were split in two equal parts: (1) The first half were considered the control and contained bricks from groups A and C (bricks untreated before inoculation) and (2) the second half of the inoculum was named the "inoculums treated" which contained bricks from groups B and D (bricks which were treated before inoculation). Samples of the inoculums were spread plated onto xylose

lysine desoxycholate (XLD) agar (Neogen, Lansing, MI) for enumeration at inoculation time and at the time of removal of the samples.

Bricks were submerged in the inoculums for 24 h at 39 °C. After 24 h the bricks were removed from inoculums and held for 30 min. in a sterile laminar flow cabinet (Nuaire Inc., Plymouth, MN) to allow drying of excess inoculum from the brick surface. Swabs were then used to sample the entire surface of each brick. The swabs were then placed in 10 mL of sterile peptone water (Acumedia Manufacturers Inc., Lansing, MI) tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes. After swabbing the surface, bricks were broken in half and the inner surfaces of both halves were swabbed. The swab was then placed in 10 mL autoclaved peptone water tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes and 0.1 mL of the sample was spread plated onto XLD agar. Plates were incubated for 24 h at 39 °C and the results were recorded as $\log_{10}$ CFU/ sq. cm with the exception of inoculum samples, which were recorded as $\log_{10}$ CFU/ mL.

3.3.4 – Statistical analysis

Completely randomized design was used to assign concrete blocks to the four treatment groups. Three replications of this experiment were performed and the averages of the survival populations ($\log_{10}$ CFU/ sq. cm) of various strains of *Salmonella* were analyzed using analysis of variance (ANOVA) with SAS PROC GLM procedures (2002-03 SAS Institute, Gary, NC). Statistical significance was reported at a p-value of less than or equal to 0.05 ($p<0.05$).

3.4 – Results and Discussion

As evident from figure 1, biofilm formation was observed by all five *Salmonella* strains tested in this study. Biofilm contents and architectures are highly heterogeneous and variable, suggesting that bacterial phenotypes and physiology are also variable. They depend not only on the bacterial strains that form the biofilm but also on the material of the surface and on the growth and environmental conditions (Scher et al., 2004; Joseph et al., 2001). In this study several antimicrobial characteristics of BioSealed for Concrete™ were evaluated such as bactericidal effects, prevention of bacterial attachment, prevention of biofilm formation and removal of formed biofilms. Group A was our control group and served as a base of comparison to all other treatments. Treatment B was applied to evaluate the residual effects of the product and determine if the residual effects of the product were able to prevent biofilms formation, whereas treatments C and D simulated situations of most present day food processing plants i.e. never had this type of product applied before. Treatment D allowed us to evaluate if previous treatment would have any additional or different effect from first time applications of this product.

3.4.1 – External brick surfaces

Analysis of Variance (ANOVA) of survival populations of *Salmonella* ($\log_{10}$ CFU/ sq. cm) did not show any significant difference ($p>0.05$) following treatments A and B suggesting that there is no evidence of the product producing any residual effect that would prevent the attachment of bacteria and formation of new biofilms for all the strains of *Salmonella* tested in this study except for *S.* Kentucky. When comparing the survival populations of *Salmonella* Typhimurium on the external surface no significant differences ($p>0.05$) between treatments A and B; or treatments C and D were observed

(table 2). Treatment C resulted in a significant reduction of the *S.* Typhimurium population ($p<0.05$; ca. 3.78 $\log_{10}$ CFU/ sq. cm) as compared to treatment A, whereas treatment D resulted in significant reduction ($p<0.05$; ca. 2.9 $\log_{10}$ CFU/ sq. cm) of the *S.* Typhimurium populations when compared to treatment B. *S.* Heidelberg showed similar results on the external surface of the bricks as *S.* Typhimurium (table 2). Results showed a significant reduction ($p<0.05$; ca. 4.22 $\log_{10}$ CFU/ sq. cm) in the *S.* Heidelberg populations following treatment C as compared to treatment A while treatment D resulted in significantly reducing ($p<0.05$; ca. 3.45 $\log_{10}$ CFU/ sq. cm) the populations of *S.* Heidelberg as compared to treatment B. Similar to *S.* Typhimurium and *S.* Heidelberg, results indicated that Bioseal for Concrete™ has potent antimicrobial effect on *S.* Seftenberg and *S.* Enteritidis. A 2.96 $\log_{10}$ CFU/ sq. cm reduction was observed when comparing treatments A and C; and a 3.77 $\log_{10}$ CFU/ sq. cm reduction was observed when comparing treatments B and D for *S.* Enteritidis trial. Reduction in the survival populations of *S.* Seftenberg were observed to be greater than *S.* Enteritidis; 3.18 $\log_{10}$ CFU/ sq. cm reduction when comparing groups A and C; and a 3.49 $\log_{10}$ CFU/ sq. cm reduction when comparing groups B and D.

Although, the antimicrobial effects of Bioseal for Concrete™ on *S.* Heidelberg and *S.* Typhimurium were similar to those on *S.* Enteritidis and *S.* Seftenberg, treatment B resulted in slightly greater average survival populations of the bacteria than treatment A for the later two strains of *Salmonella*. Although the difference is not significantly different ($p>0.05$), the higher recovery of the pathogen from the surface of the concrete blocks as a result of prior application of Bioseal for Concrete™ indicated loose attachment and/ or the lack of bacterial attachment hence making it more susceptible to

standard sanitation procedures. This could lead to more effective sanitation in the processing plants. Survival population (log_{10} CFU/ sq. cm) of *S.* Kentucky was different from the other strains of *Salmonella*. Bioseal for Concrete™ was an effective ($p<0.05$) antimicrobial against *S.* Kentucky and a 3.56 log_{10} CFU/ sq. cm reduction was observed when comparing treatments A and C; and a 2.4 log_{10} CFU/ sq. cm reduction was observed when comparing treatments B and D. Throughout the study treatments A and B showed similar results and no significant difference ($p>0.05$) was observed between these two groups on *S.* Kentucky. The difference observed between *S.* Kentucky and all the other strains tested was that the survival populations (log_{10} CFU/ sq. cm) in groups B and C were not significantly different ($p>0.05$) indicating that treating concrete with Bioseal for Concrete™ prior to or post bacterial challenge does not change bacterial colonization. These results suggested that there could be a possible residual effect of the Bioseal for Concrete™ on *S.* Kentucky while this was not evident on the other four strains. In addition to the possible residual effects of Bioseal for Concrete™, significant differences ($p<0.05$) in the survival populations of *S.* Kentucky between treatment B and D indicate a cumulative bactericidal effect on the concrete blocks. These results indicate that Bioseal for Concrete™ does not necessarily need to be applied on newly built facilities thus being effective under existing conditions in processing plants.

3.4.2 – Internal brick surfaces

Analysis of variance (ANOVA) of the survival populations (log_{10} CFU/ sq. cm) of *Salmonella* spp. from the internal surfaces of concrete blocks varied greatly among strains (table 3). The detection level for this study was less than 5 CFU/ sq. cm (ca. 0.7 log_{10} CFU/ sq. cm). Following treatments C and D the survival populations (log_{10} CFU/

sq. cm) of *Salmonella* Typhimurium, *S.* Heidelberg and *S.* Enteritidis were below detection limit (< 0.7 $\log_{10}$ CFU/ sq. cm), whereas no recoverable populations of *S.* Seftenberg were observed as a result of treatment D. Due to the lack of recovery of any survival populations of *S.* Enteritidis and Seftenberg the data for these two strains is not shown in this paper. The lack of recoverable populations of these strains of *Salmonella* could be due to the low initial levels of inoculum on the interior surface of the concrete blocks. The survival populations ($\log_{10}$ CFU/ sq. cm) of *S.* Heidelberg and *S.* Typhimurium were significantly lower ($p<0.05$) when comparing treatment A to treatments C and D, but the actual levels of reductions could not be enumerated due to the low initial counts of bacteria on the interior surfaces. This indicates that although Bioseal for Concrete™ does show antimicrobial characteristics the true extent of this could not be well evaluated because of the lack of penetration of *Salmonella* into the concrete blocks. No significant differences ($p>0.05$) were observed in the survival populations of all the strains of *Salmonella* when comparing treatment B to groups C and D. The lack of any antimicrobial effects on the internal surface of the concrete blocks could be attributed either to the inability of Bioseal for Concrete™ to penetrate into the concrete pores or due to the lower populations of viable *Salmonella* on the internal surfaces of cement blocks to evaluate the magnitude of reduction. *S.* Kentucky was the only strain which presented significant difference ($p<0.05$) between treatment A and all other treatments. The significantly lower ($p<0.05$) populations of *S.* Kentucky as a result of the application of Bioseal for Concrete™ (treatment C and D) suggest that this strain is probably more susceptible than the other strains tested even in the presence of lower concentrations of the product. The lack of antimicrobial activity on the interior of the

concrete blocks could also have been due to the slow migration rate of Bioseal for Concrete™ from the exterior to the inside of the blocks.

In this study the effectiveness of Bioseal for Concrete™ to reduce populations of different strains of *Salmonella* from the external surfaces of concrete blocks ranged from 3.06 to 4.22 $\log_{10}$ CFU/ sq. cm. This is comparable to other studies where quaternary ammonium compounds were shown to be effective against *Salmonella* attachment on stainless steel (type 304), rubber (type 7S15) and plastic (polytetrafluorethylene) (Sinde and Carballo, 2000). Results from the present study showing effectiveness in reducing *Salmonella* populations on the external and internal surfaces of concrete blocks are in concurrence with results reported by Joseph et al. (2001) and Ramesh et al. (2002). Joseph et al. (2001) in their research reported complete inactivation of *Salmonella* biofilm cells (6 log reduction) on cement after iodophor (50 ppm – 25 min) exposure. Ramesh et al. (2002) evaluated different groups of sanitizers in reducing viable counts of *Salmonella* cells from biofilms attached on galvanized steel and found averages of 7.63-log reduction for chlorinated compounds (500 ppm – 2 minutes) in the absence of organic matter, however, reported only a 2 log reduction when organic matter was present. In the same study, iodine compounds evaluated resulted in an average of 7.3-log reduction in the absence of organic matter and a 2.14-log reduction in the presence of organic matter. Leyer and Johnson (1997) reported a 5-log reduction of an original 7-log population when using iodine as a sanitizer in planktonic cells of *S.* Typhimurium. The variation in the reduction levels of different strains of *Salmonella* to Bioseal for Concrete™ is an indication of differences in the susceptibility of the different strains as suggested by Joseph et al. (2001). In their study Joseph et al. (2001) found great variation among the

Salmonella cultures susceptibility to hypochorites. At identical concentrations (100 ppm) the researchers reported a 5 log reduction for *Salmonella weltevreden* after cement was exposed to hypochlorite for 15 minutes whereas no cells were detected for *Salmonella* FCM 40. Korber et al. (1997), when evaluating the susceptibility of *Salmonella* Enteritidis to disinfectants in glass biofilms, found that 10% trisodium phosphate was able to inactivate all the cells from *Salmonella* biofilms after 15 seconds while Wang et al. (1997) reported a 2-log reduction of *Salmonella* Typhimurium using same concentrations of trisodium phosphate on chicken skin.

Peroxygens are another group of disinfectants largely used in the food industry and have been reported to reduce *Salmonella* Typhimurium biofilms by 2.96 log on cement by fogging the room (Dunowska et al., 2005). Results from our study indicating up to 4.22 $\log_{10}$ CFU/ sq. cm reduction suggest a higher effectiveness of BioSealed for Concrete™ on *Salmonella* when comparing it to results from the study conducted by Dunowska et al. (2005). It is well documented that acid adapted bacteria are more resistant to antimicrobials than non-adapted cells (Leyer and Johnson, 1997 and 1993). Variation in the results from multiple studies that have evaluated various disinfectants and multiple strains of *Salmonella* indicate that the effectiveness depends on several factors including the type of surface, contact time of the disinfectants with the surface, concentration of the disinfectant, temperature, and the type of strains of the pathogen. From this point of view further studies need to be conducted to establish application parameters to clarify the antimicrobial properties of BioSealed for Concrete™ against biofilms.

3.5 -- Conclusion

BioSealed for Concrete™ proved to be a potent antimicrobial with immediate bactericidal effects and facilitating biofilm removal. Results from this study indicate that BioSealed for Concrete™ can be used as an alternative in food processing plants which have persistent and recurrent *Salmonella* spp. biofilm issues. Although antimicrobial capabilities of BioSealed for Concrete™ are shown in this study, its use should not be substituted for good manufacturing practices and/ or efficient cleaning and sanitizing procedures. In sight of the current industry efforts to control biofilms in the poultry processing environment and results from this study demonstrating bactericidal effects of BioSealed for Concrete™, further research needs to be conducted to determine the mode of action, concentration, and time of contact of this concrete sealant to be effective against bacterial biofilms.

3.6 -- References

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Table 1: Behavior of *Salmonella* inoculums (log_{10} CFU/ml) throughout the trial

Strains	Typhimurium	Heidelberg	Enteritidis	Seftenberg	Kentucky
Inoculum A[1]	9.64	9.87	9.28	9.37	11.51
Inoculum B	10.07	10.40	9.43	9.94	9.98
Inoculum C	8.81	9.58	8.99	9.32	9.92
Inoculum D	8.53	8.75	8.15	8.77	10.24

[1]A = Initial inoculums before introducing cement blocks; B = Inoculums at removal time without any cement blocks; C = Inoculums at removal time containing cement blocks with no BioSealed for Concrete™; D = Inoculums at removal time containing cement blocks with BioSealed for Concrete™.

Table 2: Survival populations (Log_{10} CFU/ sq. cm) of *Salmonella* strains on the external surfaces of brick blocks

Treatments	Strains				
	Typhimurium	Heidelberg	Enteritidis	Seftenberg	Kentucky
A[1]	4.45[a]	5.49[a]	4.69[a]	3.86[a]	5.22[a]
B	4.29[a]	4.09[a]	5.02[a]	4.29[a]	3.43[a,b]
C	0.67[b]	1.27[b]	1.73[b]	0.68[b]	1.66[b,c]
D	1.39[b]	0.64[b]	1.25[b]	0.80[b]	1.03[c]
P Value	0.0220	0.0077	0.0347	0.0286	0.0196

[1] A = No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation

Superscripts(a, b and z) indicate significant difference ($p<0.05$) within a column.

Table 3: Survival populations (Log_{10} CFU/ sq. cm) of *Salmonella* strains on the internal surfaces of brick blocks

Treatments	Strains		
	***S.* Typhimurium**	***S.* Heidelberg**	***S.* Kentucky**
A[1]	1.20[a]	0.96[a]	1.56[a]
B	0.79[ab]	0.70[ab]	0.70[b]
C	ND[2 b]	ND[b]	ND[b]
D	ND[b]	ND[b]	ND[b]
P value	0.0923	0.1363	0.0685

[1] A = No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation

[2] ND = Non detectable; detection limit is less than $\log_{10}$ 0.69 CFU / cm^2

Superscripts(a and b) indicate significant difference ($p<0.05$) within a column.

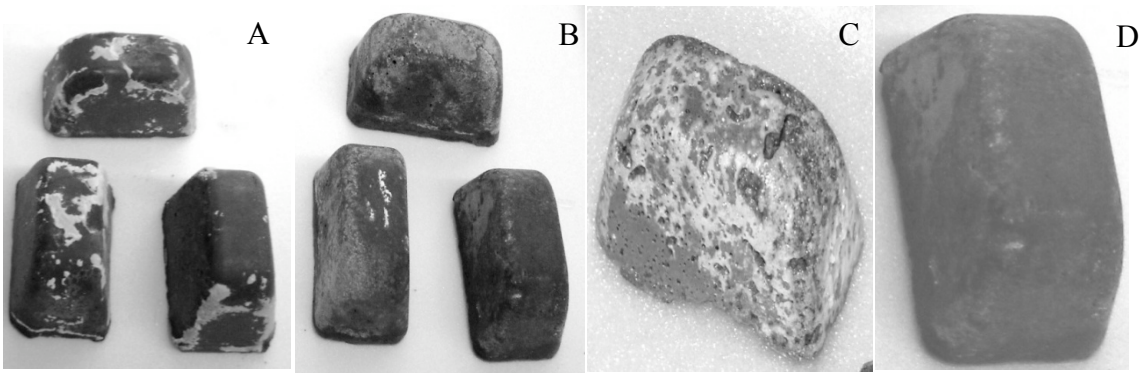

Figure 1: Bricks with different treatments after 24 h of incubation at 39 °C. A: Bricks not treated with BioSealed for Concrete™ .B: Bricks treated with BioSealed for Concrete™ before inoculation. C: Bricks treated with BioSealed for Concrete™ after inoculation. D: Bricks treated with BioSealed for Concrete™ before and after inoculation.

4 – EFFICACY OF BIOSEALED FOR CONCRETE™ AGAINST SURVIVAL OF *LISTERIA* SPECIES ON CONCRETE SURFACES

(Submitted to Journal of Food Protection for Publication, May 2009)

4.1 -- Abstract

Listeria monocytogenes is an important foodborne pathogen isolated from food processing facilities and implicated in numerous foodborne. In this study, antimicrobial efficiency of BioSealed for Concrete™ to prevent attachment and colonization of *Listeria monocytogenes* (3C and Scott A) and *L. innocua* on concrete was evaluated. Concrete blocks were divided into four different treatments: A) No Biosealed, B) Biosealed prior to inoculation, C) Biosealed after inoculation, or D) Biosealed before and after inoculation. Cultures were prepared by inoculating *Listeria* strains into brain and heart infusion broth (BHI) and incubating at 35 °C for 24 h. Cement blocks were inoculated by submerging in BHI broths containing one of the three *Listeria* strains (ca. 10^9 CFU/ ml) and incubated at 35 °C for 24 h. External surfaces of inoculated blocks were sampled using sterile swabs and placed in 10 mL peptone water (PW). Cement blocks were broken in half and interior surfaces were swabbed to determine *Listeria* spp. levels. Following 24 h of incubation on Modified Oxford agar plates (MOX) at 35 °C, surviving populations of *Listeria* on external and internal surfaces were enumerated. Significantly lower (P<0.05) populations of all three strains were observed for treatments C and D when compared to treatment A on the external surface of cement blocks, whereas, the

survival populations on the internal surfaces varied for all three serotypes. Results show that BioSealed for Concrete™ is a potent antimicrobial and has potential to be used in combination with other sanitation procedures to control bacterial colonization on concrete surfaces.

4.2 – Introduction

Listeria monocytogenes has emerged as one of the most important and deadly foodborne pathogens, resulting in a high rate of hospitalization (88%) and the highest fatality rate (20%) of all foodborne illnesses (3, 8, 9). *L. monocytogenes* is especially pathogenic to high risk populations including newborns, pregnant women, the elderly, and people with weakened immune systems. *Listeria* spp. are Gram positive, non-sporeforming, facultative anaerobic rods that are motile when cultured at 20 - 25 °C (14, 16, 18). While the optimal growth temperatures are between 30 and 37 °C, *Listeria* are capable of growth over a temperature range of – 0.4 and 50 °C, making the organism a potential food safety concern in refrigerated foods (15). Listeriae grow best in the pH range 6-8; however, they will grow in a wide range of pH (4.1 – 9.6) (14). Because *Listeria* can also grow at low temperatures, it is classified as a psychrotroph and studies show that *Listeria* cells grown at low temperatures were able to induce the production of exopolysaccharides (30). *L. innocua* and *L. monocytogenes* are very close related genetically, making *L. innocua* of special interest to food microbiologists since it is frequently used as a surrogate to *L. monocytogenes*. According to Jay (14), *L. innocua* is represented by only 2 serovars; it is considered to be a nonpathogenic variant of *L. monocytogenes* and their nutritional requirements are typical of other Gram-positive

bacteria. The food hazards posed by *L. monocytogenes* are especially due to its ability to grow over a broad temperature range, a characteristic based on the ability to modify its membrane composition in order to maintain membrane fluidity (1, 21).

Although initial outbreaks of listeriosis were linked to consumption of coleslaw, raw vegetables, milk and Mexican-style cheese, the consumption of undercooked chicken and uncooked frankfurters has been strongly linked epidemiologically to an increased risk of listeriosis (34). It is well known that a great variety of fresh food products of animal and plant origin may contain *L. monocytogenes* (1, 14): this organism has been isolated from many different foods such as milk and dairy products, fresh and frozen meat – including beef, pork, poultry and seafood, fruits and vegetables (16, 31). Contamination by *L. monocytogenes* in raw chicken is high in comparison with other foods, which is a potential health hazard because of the chances of cross contamination to other foods in the home and the possibility of the microorganism surviving in processed chicken (2, 11, 31). In addition, raw chicken must be considered as one of the many sources of contamination to further processing facilities as the presence of *Listeria* spp. on incoming raw meat ingredients can work as a continuous source of contamination for other processing plants (11, 12). There are several opportunities for this organism to spread and colonize in the industrial environment following its entrance in a processing and further processing poultry facility. Floors and drains have been implicated as the primary source of *L. monocytogenes* in poultry processing plants (9, 21, 22) and the persistence of this microorganism in the hostile processing environment is enhanced by their ability to attach to surfaces and form biofilms. This unique survival property makes the presence of *Listeria* difficult to control and remove from processing environments

(23). Because of the ubiquitous nature of the organism, it is important in a food processing operation to prevent contamination from *L. monocytogenes* and prevent recontamination of foods that are ready-to-eat (RTE) finished products. The hardy nature of *L. monocytogenes* coupled with its ability to proliferate in cold, wet environments is ideal for biofilm formation (10, 14).

Poultry products have been widely implicated in *L. monocytogenes* outbreaks. The United States Department of Agriculture (36) has dealt with numerous recalls and outbreaks that resulted from *L. monocytogenes* contamination. Over a 21-month period in the United States, 19.3% of 3,700 raw broiler carcasses were tested positive to *L. monocytogenes* (14). Several studies have demonstrated that the processing environment is a major source of *Listeria* contamination of raw and cooked poultry products. These studies indicate that the incidence of *Listeria* within the product increases from the beginning to the end of processing, indicating cross contamination from the environment (22). When contamination of poultry occurs after cooking, there is a high probability that this product will carry this pathogen to the market place reaching the consumer. Research has shown that *Listeria* enters commercially processed foods as post-processing contaminants (9). The United States government has designated the presence of *L. monocytogenes* as an adulterant. This means that any RTE food that contains this organism will be considered adulterated, and, thus be subjected to recall and/or seizure (14, 31).

Biofilms have developed into a significant issue for public health because bacteria within them are less susceptible to antimicrobial agents and treatments (20, 33). Bacterial biofilms are microbially derived sessile communities characterized by cells that are

attached to a substratum, to an interface, or to each other (33). Biofilms formed in food processing environments are of significant importance as they have the potential to act as a chronic source of microbial contamination that can eventually lead to food spoilage or transmission of diseases (35). Biofilms lead to serious hygienic problems and huge economic losses due to food contamination and recalls. Biofilms are self-regulating and as they age individual cells or parts of the biofilm may release or detach from the primary microbial community. The periodic detachment of relatively large particles of biomass from the biofilm (sloughing) and individual cell release leads to contamination and colonization of new substrates (12, 20, 27).

Control of biofilms represents one of the most persistent challenges within food and industrial environments where these microbial communities are problematic (20, 33). The essential aspects in controlling biofilm formation and minimizing the biotransfer potential on the food processing equipment and environments include proper cleaning and sanitation procedures. Regular sanitation programs typically consist of removal of gross debris, rinsing, presoaking in detergent, rinsing, disinfection and final rinsing. Bacterial cells are more resistant to environmental stresses such as nutritional deprivation and oxidative stress when in a biofilm environment. In addition to this, cells within a biofilm are more resistant to antimicrobial agents than free cells (19, 32, 33). So far, very few studies have reported specific reasons for this resistance; however there are two hypotheses that are widely accepted by the scientific community. The first hypothesis refers to the slow or incomplete penetration of antimicrobial substances into the biofilm environment. This retarded penetration is probably due to the polysaccharide matrix that serves as a shield to the cells, preventing the antimicrobial substances from coming into

contact with the bacterial cells. The second hypothesis suggests the occurrence of an altered microenvironment within the biofilm, which modifies the properties of the bacteria cells or the antimicrobial itself (19, 33). In addition, it has been reported that bacteria within biofilms have reduced metabolic activity mainly due to reduced nutrient and oxygen supply and it is this reduction in metabolic activity that has been correlated to resistance (12, 32). Since removal of biofilms is a very difficult and demanding task, current sanitation programs are usually not sufficient to remove biofilms. Thus, the food industry has been looking for cost efficient cleaning and sanitation alternatives to facilitate biofilm removal and to prevent new biofilm formation on inert surfaces. These new strategies usually include physical and chemical methods which interfere with bacterial attachment, colonization and biofilm development.Most of the preceding research on the attachment of *Listeria* to inert surfaces has been done on a few classic materials such as stainless steel and rubber (1). But it has been reported that several other materials present in poultry processing plants, such as door handles, floors, squeegees, gloves and drains, can harbor *Listeria* (22, 23). Concrete is a major component of the building material in a food processing plant and due to the high volumes of foods being processed on a daily basis, floors, ceilings, and drains receive a great amount of organic matter. Organic matter in the poultry industry is a result of usual processing steps such as bleeding, scalding, eviscerating and feather-picking, and this organic matter has the potential to serve as an initial source of nutrients to microorganisms allowing them to colonize on and / or in concrete. Concrete is a microporous, microstructure-sensitive construction material, and the pores in concrete are randomly sized, arranged, and connected forming a capillary system that allows water and other substances to traffic

freely (37). These free flowing liquids in concrete can serve as carriers of microorganisms that can be a source of biofilm formation and cross-contamination onto food products.

To understand and interpret the behavior of a composite element such as concrete, knowledge of the characteristics of its components is necessary. Concrete is produced by the collective mechanical and chemical interaction of a large number of constituent materials. Disintegration of concrete due to cycles of wetting, freezing, thawing, drying, chemicals and the propagation of the resulting cracks is of great importance for the food industry as it can serve as an attachment site for bacteria to form niches (28). These niches have the potential to be permanent sources of direct and/ or indirect contamination during food processing due to water splashing at the time of sanitation, staff shoes, and clothes. Therefore, the food industry has placed a great deal of effort on the reduction of niches and trying to avoid the formation of biofilms. There is no single action which will reduce or eliminate biofilms from industrial environments. Several actions must be taken collectively to prevent the formation of biofilms and eliminate these chronic sources of contamination. The objective of this study is to determine the efficiency of BioSealed for Concrete™ (GreenSealed Solutions, Inc. – Georgia) to prevent *Listeria spp.* attachment on concrete surfaces.

4.3 – MATERIALS AND METHODS

4.3.1 – Bacterial Cultures

In this study three different serotypes of *Listeria* spp. were used: *Listeria monocytogenes* 3C, *Listeria monocytogenes* Scott A and *Listeria innocua*. They were all

independently cultured in Brain-Heart Infusion (BHI; Acumedia Manufacturers, Inc., Lansing, MI) broth and incubated at 35 ˚C for 24 h prior to being used to challenge the concrete bricks. The length of incubation and inoculation of the bacterial cultures were based on 24 h growth curves that were performed in the laboratory (data not shown). At time of inoculation the average count of inoculums varied according to the strain (table 1).

4.3.2 – Concrete Bricks Preparation

Quikrete® (Quickrete – Georgia) powder mix was used to produce concrete bricks in commercial sized ice cube trays, as per manufacturer's directions. Ice cube sized bricks were made to simulate commercial concrete blocks for experimental purposes in the laboratory.

4.3.3 – Sampling

Concrete bricks were divided into four groups: (A) Bricks which were not treated with BioSealed for Concrete™ (control), (B) Bricks treated with BioSealed for Concrete™ before inoculation, (C) Bricks treated with BioSealed for Concrete™ after inoculation, and (D) Bricks treated with BioSealed for Concrete™ before and after inoculation. The inocula were split in two equal parts: (1) The first half were considered the control inoculums and contained bricks from groups A and C (bricks untreated before inoculation) and (2) The second half of the inocula were named the "treated" inocula which contained bricks from groups B and D (bricks which were treated before inoculation). Following manufacturer's directions, BioSealed for Concrete™ was sprayed on the surface of the bricks using a paint spray (Wagner 5.4 GPH, Wagner Spray Tech Corporation, Plymouth, MN). Samples of the inocula were spread plated onto Modified

Oxford (MOX) agar (Neogen, Lansing, MI) for enumeration at inoculation time and time of removal of the samples.

Bricks were submerged in the inocula for 24 h at 35 °C. After 24 h the bricks were removed from the inocula and held for 30 min. in a sterile laminar flow cabinet (Nuaire Inc., Plymouth, MN) to allow drying of excess inoculum from the brick surface. Sterile swabs were then used to sample the entire surface of each brick. The swabs were then placed in 10 mL sterile peptone water (Acumedia Manufacturers Inc., Lancing, MI) tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes. After swabbing the surface, bricks were broken in half and the inner surfaces of both halves were swabbed. The swab was then placed in 10 mL autoclaved peptone water tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes. All samples were spread plated onto MOX agar. Plates were incubated for 24 h at 35 °C and results were recorded as $\log_{10}$ CFU/ sq. cm with the exception of the inoculum samples, which were recorded as $\log_{10}$ CFU/ mL.

4.3.4 – Statistical Analysis

Completely randomized design was used to assign concrete blocks to the four treatment groups. Three replications of this experiment were performed and averages of the survival populations ($\log_{10}$ CFU/ sq. cm) of various serotypes of *Listeria* spp. were analyzed using analysis of variance (ANOVA) with SAS PROC GLM procedures (2002-03 SAS 9.1 Institute, Gary, NC). Statistical significance was reported at a *P*-value of less than or equal to 0.05 ($P \leq 0.05$).

4.4 – Results and Discussion

4.4.1 – External brick surfaces

Analysis of Variance (ANOVA) of survival populations of *L. monocytogenes* serotype 3C (log_{10} CFU/ sq. cm) did not show any significant difference ($P>0.05$) following treatments A and B (Table 2). Treatment C (ca. 1.7 log_{10} CFU/ sq. cm) and D (ca. 1.52 log_{10} CFU/ sq. cm) resulted in significantly lower ($P<0.05$) survival populations of *L. monocytogenes* serotype 3C when compared to treatment A, whereas, results show no significant difference ($P>0.05$) between treatments B, C and D (Table 2). No significant ($P>0.05$) reduction in the populations of *L. monocytogenes* serotype 3C when comparing treatments A and B suggest the lack of a possible residual effect of BioSealed for Concrete™ . Results from the analysis of survival populations of *L. monocytogenes* serotype 3C on the external surface of brick surfaces indicate a listericidal effect of BioSealed for Concrete™. Since treatment C was applied after the bricks were challenged with different strains of the pathogen and significant reduction ($P<0.05$) was observed when comparing it to group A, these results also suggest that the product does not have to be applied on newly built facilities thus being effective under existing conditions in processing plants. However, as no significant difference ($P>0.05$) was found when comparing treatments A and B there is no suggestion that the product prevents new bacterial attachment.

Similar to *L. monocytogenes* 3C, BioSealed for Concrete™ was an effective antimicrobial against *L. monocytogenes* Scott A (Table 2). A 1-log reduction was observed when comparing treatments A and C, and a 0.82 log_{10} CFU/sq. cm reduction was observed when comparing treatments B and D. Throughout the *L. monocytogenes* Scott A study, treatments A and B showed similar results to *L. monocytogenes* 3C and no significant difference ($P>0.05$) was observed between these two groups (A and B). In

addition, because survival populations (log_{10} CFU/ sq. cm) between treatments B and C were not significantly different ($P>0.05$), it suggests that treating concrete with the product prior to or post bacterial contamination does not change bacterial colonization behavior. Also, significant differences ($P<0.05$) in the survival populations of *L. monocytogenes* Scott A between treatments B and D indicate a cumulative bactericidal effect on the concrete blocks.

In this study, *Listeria innocua* was the most susceptible *Listeria* serotype tested. Treatments B, C and D had significantly lower ($P<0.05$) viable counts than the control group. A reduction of 0.7 log_{10} CFU/ sq. cm., 1.26 log_{10} CFU/ sq. cm. and 1.4 log_{10} CFU/ sq. cm. was observed when comparing treatment A to treatments B, C and D, respectively (Table 2). Once again, these results indicate the bactericidal effects of BioSealed for Concrete™ and the possible residual effect caused by this product. Unlike the two other serotypes tested in this study, groups A and B were significantly different ($p<0.05$) from each other. These results indicate that the product has the ability to prevent bacterial attachment, even though in small proportions.

4.4.2 – Internal brick surfaces

ANOVA of the survival populations (log_{10} CFU/ sq. cm) of *L. monocytogenes* 3C for the internal surfaces of concrete blocks did not show any significant difference ($P>0.05$) following treatments A, B and C, and treatments B, C and D were also not significantly different ($P>0.05$; Table 3). *L. monocytogenes* Scott A had similar results to *L. monocytogenes* 3C. *L. innocua* was the most susceptible serotype tested. Significant reduction ($p<0.05$) can be observed when comparing all treated groups (B, C and D) to the control group (A) (Table 3). This lack of a significant difference found for *L.*

monocytogenes 3C and *L. monocytogenes* Scott A cannot be attributed to the inability of the product to penetrate deep into concrete pores. If the product was unable to penetrate into concrete pores, no significant difference would have been found in the *L. innocua* trial, either. Therefore, what these results suggest is that a secondary reaction between the product and the concrete occurred, reducing its bactericidal effect. That would justify why *L. innocua* was the only susceptible strain tested even in the presence of lower active concentrations of the product. It is also observed in this study that the product does not seal concrete pores as high numbers of *Listeria* spp. were recovered from the internal surfaces of concrete blocks (Table 3).

Biofilm contents and architectures are highly heterogeneous and variable, suggesting that bacterial phenotypes and physiology are also variable. They depend not only on the bacterial strains that form the biofilm but also on the surface material and on the growth and environmental conditions (17, 33). In this study the antimicrobial characteristics of BioSealed for Concrete^{TM} were evaluated in addition to its ability to prevent biofilm formation. Treatment A was our control group and served as a base of comparison to all other treatments. Treatment B was applied to evaluate the residual effects of the product and if the product was able to prevent biofilms formation, whereas Treatments C and D simulated situations of most present day food processing plants i.e. never had this type of product applied before. Treatment D also allowed us to determine if previous treatment would have any additional or different effect from first time applications of this product.

In this study, as evident from Table 2, great variability concerning antimicrobial susceptibility can be observed among the different *Listeria* serotypes. This variability has

been reported in previous studies comparing biofilm formation behavior and serotype susceptibility to antimicrobials (3, 4). Before comparing BioSealed for Concrete™ with other sanitizers available in the market, it is important to consider three points. The first is that most studies of *Listeria* susceptibility to antimicrobials are performed with individual serotypes of *Listeria* and this study is a good proof of this great variability among serotypes, thus conclusions about *Listeria* susceptibility to antimicrobials should be made individually and not extended to the whole genus. The second point is that the efficiency of a certain antimicrobials depends on several factors, including length of contact, concentration of the antimicrobial and temperature. Therefore, it is difficult to compare antimicrobials without considering these factors. The third consideration is that the culture state of the cells is very important to evaluate its resistance to antimicrobials. It is well known that acid adapted bacteria are more resistant to antimicrobials in general than non-adapted cells (24, 25). All of these factors must be taken into consideration before comparing results from different studies.

The major types of sanitizers used in the food industry are halogens, peroxygens, acids, and quaternary ammonium compounds (5, 13, 15). Bower and Daeschel (5) reported that surfaces with adsorbed antimicrobial were found to decrease the incidence of surface contamination by *L. monocytogenes* on model food contact surfaces. Bracket (6) reported that 100 ppm of chlorine for 20 minutes was necessary to completely inactivate 8 logs of planktonic cells of *L. monocytogenes* Scott A. Byun et al. (7) reported that to completely inactivate a bacterial suspension of *L. innocua*, the suspension should be exposed to 200 ppm of chlorine for 30 seconds. And when the same culture was in a biofilm, the same concentration of chlorine for the same amount of time was

only able to cause a 2.4 log reduction when the cells were attached to polypropylene. Under the same conditions, a 1.2 log reduction and a 0.6 log reduction was observed by the same authors when the biofilm was attached to polyester and polycarbonate respectively (7). Norwood and Gilmour (29) reported that exposure to 1000 ppm free chlorine for 20 minutes was required to reduce *L. monocytogenes* biofilm attached to stainless steel by a two-log cycle; however, planktonic cells were eliminated by an exposure to 10 ppm free chlorine for 30 seconds. Chmielewski and Frank (10) also reported that peroxide-based sanitizers are found to be more effective against *L. monocytogenes* in a biofilm matrix that hypochlorite. However, they also reported that *L. innocua* showed resistance to peroxide sanitizers (5). Several authors have reported the low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds (20, 26). Research has demonstrated that high concentrations of quaternary ammonium compounds for a long period of time are required to inactivate *L. monocytogenes* biofilms. Chmielewski and Frank (10) stated that on previous research 400 ppm of quaternary ammonium compounds for 5 minutes of contact time was required to inactivate *L. monocytogenes* biofilm on chitin, while on other studies even when the biofilm was exposed to 800 ppm of quaternary ammonium compounds for 20 minutes it was still not completely inactivated. Therefore, BioSealed for Concrete™ has showed to be as efficient as the antimicrobials regularly used in processing plants daily sanitation operations.

4.5 -- Conclusion

BioSealed for Concrete™ proved to be a potent antimicrobial with immediate bactericidal effects that has potential to facilitate biofilm removal. Results from this study indicate that BioSealed for Concrete™ can be used as an alternative in food processing plants that have persistent and recurrent *Listeria* spp. biofilm issues. Although antimicrobial capabilities of BioSealed for Concrete™ are shown in this study, its use should not be substituted for good manufacturing practices and/ or efficient cleaning and sanitizing procedures. The factors required for biofilm formation in listeriae species are still unknown, and although some progress has been made in this area, understanding the full mechanism of *Listeria* colonization to inert surfaces is a key factor on the process of attempting to prevent its occurrence.

4.6 -- References

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Table 1: *Listeria* inoculum levels (log_{10} CFU/ml)

Treatments	*L. monocytogenes* 3C	*L. innocua*	*L.* Scott A
Inoculum A	10.27	10.41	11.00
Inoculum B	7.43	8.82	8.17
Inoculum C	8.87	9.19	10.10
Inoculum D	8.79	9.77	10.10

A= Initial inoculums before introducing cement blocks; B = Inoculums at removal time without any cement blocks; C = Inoculums at removal time containing cement blocks with no BioSealed for Concrete™; D = Inoculums at removal time containing cement blocks with BioSealed for Concrete™.

Table 2: Survival populations (Log_{10} CFU / sq. cm) of *Listeria* strains on the external surfaces of brick blocks

Treatments	Strains		
	***L. monocytogenes* 3C**	***L.* Scott A**	***L. innocua***
A	5.49^{a}	6.45^{a}	6.03^{a}
B	4.65^{ab}	6.07^{ab}	5.33^{b}
C	3.79^{b}	5.45^{bc}	4.77^{bc}
D	3.97^{b}	5.25^{c}	4.63^{c}
***P*-value**	0.0089	0.0272	0.0210

A= No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation. Superscripts (a, b and c) indicate significant difference ($P<0.05$) within a column.

Table 3: Survival populations (Log_{10} CFU / sq. cm) of *Listeria* strains on the internal surfaces of brick blocks

Treatments	Strains		
	***L. monocytogenes* 3C**	***L.* Scott A**	***L. innocua***
A	2.64^{a}	3.95^{a}	2.85^{a}
B	2.28^{ab}	3.18^{ab}	1.92^{b}
C	1.82^{ab}	2.72^{bc}	1.69^{b}
D	1.40^{b}	2.01^{c}	1.47^{b}
***P*-value**	0.0188	0.0183	0.0188

A= No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation. Superscripts (a, b and c) indicate significant difference ($p<0.05$) within a column.

5 – SPOREFORMERS SUSCEPTIBILITY TO TOPICAL SEALANT ON CONCRETE SURFACES

(A Paper to be Submitted to Journal of Applied Microbiology for Publication)

5.1 – Abstract

Clostridium and *Bacillus spp.* can survive traditional sanitation and disinfection procedures due to their spore forming ability. In this study, the antimicrobial efficiency of BioSealed for Concrete™ to prevent bacterial attachment and colonization of *C. perfringens* and *B. subtilis* on concrete cement blocks was evaluated. Cement blocks were divided into four different treatments: A) No Biosealed application, B) Biosealed applied before inoculation, C) Biosealed applied after inoculation, or D) Biosealed applied before and after inoculation. The cultures were prepared by inoculating *C. perfringens* and *B. subtilis* into brain and heart infusion broth (BHI) and incubating at 37 °C for 24 h (*C. perfringens* was incubated in anaerobic chamber). Cement blocks were inoculated by immersion in BHI containing one of the cultures and incubated at 37 °C for 24 h (ca. 10^9 CFU/ ml). External surfaces of the blocks were swabbed using sterile swabs and placed in 10 mL 0.1% peptone water. The blocks were then broken in half and interior surfaces were swabbed to determine viable counts. Samples were serially diluted and spread plated on TSC (*C. perfringens*) and MYP (*B. subtilis*) agar and incubated for 24 h at 37 °C. Experiments were performed in triplicates and results were analyzed using SAS. On the external surface of the blocks, significantly lower (P<0.05) populations of both

microorganisms were observed for treatments C and D when compared to treatments A and B. When comparing treatments A and C; as well as groups B and D; the product was shown to be efficient for biofilm removal on concrete surfaces. No significant difference (P>0.05) was found when comparing groups A and B suggesting that the product has poor residual effect. Results from this study indicated that Biosealed for Concrete™ is a potent antimicrobial and has the potential to be used in combination with other GMP's and other sanitation practices to control bacterial colonization on concrete surfaces.

5.2 -- Introduction

It is well known that spores (consequently sporeformers) are much more resistant than cells to a variety of chemical compounds and physical treatments, including cross-linking agents such as glutaraldehyde, oxidizing agents, phenols, formaldehyde, chloroform, octanol, alkylatin agents including ethylene oxide, iodine, and detergents, as well as to pH and temperature extremes, and lytic enzymes such as lysozyme (Slepecky and Hemphill 1992; Setlow and Johnson 2001). The spore metabolic dormancy is undoubtedly one factor in its ability to survive extremely long periods in the absence of nutrients and hostile environments. Despite the spore's extreme dormancy, if given the appropriate stimulus the spore can rapidly return to life via spore germination causing serious problems to the food industry due to shelf life reduction and contamination of foods by pathogens (Setlow and Johnson 2001).

The two bacteria used in this study were *Bacillus subtilis* and *Clostridium perfringens*. The first one, *B. subtilis,* is neither an important pathogen nor an important agent of poultry spoilage. However its natural transformability, as well as an abundance

of molecular biological and genetic information has made *B. subtilis* the organism of choice for mechanistic studies on sporulation, spore germination and spore resistance (Setlow and Johnson 2001). In addition, several researchers to study the physiopathology, behavior, sporulation and resistance of *B. anthracis* have been using *B. subtilis* as a surrogate since its low pathogenic profile.

Clostridium perfringens is an important pathogen that causes a wide variety of human and veterinary diseases. *C. perfringens* has two main characteristics that contribute to its ability to cause foodborne disease. First, its low generation time (reportedly <10 minutes for vegetative cells) allows *C. perfringens* to quickly multiply in food (McClane 2001; Setlow and Johnson 2001). Secondly, in addition to its ability to survive in incompletely cooked foods due to their relative heat tolerance of its vegetative cells, *C. perfringens* has the ability to form spores which are resistant to environmental stresses such as radiation, desiccation, and heat; the ability to form such resistant spores facilitates of survival of *C. perfringens* in incompletely cooked or inadequately warmed foods (McClane 2001).

Bacterial biofilms are defined as microbially derived sessile communities characterized by cells, often of different species, that are attached to a substratum, to an interface, or to each other (Ofek et al. 2003; Costerton 2004). At this point, however, it is not clear to what extent biofilms at these different interfaces share metabolic or physiological traits (Costerton 2004). Biofilm cells are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription (Scher et al. 2005). Nutrient rich conditions are often found in food processing plants due to residues of proteins and fats being deposited on

surfaces which allow colonization and maintenance of microbial populations within the food industry (Lindsay et al. 2006).

A biofilm is a dynamic microenvironment, such that the microorganisms divide or are released and shed into the medium and its organic constituents are turned over (Ofek et al. 2003). Biofilms are self-regulating, and as they age, individual cells or parts of the biofilm may release or detach from the primary microbial community. The detachment process is an important component of the biofilm life cycle and plays a fundamental role in dissemination and contamination and ultimately long term survival in either natural or artificial settings (Purevdorj-Gage and Stoodley 2004).

Several authors have reported that bacterial cells are more resistant to environmental stresses such as nutritional deprivation and oxidative stress when in a biofilm environment. In addition, when in a biofilm, the cells are more resistant to antimicrobial agents and antibiotics than free cells (Scher et al. 2005; Kim and Wei 2007; Sandasi et al. 2008).

Concrete is present in the food industry especially in flooring, walls and ceilings. During processing, concrete receives a great amount of organic matter. The organic matter in the poultry industry is a result of usual processing steps such as bleeding, scalding, eviscerating and feather-picking. This organic matter has the potential to serve as an initial source of nutrients to microorganisms allowing them to colonize on and / or in concrete. Concrete is a microporous, microstructure-sensitive construction material, and the pores in concrete are randomly sized, arranged, and connected (Yang et al. 2004). These pores form capillary systems in concrete allowing water and other substances to traffic freely in concrete structures. When liquids flow freely on concrete, they may serve

as carriers in the transport of microorganisms such as bacteria. To understand and interpret the behavior of composite element such as concrete, knowledge of the characteristics of its components is necessary. Concrete is produced by the collective mechanical and chemical interaction of a large number of constituent materials. Disintegration of concrete due to cycles of wetting, freezing, thawing, drying, chemicals and the propagation of the resulting cracks is a matter of great importance for the food industry (Nawy 1996). The disintegration of concrete will serve as great attachments sites for bacteria to form niches. These niches will work as permanent sources of contamination for whatever food is being processed. Contamination of food may occur from direct contact of food to concrete surfaces or indirect contact (water splashes during sanitation, staff shoes and clothes).Biofilms have developed into a significant issue for public health as they are less susceptible to antimicrobial agents and treatments (Scher et al. 2005). Inorganic interfaces are rapidly colonized by microorganisms sometimes posing serious problems for the industry and hygiene in general (Brisou 1995).

For this study BioSealed for Concrete^{TM} was used which is a product manufactured and commercialized by GreenSealed Solutions, Inc. This is product is a hydrosilicate catalyst in a colloidal liquid base with an extremely alkaline pH (12), odorless appearing white/opaque. BioSealed for Concrete^{TM} is recommended to be applied using spray apparatus and, no special ventilation conditions nor breathing equipment are required. The main difference between BioSealed for Concrete^{TM} from other typical topical sealants is that BioSealed for Concrete^{TM} couples an antimicrobial with a gel barrier to eliminate pathogens. In addition, its deeper penetration into concrete (7 inches against 3 inches from typical topical sealants) closes down concrete capillary

system sealing out water, moisture, pathogens and contaminants. BioSealed for Concrete™ permanently stops water migration and halts contaminants ingress from any direction with the benefit of preventing alkali-silicate reactions to thereby reduce concrete disintegration and abrasion.

Biofilms formed in food processing environments are of significant importance because when viable cells are transferred to food, they could have a serious negative impact on the storage quality and safety of that food (Hood and Zottola 1995; Stepanović et al. 2004). As a consequence, biofilms lead to serious hygienic problems (presence of foodborne pathogens leading to foodborne illness) and economy losses due to food spoilage (shelf life reduction) and potential recalls (Lindsay et al. 2006). Therefore, the food industry has placed a great deal of effort on the reduction of niches and trying to avoid the formation of biofilms. There is no single action which will reduce or eliminate biofilms from industrial environments. Several actions must be taken collectively to prevent the formation of biofilms and eliminate these chronic sources of contamination. This study focuses on the attachment of sporeformers on concrete and its potential to form biofilms. The objective of this study is to determine the efficiency of BioSealed for Concrete™ (GreenSealed Solutions, Inc. – Georgia) as an antimicrobial and its ability to prevent biofilm formation by *B. subtilis* and *C. perfringens*.

5.3 – Materials and Methods

5.3.1 – Bacterial Cultures

Bacillus subtilis and *Clostridium perfringens* were independently cultured in Brain Heart Infusion (BHI) broth (Acumedia Manufacturers Inc., Lansing, MI) and incubated at 37 °C for 24 h (*C. perfringens* was incubated using anaerobic chambers)

before being used to challenge the concrete bricks. The length of incubation and inoculation of the bacterial cultures were based on 24 h growth curves that were performed in the laboratory (data not shown). At the time of inoculation the average count of the inocula varied based on the bacterium (table 1).

5.3.2 – Concrete Bricks Preparation

Quikrete® (Quickrete, Georgia) powder mix was used to produce concrete bricks in commercial sized ice cube trays, as per manufacturer's directions. Miniature bricks were made in ice cube trays in order to simulate concrete blocks to conduct laboratory experiments.

5.3.3 – Sampling

Concrete bricks were divided into four groups. (A) Bricks which were not treated with BioSealed for Concrete™, (B) Bricks which were treated with BioSealed for Concrete™ before inoculation, (C) Bricks which were treated with BioSealed for Concrete™ after inoculation, and (D) Bricks which were treated with BioSealed for Concrete™ before and after inoculation. The inocula were split in two equal parts: (1) The first half were considered the control and contained bricks from groups A and C (bricks untreated before inoculation) and (2) the second half of the inoculum was named the "inocula treated" which contained bricks from groups B and D (bricks which were treated before inoculation). Following manufacturer's directions, BioSealed for Concrete™ was sprayed on the surface of the bricks using a paint spray (Wagner 5.4 GPH, Wagner Spray Tech Corporation, Plymouth, MN). Samples of *C. perfringens* inocula were spread plated onto tryptose sulfite cycloserine (TSC) agar (Oxoid Ltd., Hampshire, England) for enumeration at inoculation time and at the time of removal of

the samples. TSC plates were then incubated anaerobically at 37 °C for 48 h. Samples of *B. subtilis* inocula were also spread plated for enumeration at inoculation time and at the time of removal of the samples onto mannitol-egg-yolk-polymyxin (MYP) agar (Becton, Dickinson and Company, Sparks, MD). MYP plates were then incubated at 37 °C for 24 h.

For the vegetative cells study, bricks were submerged in the inocula for 24 h at 37 °C, and once again *C. perfringens* cultures were incubated in anaerobic chambers. After 24 h the bricks were removed from inocula and held for 30 min. in a sterile laminar flow cabinet (Nuaire Inc., Plymouth, MN) to allow drying of excess inoculum from the brick surface. Swabs were then used to sample the entire surface of each brick. The swabs were then placed in 10 mL of sterile peptone water (Acumedia Manufacturers Inc., Lansing, MI) tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes. After swabbing the surface, bricks were broken in half and the inner surfaces of both halves were swabbed. The swab was then placed in 10 mL autoclaved peptone water tubes and vortexed for 30 seconds. Serial dilutions were made from these initial tubes and 0.1 mL of the sample was spread plated onto TSC agar (*C. perfringens*; incubated anaerobicaly) and MYP agar (*B. subtilis*). Plates were incubated for 24 h at 37 ˚C and the results were recorded as $\log_{10}$ CFU/ sq. cm with the exception of inoculum samples, which were recorded as $\log_{10}$ CFU/ mL.

To evaluate the spore resistance in this study, after the bricks stayed submerged in the inocula at 37 ˚C for 24 h, they were exposed to a temperature of 70 ˚C for 15 minutes while still immersed to induce sporulation of the cells. After that, the same procedure of drying and swabbing was repeated as described above. Sporulation of the cells was

confirmed by gram staining and observation of these samples under contrast microscope (unstained refractory structures). Sporulation was also verified by the Schaffer-Fulton spore staining technique using malachite green followed by microscopic observation.

5.3.4 – Statistical analysis

Completely randomized design was used to assign concrete blocks to the four treatment groups. Three replications of this experiment were performed and the averages of the survival populations ($\log_{10}$ CFU/ sq. cm) of *C. perfringens* and *B. subtilis* were analyzed using analysis of variance (ANOVA) with SAS PROC GLM procedures (2002-03 SAS 9.1 Institute, Gary, NC). Statistical significance was reported at a *P*-value of less than or equal to 0.05 ($P<0.05$).

5.4 – Results and Discussion

5.4.1 – External brick surfaces

Analysis of Variance (ANOVA) of survival populations of *Bacillus subtilis* ($\log_{10}$ CFU/ sq. cm) did not show any significant difference ($P>0.05$) following treatments A and B (Table 2) on neither of the trials (vegetative vs. spore). Also, no significant difference ($P>0.05$) was observed when comparing treatments C and D. During the vegetative trial in this study, treatment C and D resulted in significantly lower ($P<0.05$) survival populations of *B. subtilis*, 2.96 $\log_{10}$ CFU/ sq. cm and 2.7 $\log_{10}$ CFU/ sq. cm respectively, when compared to treatment A reflecting the bactericidal properties of the product. The spore trial when compared to the vegetative trial shown the same pattern but on a smaller proportion meaning that significant reduction ($P<0.05$) was observed when comparing treatment A to treatments C (ca. 1.09 $\log_{10}$ CFU/ sq. cm) and D (ca. 1.53 $\log_{10}$

CFU/ sq. cm). Therefore, this data is in accordance to what researchers have reported about spores being more resistant to antimicrobials than vegetative cells, especially when in biofilm environments. Since treatments A and B were not significantly different (P>0.05) during the trials, there is no suggestion that the product may have a residual effect for either conditions (spore vs. vegetative).

Similar to *B. subtilis*, BioSealed for Concrete^{TM} was an effective antimicrobial against *Clostridium perfringens* (Table 2). The product's bactericidal properties can be observed for this bacterium since significant reduction (P<0.05) was reported when comparing treatment A to treatments C (ca. 1.19 $\log_{10}$ CFU/ sq. cm) and D (ca. 1.86 $\log_{10}$ CFU/ sq. cm) during vegetative cells trials. Once again, similar to the *B. subtilis* study, the spore trial when compared to the vegetative trial are very much alike where significant reduction (P<0.05) can be observed when comparing treatment A to treatments C (ca. 1.04 $\log_{10}$ CFU/ sq. cm) and D (ca. 1.7 $\log_{10}$ CFU/ sq. cm). Throughout this study no significant difference (P>0.05) between treatments A and B, nor C and D were observed. In both cases, *C. perfringens* and *B. subtilis* – spores and vegetative trials, because survival populations ($\log_{10}$ CFU/ sq. cm) between treatments B and C were not significantly different (P>0.05), it suggests that treating concrete with the product prior to or post bacterial contamination does not change bacterial colonization behavior.

5.4.2 – Internal brick surfaces

ANOVA of the survival populations ($\log_{10}$ CFU/ sq. cm) of *B. subtilis* for the internal surfaces of concrete blocks did not reveal any significant difference (P>0.05) following treatments A, B and C (P>0.05; Table 3) for neither trials. Since lower viable

populations of *B. subtilis* were recovered it is difficult to evaluate the true magnitude of the reduction caused by the product. Similar results can be observed in *C. perfringens* spores trials, where no significant difference can be observed between treatments A and B, and C and D ($P>0.05$; Table 3). On the other hand, the vegetative trials, significant reduction ($P<0.05$) can be observed when comparing treatment A to treatments B (ca. 0.45 $\log_{10}$ CFU/ sq. cm), C (ca. 0.46 $\log_{10}$ CFU/ sq. cm) and D (ca. 0.83 $\log_{10}$ CFU/ sq. cm). Once again, these results to be analyzed carefully since the low recovery rate from the internal surfaces limit our judgment of the antimicrobial effects of the product.

The lack of a significant difference found for *B. subtilis* and *C. perfringens* (spores trial) should not be attributed to the inability of the product to penetrate deep into concrete pores. If the product was unable to penetrate into concrete pores, no significant difference would have been found in the *C. perfringens* vegetative trial, either. Therefore, what these results suggest is that a secondary reaction between the product and the concrete occurred, reducing its bactericidal effect.

Before comparing the antimicrobial efficiency of BioSealed for Concrete™ with other antimicrobials extensively used by the food industry it is important to consider that the efficiency of most antimicrobials depends on several factors, including length of contact, concentration of the antimicrobial and temperature which will largely influence their bactericidal effects.

The major types of sanitizers used in the food industry over the year have been halogens, peroxygens, acids, and quaternary ammonium compounds (Bower and Daeschel 1999; Gandhi and Chikindas 2007, Johnson et al. 1990). Lindsay and Holy (1999) evaluated the different responses of planktonic and attached (stainless steel and

polyurethane) *B. subtilis* to different sanitizer treatments. Their study revealed that a mixture of peracetic acid and hydrogen peroxide was the most effective sanitizer against *B. subtilis*. A 2.0 log reduction was obtained when *B. subtilis* was exposed to 170 ppm during 5 minutes to this combination of peracetic acid and hydrogen peroxide. In the same study, when attached *B. subtilis* was exposed to 35 ppm of iodophor or to 1000 ppm of chlorhexidine gluconate during 5 minutes, a 1 log reduction was observed (Lindsay and Holy 1999). Our data suggest an increased bactericidal activity of BioSealed for Concrete™ since and average of 2.83 log reduction was observed with an immediate contact product. Again, these comparisons should be made extremely carefully, since exposure conditions and material used (concrete vs stainless steel and polyurethane) were different.

Little or no information is available on *C. perfringens* susceptibility to sanitizers when in biofilms or even free cells. Taormina and Dorsa (2007), to evaluate the hot water and sanitizer dip treatments of knives, used *C. perfringens* cultures to contaminate stainless steel knives. In their study, after dipping the knives for 1 second - loosely attached bacteria, 400 ppm of quaternary ammonium promoted a 2.04 log reduction while acid quaternary ammonium (440 ppm) and peracetic acid (700 ppm) induced a 1.96 and 1.50 log reductions respectively (Taormina and Dorsa 2007). Shetty et al. (1999) evaluated the bactericidal activity of a mixture of oxidizing compounds – hypochlorous is the main component at a concentration of 144 mg/L. In their study they used spores suspension of *C. difficile* in the presence and absence of organic matter. A 4 log reduction was obtained when the spore suspensions were exposed during two minutes. However, organic load was able to completely inactivate the sanitizing mixture.

5.5 – Conclusion

BioSealed for Concrete™ proved to be a potent antimicrobial with immediate bactericidal effects that has potential to facilitate biofilm removal. Results of this study can be used as an information resource since *B. subtilis* and *C. perfringens* are reference organisms for spores' antimicrobial susceptibility studies and there is lack of information about these microorganisms when attached onto concrete surfaces. Although antimicrobial capabilities of BioSealed for Concrete™ are shown in this study, its use should not be substituted for good manufacturing practices and/ or efficient cleaning and sanitizing procedures.

5.6 – References

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Table 1: *Bacillus subtilis* and *Clostridium perfringens* inoculums levels (log_{10} CFU/mL)

Treatments	*B. subtillis*		*C. perfringens*	
	Vegetative	Spores	Vegetative	Spores
Inoculum A	7.00	8.08	7.07	8.85
Inoculum B	7.85	7.93	7.83	7.67
Inoculum C	6.94	6.85	8.50	6.55
Inoculum D	6.79	6.68	6.46	6.72

A = Initial inoculums before introducing cement blocks; B = Inoculums at removal time without any cement blocks; C = Inoculums at removal time containing cement blocks with no BioSealed for Concrete™; D = Inoculums at removal time containing cement blocks with BioSealed for Concrete™.

Table 2: Survival populations (log_{10} CFU / sq. cm) of *Bacillus subtilis* and *Clostridium perfringens* on the external surfaces of brick blocks

Treatments	*B. subtillis*		*C. perfringens*	
	Vegetative	Spores	Vegetative	Spores
A	5.53[a]	2.23[a]	3.76[a]	2.59[a]
B	4.26[a]	1.37[ab]	2.97[ab]	1.87[ab]
C	2.57[b]	1.14[b]	2.57[bc]	1.55[bc]
D	2.83[b]	0.70[b]	1.90[c]	0.89[c]
***P* value**	0.0051	0.0243	0.0071	0.0082

A = No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation

Superscripts (a, b and c) indicate significant difference ($P<0.05$) within a column.

Table 3: Survival populations (log_{10} CFU / sq. cm) of *Bacillus subtilis* and *Clostridium perfringens* on the internal surfaces of brick blocks

Treatments	*B. subtillis*		*C. perfringens*	
	Vegetative	Spores	Vegetative	Spores
A	0.95^{a}	0.81^{a}	1.66^{a}	0.70^{a}
B	0.70^{a}	0.70^{a}	1.21^{b}	0.70^{a}
C	0.70^{a}	0.70^{a}	1.20^{b}	ND^{b}
D	ND^{b}	ND^{b}	0.83^{c}	ND^{b}
***P* value**	0.3806	0.4411	0.2036	0.0015

A = No BioSealed for Concrete™ application; B = BioSealed for Concrete™ applied before bacterial inoculation; C = BioSealed for Concrete™ applied after bacterial inoculation; D = BioSealed for Concrete™ applied before and after bacterial inoculation

Superscripts (a, b and c) indicate significant difference ($P<0.05$) within a column.

6 – CONCLUSIONS

The food industry is in constant search for new chemicals, techniques and procedures to kill microorganisms and prevent them from colonizing the industrial environment. These innovative products and procedures are not only an attempt to substitute pre-existing ones that no longer work due to microbial resistance, but also to assist pre-existing routines by increasing sanitation and disinfection efficiency. One such product that aims to accomplish these goals is BioSealed for Concrete™.

The goal of the experiments within the preceding chapters was to determine the susceptibility of different bacteria species to BioSealed for Concrete™. BioSealed for Concrete™ proved to be a potent antimicrobial with immediate bactericidal effects and facilitating biofilm removal for all microorganisms tested in the research presented in the previous pages. Results indicate that this product may be used as an alternative chemical in food processing plants that have persistently failed to reach inspection agencies standards. The antimicrobial properties of this product should not be substituted for good manufacturing practices nor efficient cleaning and sanitizing procedures. Extension of the antimicrobial properties of BioSealed for Concrete™ should be made carefully, since in the research within this thesis susceptibility variation was found even within the same specie of bacterium tested.

7 – FURTHER STUDIES

In sight of the current industry efforts to control and prevent the formation of biofilms in the food processing environment it is essential to determine the mechanisms of biofilm formation and functionality. When more information is available about these mechanisms, it will be easier to tackle specific aspects of these events and thus be more efficient in the prevention and removal of biofilms.

The research presented within this thesis refers exclusively to microbial susceptibility to the product, however its effectiveness as a topical concrete sealant was not studied. Therefore, further research must be performed to investigate other functional properties of this product.

In addition, this research was conducted in the laboratory under a controlled situation. However, the food processing environment contains concrete which is larger in volume and size than that used in this study which might influence the product's functionality. Other factors which may cause variation to these results such as organic matter, other chemicals and temperatures should be studied. Therefore, further research should include research evaluating the product's efficiency under real processing conditions.