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ANTIMICROBIAL EFFECTS OF COPPER AND BRASS IONS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AT DIFFERENT TEMPERATURES, PH AND NUTRIENTS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in

The Department of Food Science

by
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August 2005

Dedicated to
My Mother,
Maryam Abdulla Al Shehi

ACKNOWLEDGEMENTS

First, I would like to thank my family with all love and appreciation, especially my mother, Maryam Al Shehi, without her love, encouragement and prayers I would not have reached this far. Also I would like to thank my best friend Mona Al Suwiadi for her encouragement and support.

Also I would like to thank the second mother, my country the United Arab Emirates that financed all my studies since I was sent to graduate school and provide all the needs for me to reach this level.

A special thanks to my major advisor Dr. Marlene Janes, who supported me with her advice, guidance and care all the time, and encouraged me to finish my study on time, and to my committee members Dr. Witoon Prinyawiwatkul who took care of me since I applied in the Food Science Department and provided help to me when I had a hard time with my studies, Dr. Jon Bell I am appreciated his help in the part of processing plant experiment and Dr. Donal Day during the entire study. I appreciate all the help that Dr. Richelle Beverly provided to me in the microbiology laboratory.

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ABSTRACT

Listeria monocytogenes has been recognized as a human pathogen since 1929. This pathogen is found in many foods and listeriosis infections affect approximately 2,500 people in the United States each year, according to the Centers for Disease Control and Prevention. Of those infected with *L. monocytogenes* approximately 500 die as a result of the illness. *Listeria monocytogenes* is a bacterium, commonly found in water, soil, plant material, animals and human. Today, different methods are used by food manufacturers, to reduce the risk of *Listeria monocytogenes*, such as antimicrobial agents, heating, irradiation, and fermentation. The ability of the bacteria to grow at temperatures as low as 3˚C permits multiplication in refrigerated foods. The purpose of this study was to determine the antimicrobial effect of copper ions against *Listeria monocytogenes* on the surface of copper, brass and concrete coated with polyurethane containing different concentrations of copper ions. The utilization of pH, nutrients and temperatures were applied. Copper alloys antimicrobial effect in two different crawfish processing plants was also evaluated. The amount of copper ions released into raw and cooked shrimp at different temperatures was also assessed. Our study has been successful in understanding the survival of *Listeria monocytogenes* at different copper ions concentrations under different temperatures, pH and nutrients. It has also been observed that the use of different copper ions concentrations haves great potential as antimicrobial agents that can be employed by food processors.

CHAPTER 1

INTRODUCTION

Listeria monocytogenes has been recognized as a human pathogen since 1929 (Jay, 2000). In 1927, several unusual deaths occurred in gerbils near Johannesburg, South Africa, a new microorganism was discovered and the disease was named the Tiger River disease. Later the name was changed to Listerella dedicating it to Lord Lister, one of the most distinguished bacteriologists (Pirie, 1927). Listeriosis infections affect approximately 2,500 people in the United States each year, according to the Centers for Disease Control and Prevention (CDC, 2000). Of those infected with *Listeria monocytogenes,* approximately 500 die as a result of the illness. *Listeria monocytogenes* is a bacterium, commonly found in water, soil, plant material, animals and human. Another important source of this pathogen is food. The symptoms of listerosis are spontaneous abortion in pregnant women, meningitis in newborn infants, and meningocephalitis in immunocompromised adults (FSIS, 1999). In the1980's there were several listeriosis outbreaks linked to the consumption of raw vegetables, cheese, raw and cooked poultry products, egg products, raw meat, and seafood products (FSIS, 1999). The largest outbreak of listeriosis occurred in Los Angles in 1985 and affected 93 pregnant women (Ryser and Marth, 1999). The outbreak was associated with the consumption of soft cheese produced locally in California. Another outbreak occurred in Switzerland during 1983 to 1987 with 122 cases of listeriosis affecting 65 pregnant women and their infants from soft cheese consumption (Ryser and Marth, 1999).

Today, different methods are used by food manufacturers, to reduce the risk of *Listeria monocytogenes*, such as use of antimicrobial agents, heating, irradiation, and fermentation. Because of the widespread nature of the organism and the ability of this bacterium to grow at temperatures as low as 3˚C, it is important to make every effort to prevent contamination of food products with *Listeria monocytogenes* during the food processing operation and prevent

recontamination of ready-to-eat (RTE) products after processing. *Listeria monocytogenes* contamination of cooked meat products occurs when a product or food contact surface is contaminated between the cooking and packaging stages (e.g. during slicing or peeling operations). However, there may be other sources whereby the organism is introduced into the processing area from poor non-hygienic practices of food handlers. The most common non-hygienic practices involves preparation of food with dirty hands, inadequate cleaning of processing surfaces, wiping the nose while preparing food, handling of garbage while processing food, leaving leftovers or ready-to-eat foods at ambient temperatures for excessive time periods, and failure to use separate hand and dish towels (FSIS, 2001).

Today, a major food safety concern is the contamination after thermal processing. Some examples of food products that were contaminated after processing are hot dogs and luncheon meats (FSIS, 2001). In 1998, one of the largest outbreaks of *Listeria monocytogenes* occurred due to the ingestion of hot dogs produced by a single manufacturer. This outbreak resulted in 15 adult deaths, 6 stillbirths, and the recall of one million pounds of product (FSIS, 2001). Effective control of *Listeria monocytogenes* is challenging and requires intensive management and resources. Even though the risk of listeriosis is relatively low, the consequences are devastating for both the consumer and the processor. *Listeria monocytogenes* is often present in raw ingredients, and since *Listeria monocytogenes* is heat resistant, adequate cooking is important to assure destruction of the organism (FSIS, 2001).

In food processing plants, microorganisms can attach to solid surfaces along with various nutrients, minerals, and organic matter to form microcolonies or biofilms on food contact equipment surfaces. Biofilms are a threat to the food industry because they are difficult to remove or inactivate, and can contaminate foods even after routine cleaning. The attached

bacteria in a biofilm are resistant to sanitizers and other antimicrobial agents compared to the planktonic or free-living bacteria (Ravishankar, 2002). Proper detection and control methods for biofilms are necessary for a safe food processing operation (Ravishankar, 2002).

In the food processing environment, biofilms are sources of pre- or post- processing contamination. Chemical cleaning with detergents, accompanied by manual or mechanical scrubbing of food contact surfaces, and application of sanitizer, is an effective way to control biofilms (Ravishankar, 2002). When designing food contact equipment, the appropriate type of material should be carefully selected to prevent microbial adhesion, and diligence should be given to the cleanliness of the equipment. These cleaning practices help to maintain a safe food processing operation free of biofilm problems and in turn create a safer food supply (Ravishankar, 2002). Once food or a surface in a food processing plant is contaminated, the bacteria can form colonies, and eventually biofilms. For example, a counter used for cutting meat may become infected with a microorganism which might attract other microorganisms to the initially adhered microorganism, and could eventually form a biofilm (Marshall, 1992).

Many researchers are studying ways in which biofilms form on surfaces. Others are exploring host response to biofilms, while some are investigating ways to penetrate the layers of microorganisms in hopes of killing them. Although biofilms can be threats, they can also be manipulated beneficial purposes. An example of a benefit is the way in which biofilms can help depollute water (Anwar and Costerton, 1992).

Copper, which is usually found in nature in association with sulfur, has played a significant and sometimes surprising role in improving public health. It has antimicrobial properties that help to protect against infections in homes, at work, and in hospitals. Copper can help preserve the purity of drinking water (Copper Development Association, 2004). The

confirmed antimicrobial effects of copper can inhibit water-borne microorganisms, such as viruses, bacteria, infectious parasites or algae (Copper Development Association, 2004). These microorganisms can create a variety of health risks to humans, including Legionnaire's Disease, deadly *E. coli* infections (Copper Development Association, 2004).

The Center for Applied Microbiology and Research (CAMR) found that the highly toxic *E. coli* O157:H7 strain of bacteria survives for shorter periods of time on copper and brass surfaces than on stainless steel (Center for Applied Microbiology and Research, 2000). This finding has wide-ranging implications for reducing outbreaks from cross contamination of *E. coli* O157:H7 in the food processing industry (Center for Applied Microbiology and Research, 2000).

This dissertation demonstrates the effectiveness of copper, brass, or concrete surface coatings containing copper ions in reducing or destroying *Listeria monocytogenes* growing in biofilms at different temperatures, nutrients, and pH in ready-to-eat meat processing plants.

1.1 REFERENCES

Anwar, H., and J.W. Costerton. 1992. Effective Use of Antibiotics in the Treatment of Biofilm-Associated Infections. *American Society for Microbiology News*. 58:665-668.

Center for Disease Control and Prevention. 2000. CDC Surveillance Summaries, March 17, 2000.MMWR 2000; 49(No. SS-1).

Center for Applied Microbiology and Research (CAMR). 2000. http://www.copper.org/about/pressreleases/2000/DemonstratePotential.html

Copper development center. 2004. http://www.copper.org.sg/publications/cca/0309/pg06_japan_research.html

Food Safety and Inspection Service (FSIS). 2001. Controlling *Listeria Monocytogenes* in Small and Very Small Meat and Poultry Plants. United States Department of Agriculture, Washington, DC. 20250-3700.

Food Safety and Inspection Service (FSIS). 1999. *Listeria monocytogenes* and Listeriosis. United States Department of Agriculture, Washington, DC. 20250-3700.

Jay, J. M. 2000. Modern food microbiology. 6th edition. Aspen Publisher, Inc. Gaithersburg, Maryland.

Marshall, K.C. 1992. Biofilms: An Overview of Bacterial Adhesion, Activity, and Control at Surfaces. *American Society for Microbiology News*. 58:203-205.

Ravishankar, S. 2002. Microbial biofilms on food contact surfaces- detection and control. *NCFST*. 12 (3).

Pirie, J. H. 1927. A new disease of veld rodents. Tiger River Disease. *Publ. S. Afr. Inst. Med. Res*. 3:163-186.

Ryser, E. T, and E. H., Marth. 1999. *Listeria*, Listeriosis and food safety. 2nd edition. Marcel Dekker, INC. New York.

CHAPTER 2

REVIEW OF LITERATURE

2.1 *LISTERIA MONOCYTOGENES*

These bacteria belong to the genus *Listeria,* and are small Gram-positive bacilli that are found globally in soil, surface water, vegetation and throughout the food chain. They colonize a wide range of wild and domestic animals and humans, and may cause infection (listeriosis) (Colee et al., 1997). *Listeria* measure approximately 0.5 X 2-3 μm, occurring singly or in pairs which are often angled at the point of contact and may resemble diphteroids and diplococci. Filamentous forms may develop in older cultures, sometimes with loss of Gram-reaction. *Listeria* species are non-capsulate, non-sporing, and non-acid-fast, however, they produce lactic acid from glucose and other fermentable sugars (Colee et al., 1997; Walter, 2000). Six species of *Listeria* are recognized, and are characterized by the possession of antigens that give rise to 17 sevoras. The primary pathogenic species, *Listeria monocytogenes*, is represented by 13 sevoras (Walter, 2000).

Listeria antigenic structure was found to be composed of at least 14 heat-stable somatic O antigens and four heat-labile flagellar H antigens (Colee et al., 1997). These antigens can be detected by agglutination reactions when absorbed with rabbit antisera. *Listeria,* which is acquired by ingestion, has to find a way to attach itself to the intestinal mucosa. Most pathogenic bacteria that infect the intestine use their flagella to swim against the peristaltic movement of the bowel content in order to be able to penetrate the mucosal lining of the gut and adhere to the walls of the gut. *Listeria* are actively motile by means of peritrichous flagella at room temperature (20-25ºC) however, in fresh cultures such as tryptose phosphate broth incubated at 20ºC, they begin to show tumbling motility. The cells start moving by twisting and wriggling followed by rapid multiplication and dispersion in various directions (Kathariou et al., 1995). These organisms do not synthesize flagella at body temperatures (37ºC). Instead, virulence is

associated with another type of motility: the ability of the bacteria to move themselves into, within and between the host cells by polymerization of the host cell actin filaments that forms at one end of the bacterium which propels the bacteria through the cytoplasm. However, one should not totally dismiss the advantage of flagella motility for existence and spread of bacteria outside of the immediate host environment (Colee et al., 1997).

Listeria can enter the mammalian cells by attaching to their surface. The bacterium is thought to attach to the epithelial cells of the GI tract by means of D-galactose residues on the bacterial surface which adhere to D-galactose receptors on the host cells. If this analogy is correct, it works in contrast with the adhering process of most other bacterial pathogens. The bacterium displays the protein or carbohydrate on its surface and the host displays the amino acid or sugar residue to which the bacterium binds.

Listeria monocytogenes multiplies inside the host, which largely protects it against circulating immune factors such as antibodies and complement-mediated lyses. The effective host response is Cell-Mediated Immunity (CMI), involving both lymphokines (especially interferon) produced by CD4+ (T_{H1}) cells and direct lysis of infected cells by the CD8+ (T_C) cells. Both of these defense mechanisms are expressed in the microenvironment of the infected foci, which are organized as granulomas, characterized by a central accumulation of macrophages with irregularly shaped nuclei, and by peripheral lymphocytes recognizable by rounded nuclei and a narrow border of intensely staining cytoplasm (Walter, 2000).

2.2 LISTERIOSIS

The first foodborne listeriosis outbreak in United state was identified in 1981. *Listeria monocytogenes* is commonly ingested with food, and is usually a harmless transient in the intestinal tract (Colee et al., 1997). It is important to note here that cases of human listeriosis are

caused mainly by only three serotypes (4b, 1/2a and 1/2b). Most human epidemics and many sporadic cases have been caused by serotype 4b for an undetermined reason (Birgit, 2000). Those vulnerable to infection include pregnant women and their offspring, the elderly, and patients suffering from malignant conditions or debilitating diseases such as cirrhosis or diabetes mellitus. In cases of pregnancy, these symptoms are followed by a latent period during which *Listeria* progressively invades the placenta. In addition to that, infection of the fetus may result in abortion, stillbirth, premature delivery, or acute-onset disseminated infection in the newborn infant (including the form known as granulomatosis infantisepticum) with a mortality rate of 35 - 50%. Apart from that, neonatal infections can occur due to cross contamination in the nurseries resulting in acute meningitis (Steven and Lowe, 1995; Walter, 2000; Colee et al., 1997). Moreover, this bacterial infection may cause diffuse brain inflammation with neurological dysfunction manifested by myelopathy (motor and sensory signs) and encephalopathy causing confusion and reduced level of consciousness (Steven and Lowe, 1995). The incubation period varies from about 1 day to 6 weeks (Birgit, 2000).

Listeria monocytogenes is diagnosed in early stages, antibiotic treatment of pregnant women or immunocompromised individuals can prevent serious consequences of the disease. However, early diagnosis is an exception rather than a rule since the first signs of a case or an outbreak are reports of stillbirth or serious infections resembling listeriosis.

In the 1980s a number of outbreaks of listeriosis occurred, in which contaminated foods were identified to be the source of transmission. The foods included milk contaminated after pasteurization, and soft cheese contaminated with raw milk (Walter, 2000). Fresh and frozen meat, poultry, seafood products, fruits and vegetable products have also been involved in *Listeria monocytogenes* outbreaks (Walter, 2000). In a study conducted to detect the prevalence

of *Listeria monocytogenes* in milk and dairy products, it was found that the bulk tank raw milk from 260 farms in Scotland had 25 positive samples out of the160 collected in a year. The incidence of *Listeria monocyotogenes* in the retail food in the Netherlands, of the 5,779 kinds of products tested, 0.3% were positive for this pathogen (Walter, 2000). The lowest prevalence was in ice cream, and the highest was in fresh meat. In another study, 4.6% of 929 samples of soft cheese made from raw milk were positive for *Listeria monocyotogenes* (Walter, 2000). Persons who are at increased risk for listeriosis, pregnant women or immunocomprimised individuals, can be given dietary measures that can decreases risk. Also, such people should avoid foods that are epidemiologically linked with listeriosis. In addition, ready-to-eat foods and leftover foods should be cooked until steaming hot before eating (Anonymous, 1991).

2.3 DETECTION OF *LISTERIA MONOCYTOGENES* IN FOOD SAMPLES

Authorities have adopted a policy of “zero tolerance” in respect to *Listeria monocytogenes* in ready-to-eat foods. Enumeration is done by homogenizing samples in nutrient broth and plating aliquots of decimal dilutions on Listeria solid agar (LSA), or by use of spiral plating techniques (Colee et al., 1997). The differential selective Polymyxin Acriflvine Lithium Chloride Agar Medium (PALCAM) has been introduced as a companion selective agar to Oxford agar (OXA), optionally replacing LPM plus esculin and Fe^{3+} or plain LPM as the required second selective agar (Stelma et al. 1987). This substitution brings the methodology closer to that used outside the U.S. and decreases reliance on the delicate Henry illumination technique now used only optionally to confirm the purity of *Listeria* isolates. Other selective agar media are currently under consideration as alternative substitutes for LPM. The normal mouse pathogenicity test has been replaced by the more sensitive and slightly faster immunocompromised mouse pathogenicity test of Stelma et al. (1987).

PALCAM agar provides a quantitative cultivation of *Listeria monocytogenes*, while inhibiting Gram-negative and most of the Gram-positive accompanying bacteria. The selectivity of the medium results form its content of polymyxin, acriflavin, ceftacidim, and lithium chloride. *Listeria monocytogenes* breaks down the esculin in the medium to glucose and esculetin. Esculetin forms an olive-green to black complex with iron (III) ions which stains the colonies of *Listeria monocytogenes.* Mannitol-positive accompanying bacteria such as staphylococci grow as yellow colonies, if they are not inhibited (Walter, 2000).

The pH range for the ideal growth of *Listeria* is 6 to 8. In general, it was found that the minimum growth pH of the bacterium is a function of temperature used for incubation, general nutrient composition of growth substrate and the quantity of NaCl and other salts (Walter, 2000). Some studies have shown growth of *Listeria monocytogenes* in lower pH in the range of 4.4 to 5 at different temperatures (Walter, 2000). The interaction of pH with NaCl and incubation temperature has been the subject of several studies (Wijtzes et al., 1993; Miller, 1992) which concluded that the pH and NaCl effects were purely additive and not synergistic in any way (Walter, 2000).

The temperature range for growth is 3 to 43˚C. However, it was found that the mean minimum growth temperature on trypticase soy agar was 1.1˚C, with a range of 0.5 to 3.0˚C. *Listeria* grows on most ordinary media if a fermentable carbohydrate is present, but blood agar or tryptose phosphate agar is recommended (Colee et al., 1997; Walter, 2000).

The nutritional requirements of *Listeria* are typical of those for many other Gram-positive bacteria. At least four B vitamins are required, biotin, riboflavin, thiamin, and thiotic acid, and some amino acid such as cysteine, glutamine, isoleucine, leucine and valine. Growth is stimulated by Fe and phenylalanine. Also, virulent strains grew faster in the presence of iron than

did avirulent strains. Glucose and glutamine are required as primary sources of carbon and nitrogen (Premaratne et al., 1991). Unlike most Gram-positive bacteria, *Listeria* grows well on MacConkey agar. It was also found that iron is an important factor for growth, but apparently *Listeria monocytogenes* do not possess specific iron-binding compounds (Walter, 2000). *Listeria monocytogenes* was also found as a second bacterium to *Staphylococci* in the foodborne pathogens, being able to growth at a_w values <0.93 (Vannetten, 1989).

Specimens are incubated usually at 30 to 37˚C in various selective enrichment broths containing inhibitory agents. The United States Food and Drug Administration (FDA) method for milk and cheese employs a single enrichment step where the sample is selectively enriched in a suitable broth at 30˚C for 24 hours. Specimens that have been sublethally damaged by heat, cold, or chemicals, can not be reliably isolated since selective agents may inhibit their growth and recovery (Colee et al., 1997). Therefore, to overcome this, the samples are incubated in a pre-enrichment broth for 6 to 24 hour such as UVM or Fraisers broth and then transferring to selective agar plates. Several *Listeria* agars such as Oxford agar have been used, that may turn the *Listeria* colonies to black, PALCAM agar that incorporates mannitol and phenol red as a second indicator system along with enhanced haemolysis agar (EHA) that gives a direct CAMP reaction by the incorporation of sphingomyelinase in sheep blood agar (Colee et al., 1997).

2.4 RAPID METHODS FOR DETECTION OF BACTERIA

Good hygiene and effective cleaning are of fundamental importance in maintaining food safety and are essential for Good Manufacturing Practice (GMP) in food processing environment. Regulation limiting contamination of ready- to- eat foods to a zero- tolerance have been the driving force behind development of rapid tests. Therefore, rapid methods were used for

sanitation testing and measuring process control to ensure the quality of disinfection of the processing plant during the working hours (Gellin and Broome, 1989).

Adenosine Triphosphate (ATP) Bioluminescence is a well-established technology and provides an instant indication of the hygiene status of product contact surfaces. Product residues particularly foodstuffs contain huge amounts of ATP along with microbial contamination which also contains smaller amounts of ATP. After cleaning, all sources of ATP should be significantly reduced. The detection of ATP provides a direct, relevant, and reliable indicator of cleaning efficiency and hygienic status. The reagent changes from colorless to green when contamination is detected in a sample. The result is obtained in 60 seconds and is semi-quantitative i.e. the faster and the more intense the color development, the more contaminated the sample (Hygiena International, 2003).

3M Petrifilm plates are small, flat, sample-ready plates that enable food processors to perform on-site microbial testing easily and efficiently. Their exclusive design features include films coated with nutrients and gelling agent. The plates may be used to test raw materials, in-process products, finished products and the plant environment. The petrifilm plates may also be used to verify a plant's Hazard Analysis and Critical Control Point (HACCP) program (AOAC, 2000).

Polymerase Chain Reaction (PCR) has been developed commercially for detecting foodborne pathogens. Probe assays generally target ribosomal RNA (rRNA), taking advantage of the higher copy number of bacterial rRNA thereby providing a naturally amplified target and greater assay sensitivity. The basic principle of DNA hybridization is also being utilized in other technologies, such as the polymerase chain reaction (PCR) assay, where short fragments of DNA or primers are hybridized to a specific sequence or template. PCR can amplify a single copy of

DNA by a million fold in less than 2 hours; it has potential to eliminate, or greatly reduce the need for cultural enrichment. However, the presence of inhibitors in foods and in many culture media can prevent primer binding and diminish amplification efficiency so that the extreme sensitivity by PCR with pure cultures is reduced when testing foods. Some cultural enrichment is still required prior to analysis (Rose and Stringer, 1989)

The enzyme-linked immunosorbent assay (ELISA) is the most prevalent antibody assay format used for pathogen detection in foods. Usually designed as a "sandwich" assay, an antibody bound to a solid matrix is used to capture the antigen from enrichment cultures and a second antibody conjugated to an enzyme is used for detection. The walls of wells in microtiter plates are the most commonly used solid support; but ELISAs have also been designed using dipsticks, paddles, membranes, or other solid matrices (Feng, 1997).

All rapid methods require prior enrichment with several steps which take up to 48 hours. Al of concern is the significance of injured *Listeria* cells in a given food product and their potential for recovery during enrichment or in the food during long term storage.

2.5 SURVIVAL OF *LISTERIA MONOCYTOGENES* AT DIFFERENT TEMPERATURES

The temperature range of *Listeria monocytogenes* growth is of particular importance to food processors. This pathogen has features of both psychrotropic and mesophilic bacteria. *Listeria monocytogenes*, under the laboratory conditions, grow at temperatures between 3 and 45ºC, the optimal being 30 to 37ºC (Gray and Killinger, 1966).

Mechanisms that allow low-temperature growth of microorganisms involve maintenance of cellular membrane fluidity, uptake or synthesis of compatible solutes, maintenance of the structures of macromolecules, such as ribosomes, and maintenance of protein synthesis. For *Listeria monocytogenes*, adaptation of membranes to low temperatures is accomplished by

altering the branching in the methyl end of the fatty acid which are from iso branching to anteiso branching and by decreasing the fatty acid chain length, which results mainly in an increase in the amount of anteiso-C15:0 fatty acids (Annous et al., 1997).

Growth of *Listeria monocytogenes* in laboratory media at 1º C is very slow. However, when incubated at 3 to 6ºC, the growth rate of the pathogen increases to final populations of 10^8 CFU/ml after several weeks of incubation (El-Shenawy and Marth, 1989). Flasks containing Tryptose Phosphate Broth were inoculated with one of several *Listeria monocytogenes* strains and incubated at different temperatures. The bacteria exhibited average generation times of 12, 5 and 2.6 hours during incubation at 4, 10 and 15º C, respectively (Bojsen, 1972).

Henrike et al. (2002) examined the cold-shock response of *Listeria monocytogenes* by transferring these bacteria from 37°C to 10°C. *Listeria monocytogenes* was grown in BHI at 37°C at a rate of 0.6 h^{-1}. When exponentially growing cells were cold shocked at 10°C, a state phase lasting about 3 hours occurred before the growth resumed at a rate of about 0.2 h^{-1}. *Listeria monocytogenes* LO28 cells that were grown exponentially at 37°C exhibited about 50% survival after freezing. However, when cells were cold shocked for 4 hours at 10°C before freezing, the level of survival after freezing was 90%. When *Listeria monocytogenes* cells, grown at 37°C, were thawed, growth resumed after a lag period of about 2 hours, but after cold-shocked cells were thawed, growth at 37°C resumed without a lag period (Henrike et al., 2002).

Gay and Cerf (1997) studied the influence of temperature, pre-incubation temperature, and initial bacterial concentration on the survival of *Listeria monocytogenes* (Gay and Cerf, 1997). Four test temperatures (2, 6, 10 and 14°C) and two preincubation temperatures were studied (30°C or the test temperatures). The authors conducted that the survival of *Listeria*

monocytogenes (strain Scott A) at pH 4.8 is not dependent on initial bacterial concentration but on both the test and preincubation temperatures (Gay and Cerf, 1997).

Researchers have studied the thermal resistance of *Listeria ivanovii, Listeria seeligeri, Listeria welshimeri,* and *Listeria monocytogenes* (Bradshaw et al., 1991). They found that the heat resistance of *Listeria monocytogenes* appeared somewhat greater than other *Listeria* spp. in milk products although the difference was not statistically significant. Surprisingly, it has been documented that pasteurized milk and cheese made from pasteurized milk have been implicated as vehicles of *Listeria monocytogenes* transmissions in a number of listeriosis outbreaks (Fleming et al., 1985). In addition, it was recorded in a study that refrigeration of food does not prevent recovery from heat injury or growth of *Listeria monocytogenes* (Teo and Knabel, 2000). Several model studies were conducted to determine the effect of temperature, pH, and water activity as factors for controlling the microbiological safety of foods. To describe the effects of these factors on growth, two modeling equations were developed (Wijtzes et al., 1993). Researchers have studied the water activity and solute effects on growth and survival of *Listeria moncytogenes* (Miller, 1992). It was found that survival was related to the water activity level, as well as the solute employed. Other studies have found that increasing the NaCl concentration protects *Listeria monocytogenes* against the lethal effect of heat, and that low pH values increased heat sensitivity of the bacteria (Juneja and Eblen, 1999).

Increased evidence of growth and survival of *Listeria moncytogenes* at refrigeration temperatures has been demonstrated in several studies (Harrison et al., 2000). High risk-food products that may be of importance are ready-to-eat chilled foods, which require no further heating before consumption. Therefore, investigators have studied the growth of *Listeria monocytogenes* and *Y. enterocolitica* under modified atmospheres of 5% O_2: 10% CO_2: 85% N_2

and at 4 and 8˚C (Harrison et al., 2000). It was found that refrigerated, modified atmosphere, packaged foods should be maintained at 4˚C or below to ensure product safety, as the growth rate for both studied bacteria had decreased at 4˚C.

Listeria monocytogenes grows at low temperatures (-2 to 8ºC) that are used during food production and the storage of food products. The general relationship between the maximum and minimum specific growth temperature (-2 to 45ºC) was studied for *Listeria monocytogenes* (Bajard et al., 1995). These investigators showed the existence of growth rate occurring between 10 and 15ºC; below which *Listeria monocytogenes* grew faster than would be expected (Bajard et al., 1995).

2.6 THE EFFECT OF COPPER IONS IN REDUCING THE GROWTH OF BACTERIA

Copper's antimicrobial properties have been known for more than five millennia. The ancient Egyptians used copper pipes to transport water. French vintners have killed grapevine fungi using a 'Bordeaux' mixture containing copper sulfate since 1885(Rose, 1969).

Copper is significant in improving public health. Its anti-pathogen properties help to guard against infections in homes, at work, and in hospitals. Copper tubing, is widely used in plumbing because it can help preserve the purity of drinking water. Copper has antimicrobial effects that can inhibit water-borne microorganisms, such as bacteria, viruses, algae, and infectious parasites in the drinking water supply (Faundez and Figueroa, 2001).

Surfaces made from copper and brass, such as doorknobs and tabletops, can also reduce the spread of disease-causing organisms (Faundez and Figueroa, 2001). Recent research established that *Escherichia coli* O157:H7 strains, die after just a few hours on a copper surface, even under dry conditions. However, the deadly bacterium can live for over a month on stainless steel, which is a common surface material in food processing (Faundez and Figueroa, 2001).

Hospitals and clinics have also reduced the incidental transfer of microorganisms with copper-based, antibacterial paint on walls and by installing copper or brass doorknobs and fittings on doors (Rose, 1969). However, acidic foods become discolored and flavored by the copper ions. Copper ions can leach from pots, cooking surfaces, and food contact surfaces (Faundez and Figueroa, 2001).

Copper concentrations in drinking water vary according to local conditions but in most countries the recommended limit is 1 mg/ litter for domestic water supplies. The average daily dietary requirement for copper in humans has been estimated from 1 to 3 mg for adults. Copper is absorbed from the gastrointestinal tract by both active and passive transport mechanisms so that efficiency of absorption ranges from 25 to 60% and is influenced by human requirements. Absorbed copper appears first in plasma as the cupric ion loosely bound to albumin (The European Agency for the Evaluation of Medicinal Products, 1998). In this form it is available for metabolism by the liver. The liver is the major storage organ for copper where it is protein bound. Copper is then transported onwards to peripheral tissues. Since liver contains the highest concentrations of copper, it is the target organ to consider in the risk assessment of copper (The European Agency for the Evaluation of Medicinal Products, 1998).

Excess copper is excreted mainly via the bile and faeces, though urinary losses account for 0.5 to 3 % of the daily intake. In 1974 the World Health Organization, WHO, concluded that the fatal oral human dose of various copper salts is about 200 mg/kg. Chronic copper poisoning in humans is very rare, suggesting that the human body can adapt to a wide range of copper intake without detrimental effects. There are a few reports of chronic copper toxicity in human infants but the only report in adults concerns chronic effects such as pulmonary deposition and fibrosis, granulomas, and malignant liver tumors in vineyard workers. The most sensitive

parameter of copper toxicity is diarrhea frequently observed in infants after drinking contaminated water. Copper has also been implicated in the aetiology of Indian Childhood Cirrhosis in children exposed to high levels of copper in the drinking water. The direct relation of this potentially lethal liver damage to copper intoxication awaits final confirmation (The European Agency for the Evaluation of Medicinal Products, 1998).

Limited research has been done on the antimicrobial effects of copper metals against *Listeria monocytogenes*. A study conducted by the Center for Applied Microbiology & Research (CAMR, 2000) using copper, brass, and stainless steel surface found that the highly toxic *E. coli* O157:H7 strain of bacteria survived for much shorter periods of time on copper and brass surfaces than on stainless steel. This finding has wide-ranging implications for reducing outbreaks from cross contamination of *E. coli* O157 in the food processing industry.

Copper is an essential cofactor in many of the enzymatic processes taking place within the cell. However, copper is extremely reactive and for that reason it is toxic to the cell in high concentrations. Therefore, all living organisms have developed mechanisms to maintain a suitable level of intracellular copper. Copper homeostasis in most living organisms is mediated by a group of membrane-spanning proteins called CPx-type ATPases which are encoded by genes located on the chromosome (Solioz and Vulpe**,** 1996). A few cases of acquired resistance to copper have also been reported. Copper resistance genes are often located on plasmids, as opposed to the chromosomal genes that are involved in copper homeostasis and, in most cases, are transferable. Acquired and transferable copper resistance has been most thoroughly studied in Gram-negative bacteria such as the *pco* genes from *Escherichia coli* (Brown et al., 1995). Very few cases of copper resistance have been reported in Gram-positive bacteria (Leelawatcharamas, 1997.

The bactericidal activity of catechin-copper (II) in green tea complexes against *Staphylococcus aureus* compared with *Escherichia coli* was investigated in relation to the generation of hydrogen peroxide and the binding of Cu(II) ion onto the bacteria. The bactericidal activity of catechin-Cu (II) complexes against *Staphylococcus aureus* (Gram-positive) was much lower than that against *E. coli* (Gram-negative), suggesting that the binding of copper ions to the surface of bacterial cells plays an important role in the bactericidal activity of catechin-Cu (II) complexes (Hoshino et al., 2000). Gram-negative bacteria such as *E. coli* have negatively-charged lipopolysaccharide on their cell surfaces (Kigai et al., 1993), whereas Gram-positive bacteria such as *Staphylococcus aureus* do not. Sonohara et al. (1995) reported that *Staphylococcus aureus* had a more positively-charged surface than that of *E. coli*, Tichy (1975) reported that lipopolysaccharides had the ability to bind Cu (II). It was recently reported that recycling redox reactions between Cu(II) and Cu(I) were possible on the surface of *E. coli* cells, thereby generating hydrogen peroxide locally and causing damage to the cytoplasmic membrane (Hoshino and others, 1999).

A controlled experiment was performed to test the anti-bacterial properties of copper, stainless steel and zirconium. Ten pieces of each material were sealed inside a holder. In a clean booth, dental bacteria were placed in the acryl chamber with a nebuliser. The results implied that 95.5% reduction on the bacteria came from copper (Copper development center, 2003). Copper and copper-based alloys have long been know for their bacteriostatic properties, but their importance in possibly controlling foodborne bacteria in the food processing environment has not been evaluated. The most widely used copper compound is copper sulfate that is approved for use as a pesticide, germicide, feed additive, and soil additive.

2.7 FORMATION OF BIOFILM BY *LISTERIA MONOCYTOGENES*

Bacteria are often found in sessile communities known as biofilms. The organization of bacteria in biofilms can have important effects on the environment and on human activities. Biofilms are extremely resistant to treatment with biocides or detergents and show high levels of tolerance to prolonged antibiotic therapy, thus causing serious human and veterinary infections. In drinking water distribution systems and in industrial plants, bacteria can develop biofilms hundreds of microns thick, clogging filters and pipes and damaging materials. On the other hand, biofilms can perform a useful role in wastewater treatment plants and bioreactors, and in the bioremediation of polluted soils (Landini. and Zehnder, 2002).

Listeria monocytogenes like other foodborne pathogens can attach to surfaces and form biofilms. When biofilms are formed they are hard to remove by normal cleaning. It also protects the bacteria from antimicrobial or sanitizing agents and often serves as a source of recontamination for processed foods. *Listeria monocytogenes* on surfaces can be protected by presence of food components, and this protection increases as the layer of food increases. Comparison of phosphate- buffered saline (PBS) solution with various milk residues showed enhanced survival of *Listeria monocytogenes* on stainless steel and buna-n rubber with milk, and sometimes promoted growth of *Listeria* (Helke and Wong, 1994). Microorganisms embedded in biofilms are more resistant to heat, sanitizers, and other antimicrobial agents than are freely suspended cells. One of the most important mechanisms adopted by the bacteria to cling to the surface is the use of flagella. A study conducted by Vatanyoopaisarn et al. (2000) showed that at 22ºC a flagellum mutant form of *Listeria monocytogenes* attaches to stainless steel at a 10-fold level lower than wild-type cells even under conditions that prevent active motility. At 37º C when flagella were not produced, the attachment was identical, therefore, the flagella

independent of cell motility, did act as an adhesive structure during early stages of attachment under static conditions. In the area of food production, temperatures of 22 ºC and below are frequently encountered. Under such conditions *Listeria monocytogenes* will flagellate which aids in the attachment to stainless steal processing surface even if the surrounding environment dose not provide enough nutrient to allow the organism to be fully motile. Smoot and Pierson, (1998) allowed *Listeria monocytogenes* to form biofilms on stainless steel and Buna-N rubber surfaces at 10 to 45ºC for 120 min under varying pH values. The attachment to stainless steel was markedly more rapid for all temperatures, and the rate of attachment to Buna-N rubber was found to be significantly lower when cells were attached at 10ºC. Growth pH was also found to significantly affect rates of attachment to Buna-N rubber. Altering the medium pH during attachment between 4 and 9 demonstrated that rates of adhesion were slower under alkaline condition. In a mixed bacterial biofilm of *Listeria monocytogenes* and *Flavobacteruim* spp., the number of *Listeria monocytogenes* cells attaching to stainless steel increased significantly compared to the pure culture (Bremer et al., 2001).

The adhesion and subsequent development of *Listeria monocytogenes* on stainless steel was studied in the absence and in presence of a *Staphylococcus sciuri* biofilm. After 3 days of culture, *Staphylococcus* biofilm prevented the *Listeria monocytogenes* population from increasing within the biofilm, leading to an average difference of 0.9 to 2.7 CFU between the pure and mixed culture respectfully. A higher proportion of *Listeria monocytogenes* cells were observed in the planktonic phase in mixed cultures (Leriche and Carpentier, 2000).

Studies have indicated that bacteria in biofilm are more resistant to sanitizers. The antimicrobial effects of sodium hypochlorite, quaternary ammonium compound, and peroxyacetic acid on acid-adapted or nonadapted *Listeria monocytogenes* growing in biofilms on

the surface of beef was evaluated by Stopforth et al. (2002). Cells were exposed to sanitizers on days 2, 7, and 14. The pathogen formed biofilm of 5.3 log cfu/cm^2 by day 2 of storage, which was reduced to 4.6 log cfu/cm^2 by day 14. The biofilms were most sensitive to all the sanitizers on day 2, but their resistance increased during storage. Overall, there was no difference between acid – adapted and non-adapted *Listeria monocytogenes* with regard to sensitivity to sanitizers.

Chae and Schraft (2000) used thirteen *Listeria monocytogenes* strains to grow biofilms on glass surface in static conditions at 37ºC for up to 4 days. After 3 hours of incubation, bacterial cells for all 13 strains were found attached to glass slides and all strains formed a biofilm within 24 hours. The strains varied significantly in their ability to adhere to the surface. Also, a significant difference was noticed in cell numbers after 24 hour biofilm growth. Apart from that, the difference in biofilm growth was not related to variation in growth rates of planktonic cells suggesting that growth behavior of *Listeria monocytogenes* in biofilm may be different from their planktonic growth.

Hood and Zottola, 1997 studied biofilm formation in different growth media (tryptic soy broth, diluted TSB, 1% reconstituted milk and diluted meat juice) by *Salmonella typhimurim, Listeria monocytogenes, Escherichia coil* O157:H7, *Pseudomonas fragi,* and *Pseudomonas flurescens* on stainless steel. They found that the increase in the number of adherent cells over time was observed only with *Salmonella typhimurim* in diluted meat juice*, Escherichia coil* O157:H7 in tryptic soy broth and diluted meat juice, and *Pseudomonas fragi* or *Pseudomonas flurescens* in 1% reconstituted milk**.**

Two different surfaces hydrophilic (stainless steel) and hydrophobic (polytetrafluoroethylene) were examined for the growth and formation of biofilm of *Listeria monocytogenes* at 37 or 20ºC (Chavant et al., 2002). The colonization of the two surfaces was

monitored by a scanning electron microscope, or by enumeration of cells. After 5 days at both temperatures, the two strains were capable of colonizing both surfaces; however, biofilm formation was faster on the hydrophilic surface (Chavant et al., 2002).

Significant difference were found in a study demonstrating the ability of various *Listeria monosytogenes* strains to adsorb, attach, and form biofilms on a food grade stainless surface. With the exception of a single strain (Scott A), all other strains adhered as single cells and did not form biofilms. The strain demonstrating enhanced attachment produced exteracellular fibrils, whereas those that adhered poorly did not (Kalmokooff et al., 2001).

Bacteria encountered in food processing environments can be very hardy and difficult to eliminate (Wong, 1998). Bacterial attachment and subsequent survival involve interaction between other bacterial cells. *Listeria monocytogenes* can survive for prolonged periods on stainless steel and Buna N- rubber that are commonly used in food processing equipment. Temperature, relative humidity, soil, and surface affected the behavior of surface associated with *Listeria monocytogenes* (Wong, 1998). In addition, the nature of the attachment surface could affect the efficiency of sanitizers (Wong, 1998).

Temperature and moisture also affect survival of *Listeria monocytogenes* on surfaces. Palumbo and Williams (1990) suspended a mixture of several *Listeria monocytogenes* strains into either distilled water, tryptone broth, nonfat dry milk, canned milk, glycerol, light Karo syrup or beef extract, and then dried these cells suspensions on glass plates which were stored at 5 or 25ºC and 1 to 75% RH. Enhanced survival was observed at 5ºC rather than at 25ºC and at lower RH rather than higher RH.

2.8 LITERATURE REVIEW CONCLUSION

In conclusion, scientific information currently available indicates that *Listeria moncytogenes* can grow in the presence or absence of air and in food products at a pH between 4.5 and 9.2, in water activity above 0.92, and the organism is capable of proliferating at temperatures between –0.4 to 45˚C (ICMSF, 1996). It can also be concluded that *Listeria monocytogenes* can grow in the presence of high salt concentrations, and is relatively heat resistant, although the organism is killed by normal pasteurization process, and can survive for long periods of time in frozen or dried foods. Effective control of *Listeria monocytogenes* is challenging and requires extensive management and resources. Even though the risk of listeriosis is relatively low, the consequences are devastating for both the consumer and the food processor. *Listeria monocytogenes* is often present in raw ingredients. Because *Listeria monocytogenes* is heat resistant, adequate cooking is important to assure destruction of the organism. In addition, *Listeria monocytogenes* has the ability to attach to various surfaces and form biofilms and most of the time other microbes are associated with *Listeria monocytogenes* in biofilm formation on processing equipment. Post-contamination of ready-to-eat meats with *Listeria monocytogenes* is generally dependant on the properties of the biofilm, the density of the microbial population, attachment strength of the microbes, and the ability of microbes to produce an exopolysacchride.

Therefore, this study was designed to determine the effectiveness of copper, brass or concrete surface coatings containing copper ions in reducing or destroying *Listeria monocytogenes* grown in biofilms at different temperatures, relative humidity, nutrients, and pH in ready-to-eat meat processing plants.

2.9 REFERENCES

Annous, B. A., L. A. Becker, D. O. Bayles, D. P. Labeda, and B. J. Wilkinson. 1997. Critical role of anteiso-$C_{15:0}$ fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Appl. Environ. Microbiol*. 63:3887-3894.

Anonymous. 1991. *Listeria monocytogenes:* recommendations by the National Advisory Committee on microbiological criteria for foods. *Int. J. Food. Microbiol*. 14:185-246.

AOAC Official Method 992.18. 2000. MICRO-ID *Listeria*. Chapter 17.10.02, pp. 141- 144 In: Official Methods of Analysis of AOAC INTERNATIONAL. 17th Edition. W. Horwitz (ed.). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC INTERNATIONAL, Gaithersburg, MD.

Bajard, S., L. Rosso, G. Fardel, and J.P. Flandrois. 1995. The particular behaviour of Listeria monocytogenes under sub- optimal conditions. *Int. J. Food Microbi*. 29: 201-211.

Beal J. D. , S.J. Niven. Campbell, and P.H. Brooks. 2004. The effect of copper on the death rate of *Salmonella typhimurium* DT104:30 in food substrates acidified with organic acids. *Lettr. Appl. Microbio.* 38:1- 8.

Birgit, N. 2000. Microbiological criteria for *Listeria monocytogenes* in food under special consideration of risk assessment approaches. *Int. J. Food Microbiol*.62: 217-221.

Bojsen-Meller, J. 1972. Human listeriosis-diagnostic, epidemiological and clinical studies. *Acta Pathol. Microbiol.* Scand. Sec. B. (Suppl.) 229:1-157.

Bradshaw, J., J. Peeler, and R.Twedt. 1991. Thermal resistance of *Listeria Spp*. in milk. *J. Food Prot*. 54: 12-14.

Bremer, P. I. Monk, and C. Osborne. 2001. Survival of *Listeria monocytogenes* attached to stainless steel surface in the presence or absence of Flavobacterium spp..*J. Food Prot*. 64 (9): 1369-1376.

Brown, N. L., S. R. Barrett, J. Camakaris, B. T. Lee, and D. A. Rouch. 1995. Molecular genetics and transport analysis of the copper-resistance determinant (pco) from *Escherichia coli* plasmid pRJ1004. *Mol. Microbiol*. 17:1153-1166.

Chae, M. S., and H. Schraft. 2000. Comparative evaluation of adhesion and biofilm formation different *Listeria monocytogenes* strains. *Inter. J. Food Microbiol.* 62:103-111.

Chavant, P., B. Martinie, T. Meylheuc, and M. Bellon-Fantain. 2002. *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilm at different temperatures and growth phases. *Appl. Enviro. Microbiol*. 68(2): 728-737.

Colee, J.G., A. Fraser, and A.Simmons. 1997. *Listeria* and *Salmonella* in: *Practical medical microbiology.* Churchill livingstone. London.

Difco Manual. 1998. 11th ed. Difco Laboratories, Sparks, MD.

El-Gazzar, F.,and E. Marth. 1991. *Listeria monocytogenes* and listeriosis related to milk, milk preducts and dairy ingredients: A review 1. *Listeria monocytogenes*, listeriosis and responses of the pathogen to environmental conditions. *Milchwissenschaft. Milk scien. Inter*.46:14-19

El-Shenawy, M. A. and E. H. Marth, 1989. Inhibition or inavtivation of *Listeria monocytogenes* by sodium benzoate together with some organic acids. *J. Food Prot*. 52:771-776.

Faundez, G., and G. Figueroa. 2001. Evaluation of antibacterial activities of copper surfaces against Salmonella enterica and Campylobacter jejuni isolated from foods. *University of Chile preprint*

Fleming, D. W., K. Colchi, J. MnDonald, P. Brondum,, B. Hayes, M. Plikaytis, M. Holmes, A. Andurier, and C. Broome. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *National English Journal of Medicine*. 312:404-407

Feng, P. 1997. Impact of Molecular Biology on the Detection of Foodborne Pathogens. *Mol. Biotech.* 7:267-278.

Gay, M., and O. Cerf. 1997. Significance of temperature and preincubation temperature on survival of *Listeriamonocytogenes* at pH 4·8. *Lette. Appl. Microbiol.* 25 (4):257.

Gellin, B. G., and C. v. Broome. 1989. Listeriosis. *J.A.M.A*. 261:1313-1320.

Gray, M.L., and A. H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. *Bacteriol. Rev.* 30:309-382.

Harrison, W., A. Peter, and L. Fielding. 2000. Growth of *Listeria monocytogenes* and *Yersinia enterocolitica* colonies under modified atmospheres at 4 and 8˚C using model food system. *J. Appl. Microbiol.* 88:38-43.

Helke, D. M., and A. C. L. Wong. 1994. Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buna- n rubber. *J. Food Prot*. 57:963-968.

Henrike H. Wemekamp-Kamphuis, A. K. Karatzas, J. A. Wouters, and T. A. Enhanced. 2002. Levels of Cold Shock Proteins in Listeria monocytogenes LO28 upon Exposure to Low Temperature and High Hydrostatic Pressure. *Appl Environm Microbiol*. 68(2):456-463.

Hoshino, N., T. Kimura, F. Hayakawa, A. Yamaji, and T. Ando. 2000. Bactericidal activity of catechin-copper (II) complexes against *Staphylococcus aureus* compared with *Escherichia coli. Lette.Appl. Microbiol*. 31(3):213.

Hoshino, N, T. Kimura, A. Yamaji, and T. Ando. 1999. Damage to the cytoplasmic membrane of *E. coli* by catechin-copper (II) complexes . *Free Radical Biology and Medicine.* 27:1245 1250.

Hood, S. and E. A. Zottola. 1997. Adherence to stainless steel by foodborne microorganism during growth model food systems. *Int. J. Food. Microbiol*. 37:145-153.

ICMSF. 1996. Microorganisms in food 5 microbiological specifications of food pathogens. Blackie Academic and professional, an imprint of Chapman and Hill. London, UK.

Ikigai, H., T. Nakae, Y. Hara, and T. Shimamura. 1993. Bactericidal catechins damage the lipid bilayer. *Biochimca et Biophysica Acta.* 1147:132 136.

Juneja, V., and B. Eblen. 1999. Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl, and sodium pyrophospate as controlling factors. *J. Food Prot*. 62: 986-993.

Kalmokoff. M., J. W. Austin, X.-D. Wan, G. Sanders, S. Banerjee, and J.M. Farber. 2001. Adsorption, attachement and biofil formation among isolates of *Listeria monocytogenes* using model conditions. *J. Appl. Microbiol*. 91:725-734.

Kang, D. H., and D. Y. C. Fung. 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. *J. Food Prot*. 62:1346-1349.

Kang, D. H. and G. R. Siragusa. 1999. Agar underlay method for recovery of sublethally heat-injured bacteria. *Appl. Environ. Microbiol*. 65:5334-5337.

Kathariou, S., R. Kanenaka, R. D. Allen, A. K. Fork, and C. Mizumoto. 1995. Repression of motility and flagellin production at 37 degree C is stronger in *Listetia monocytogenes* than in the nonpathogenic species *Listeia innocua. Can. J. Microbiol*. 41: 572-577.

Landini,P. and A. Zehnder. 2002. The global regulatory hns gene negatively affects adhesion to solid surfaces by anaerobically grown *Escherichia coli* by modulating expression of flagellar genes and lipopolysaccharide production. *J. Bacteriology.* 184 (6):1522-1529.

Leelawatcharamas, V., L. G. Chia, P. Charoenchai, N. Kunajakr, C. Q. Liu, and N. W. Dunn. 1997. Plasmid-encoded copper resistance in *Lactococcus lactis*. *Biotechnol. Lett.* 19:639-643.

Leriche, V., and B. Carpentier. 2000. Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surface by *Staphylococcus Sciuri* biofilms. *J. Appl. Microbiol*. 88:594-605.

Miller, A. 1992. Combined water activity and solute effects on growth and survival of *Listeria monocytogenes* Scott A. *J. Food Prot*. 55:414-418.

Palumbo, S., and A. C. Williams. 1990. Effect of temperature, relative humidirty, and suspending menstrual on resistance of *Listeria monocytogenes* to drying. *J. Food Prot*. 53:377-381.

Premaratne, R. J., W. J. Lin, and E. A. Johnson. 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 57:3046-3048.

Rose, J. 1969. Copper in History & Distillation. Encyclopedia Brittanica, volume 6, Copper. http://www.aromaticplantproject.com/articles_archive/copper.html.

Rose, S.A., and M.F. Stringer. 1989. Immunological methods in: rapid methods in food microbiology: progress in industrial microbiology. M.R. Adams and C.F.A. Elsevier, New York.

Smoot, L., and M. Pierson. 1998. Influence of environmental stress on the kinetic ans strength of attachement of *Listeria monocytogenes* Scoot A to Buna- N rubberand stainless steel. *J Food Prot*. 61(10):1286-1292.

Solioz, M., and C. Vulpe. 1996. CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci*. 21:237-241

Sonohara, R., N. Muramatsu, H. Ohshima, and T. Kondo. 1995. Difference in surface properties between *Escherichia coli* and *Staphylococcus aureus* as revealed by electrophoretic mobility measurement . *Biophysical Chemistry*. 55:273- 277.

Stelma, G.N., A. L. Reyes, J. T. Peeler, D. W. Francis, J. M. Hunt, P. L. Spaulding, C. H. Johnson, and J. Lovett. 1987. Pathogenicity testing for *L. monocytogenes* using immunocompromised mice. *J. Clin. Microbiol*. 25:2085-2089.

Steven, A., and J. Lowe. 1995. *Listeria monocytogenes*, and *Salmonella* typhi infection in: *Pathology*. Mosby, Times Mirror International Publisher Ltd. London . U.K.

Stopeforth, J.D., J. Samelis, J. Sofos. P. Kandal, and G. Smith. 2002. Biofilm formation by acid-adapted and nonadapted *Listeria monocytogenes* in fresh beef decontamination washings and its subsequent inactivation with sanitizers. *J.Food Prot*. 65 (11):1717-1727.

Teo, A., and S. Knabel. 2000. Development of a simple recovery-enrichment system for enhanced detection of heat-injured *Listeria monocytogenes* in pasteurized milk. *J. Food Prot.*63: 462-472.

Tichy, S.J. 1975. The influence of some cations on bacterial endotoxins: copper. *Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene Series A—Medical III* 231:259 -268.

Vannetten, P., J. Perales, G. Curtis, and A. Mossel. 1989. Liquid and solid selective differential media for the detection and enumeration of *Listeria monocytogenes*. - *Int. Food Microbiol.,* 8: 299-316.

Vatanyoopaisarn, S., A. Nazli, and C. Dodd. 2000. Effect of flagella on initial attachement of *Listeria monocytogenes* to stainless steel. *Appl. Envirom. Microbiol*. 66 (2): 860-863.

Walter, F. 2000. Food borne Listeriosis.*Clinic. Infect. Disease*. 31: 770-775.

Wijtzes, T., P. McClure, M. Zwietering, and T.Roberts.1993. Modelling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. *Intl. J. Food Microbiol*. 18: 139-149.

Wong, A. C. L. 1998. Biofilm in food processing environments. *J. Dairy. Sci*. 81:2765-2770.

CHAPTER 3

ANTIMICROBIAL EFFECT OF COPPER AND BRASS IONS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AT DIFFERENT TEMPERATURES, PH AND NUTRIENTS

3.1 INTRODUCTION

In the 1900s, *Listeria monocytogenes* was known as a bacterium that caused illness in cattle. In recent times, it has been identified as the cause of listeriosis in humans. Healthy individuals affected by *Listeria monocytogenes* generally only experience mild flu-like symptoms (Neunlist et al., 2005). Victims of severe listeriosis are usually immunocompromised people such as cancer patients, individuals taking drugs that affect the body's immune system, alcoholics, pregnant women, and individuals infected with AIDS (Hitchins, 1998).

The greatest risk of listeriosis is from ready-to-eat products that do not require further cooking. In raw food that has to be cooked before consumption, there is less risk of *Listeria monocytogenes* to the food industry because the bacterium is destroyed on exposure to high temperatures during the cooking process. *Listeria monocytogenes* has been isolated from raw fish, cooked crabs, raw and cooked shrimp, raw lobster, and smoked fish (Ward et al., 1997). *Listeria monocytogenes* can be destroyed by thoroughly cooking and by preventing cross-contamination once the food is cooked. After ingestion of food contaminated with *Listeria monocytogenes*, incubation periods for infection are usually in the range of 4 to 21 days. *Listeria monocytogenes* can become systemic thereby invading the central nervous system, heart, eyes, or other locations in the human body (Bryan, 1999). Approximately 2,500 cases of Listeriosis are known to occur annually in the United States. However, it is likely that many of the cases are not recognized. Every year, about 500 deaths are attributed to listeriosis (FDA/CFSAN, 2003).

Apart from the fact that this pathogen is becoming increasingly dangerous, *Listeria monocytogenes* also has the ability to adapt further to its environment. *Listeria monocytogenes* can enter the food processing environment through incoming food materials such as shoes and clothing of personnel. Once *Listeria monocytogenes* is in the food-processing environment, it may adhere

itself to food contact and non-food contact surfaces such as floors and floor drains (FSIS, 2001). The USDA and FDA are working to establish routine standards to control *Listeria monocytogenes* in processing facilities of ready-to-eat (RTE) products by monitoring the plants and food regularly. If it is determined that a food is contaminated, monitoring and inspections are intensified and the product may be recalled to protect consumers (Schmidt and Rodrick, 2003).

This study evaluated the effectiveness of copper and brass sheets in reducing or destroying *Listeria monocytogenes* growing in biofilms at different temperatures, nutrient levels, and pH levels.

3.2 MATERIALS AND METHODS

3.2.1 Cultures Growth Conditions

The *Listeria monocytogenes* strain (V7 serotype ½ a) used during this study was obtained from the Center for Disease Control, Atlanta GA., USA and was originally isolated from the blood of an infected individual. The bacteria was cultivated for 16 hours in Brain Heart Infusion Broth (BHI) (Difco, Detroit, Mich., U.S.A.) at 37°C and the pure cultures were stored at -70°C and then sub-cultured twice in BHI broth at 37°C for 16 hours before using.

3.2.2 Preparation of Media

To determine if *Listeria monocytogenes* cells were injured by the copper ions, we used Oxford agar with antibiotic supplement (25 ml) poured into Petri dishes and allowing the media to solidify. To simulate biofilm production, modified Welshimer's (MW) soft agar was specially formulated for generating bacterial growth similar to that occurs in biofilms. Modified Welshimer soft agar was prepared as follows: Na_2HPO_4 (30.96 g) and KH_2PO_4 (6.56 g) were dissolved in 500 ml distilled water then autoclaved, $MgSO_4$ (0.41 g) was dissolved into distilled water then autoclaved, glucose (10 g) was dissolved into distilled water then filter sterilized, the

amino acid (L-leucine, L-isoleucine , L-arginine, L-methonine and L-valine) 0.1 g each were dissolved into 100 ml distilled water then filter sterilized, L-cysteine and L-glutamine (0.1 and 0.5 g, respectively) were dissolved into distilled water then filter sterilized, ferric citrate (0.088 g) was dissolved into distilled water then filter sterilized, vitamins (Riboflavin 0.5 mg, biotin 0.5 mg, thiamine 1.0 mg and thioctic acid 0.005 mg) were dissolved into distilled water separately and then filter sterilized. The above chemicals were mixed together on the day of the experiments with 15 g of technical agar and the volume was brought up to 1000 ml with distilled water. Modified Welshimer's (MW) soft agar pH was 6.6 (Premaratne et al., 1991).

3.2.3 Preparation of Samples

Copper and brass were cut into 3" disks (Thomas Industrial Network, User Services, 5 Penn Plaza, New York, NY 10001) sterilized by autoclaving, and placed into Petri dishes. The bacteria were grown for 16 hours in BHI broth then serially diluted to 10^7. One ml of the culture was then added to 9 ml of MW soft agar and then poured over the metal disks. In order to prevent drying of MW soft agar, each 50 cm diameter Petri dish was placed into another Petri dish that was 120 cm in diameter, containing 25 ml of sterile dH_2O.

3.2.4 Bacterial Analysis

Bacterial counts were determined by collecting the MW soft agar and then placing it into sterile bags. Ten ml of Phosphate Buffered Saline (PBS) was added to each sample then the samples were stomached for 1 minute. The samples were decimally diluted in PBS and the dilutions were plated onto Oxford media. The plates were incubated under aerobic conditions at 37°C for 24 hours and the log CFU/g was determined. At the end of each experiment, 1 ml of all treatments at non-detectable levels were inoculated into 9 ml of BHI broth and incubated for 48

hours at 37°C to make sure that all the bacteria were killed. The results in each BHI tube were negative and no *Listeria monocytogenes* were found.

3.2.5 Evaluate the Effect of Temperatures on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* Grown on the Surface of Copper or Brass Alloys

This study evaluated the effect of temperature (4, 25, or 37°C) on the reduction of *Listeria monocytogenes* when grown in MW soft agar. The bacterial counts were determined at 0, 2, 4, 6 and 8 days for 4°C and 0, 1, 2 and 3 days for 25 and 37°C.

3.2.6 Evaluate the Effect of pH on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* Grown on the Surface of Copper or Brass Alloys

A variety of pH levels (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0) were used for this experiment. The pH of MW soft agar was adjusted form 3.0 to 9.0 with either H_3PO_4 or NaOH. Nine ml of the agar inoculated with 1 ml of diluted *Listeria monocytogenes*, than poured on the surface of the copper or brass and stored at 4°C. Bacterial counts were determined after 24 hours of incubation.

3.2.7 Evaluate the Effect of Different Nutrients on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* Grown on the Surface of Copper or Brass Alloys

The levels of components in the MWB were varied to determine the effect of growth nutrients on the antimicrobial activity of copper ions against *Listeria monocytogenes*. Glucose was evaluated at concentrations of 1.0, 10, and 20 g/L. The glucose in MW soft agar was also replaced with cellobiose, fructose, or mannose at a concentration of 10 g/L. Phosphate levels were either reduced by one-fold or increased by five-fold from the control levels in the MW soft agar. The levels of amino acids (L-leucine, L-isoleucine, L-arginine, L-methonine, L-cysteine, L-glutamine and L-valine) were either decreased by one tenth or increased by five-fold from the control MW soft agar. Amino acids were also replaced in MW soft agar with 17.0 g/L of tryptone (Kim and

Frank, 1995). The samples were stored at 37°C and bacterial counts were determined every 24 hours for 2 days.

3.2.8 Statistical Analysis

The effects of temperatures, pH and nutrients on the antimicrobial activity of copper ions against *Listeria monocytogenes* grown in MW soft agar on the surface of copper or brass metals were analyzed by statistical comparisons of all pairs using Student's t-test, following one way analysis of variance (ANOVA) (SAS® Version 9.1.2, SAS Institute Inc., Cary, N.C., USA). The level of statistical significance was chosen to be 0.05. All experiments were repeated 3 times.

3.3 RESULTS

3.3.1 Evaluate the Effect of Temperatures on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* Grown on the Surface of Copper or Brass Alloys

The first 3 figures show the effects of temperatures on the antimicrobial activity of copper and brass (brass contains 60% copper, 30% zinc and 1-2% lead) against *Listeria monocytogenes.* It can be seen that during the first 4 hours *Listeria monocytogenes* counts, on the surface of copper and brass, were significantly lower than the controls at 4°C (Figure 3.1). Later, the *Listeria monocytogenes* counts were further reduced from 8 thru 24 hours, for both metals, by 4 log compared to the control; furthermore, by 72 hours, *Listeria monocytogenes* counts had reached non-detectable levels on the surface of copper and brass metals.

At 25°C *Listeria monocytogenes* counts, on the surface of copper and brass, were reduced by 2 logs in 2 hours and by 4 logs between 4 and 6 hours compared to the control counts (Figure 3.2). From 8 hrs to 24 hours, *Listeria monocytogenes* counts had reached non-detectable levels while the control was about 6 logs CFU/g. (Figure 3.2).

Figure 3.3 shows the antimicrobial effects of copper and brass against *Listeria monocytogenes* at 37°C. From 2 to 4 hours, *Listeria monocytogenes* counts on the surface of both metals were significantly lower as compared to the control counts at 37ºC. *Listeria monocytogenes* counts had reached non-detectable levels in MW soft agar on the surface of copper and brass by 6 hours through 24 hours at 37ºC (Figure 3.3).

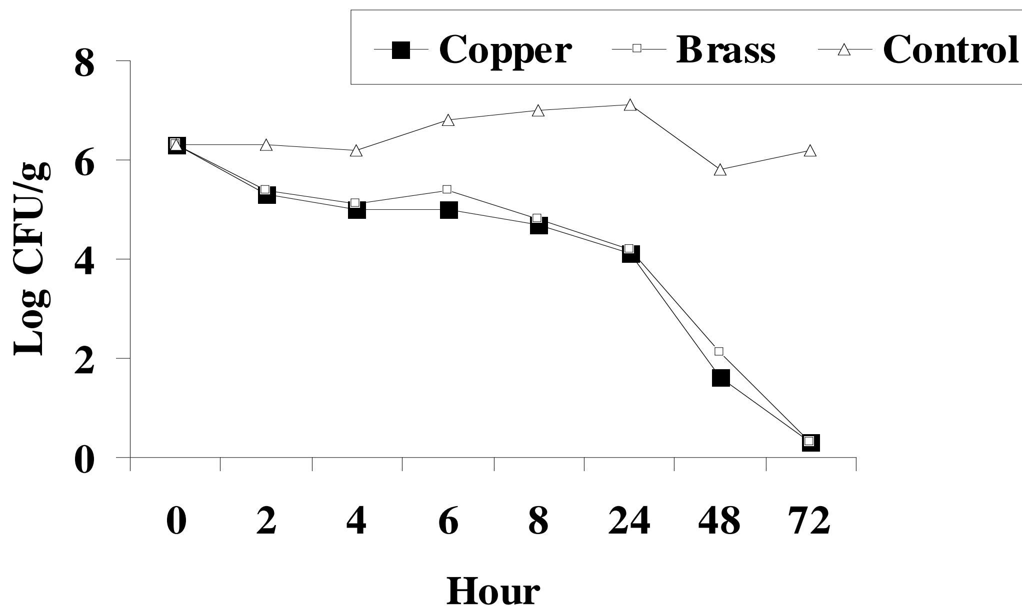

Figure 3.1. ***Listeria monocytogens*** **in MW soft agar poured on the surface of copper and brass alloys that were incubated at 4ºC**

3.3.2 Evaluate the Effect of pH on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* Grown on the Surface of Copper or Brass Alloys

This portion of the study evaluated the effect of pH on the antimicrobial activity of copper and brass alloys. *Listeria monocytogenes* counts on the surface of brass and copper were reduced to non-detectable levels within 2 hours in MW soft agar with a pH of 3 (Figure 3.4). At a pH of 4, *Listeria monocytogenes* counts on the surface of copper and brass reached non-

detectable levels within 2 hours, whereas it took 24 hours for the control *Listeria monocytogenes* counts to reach the non-detectable levels (Figure 3.5).

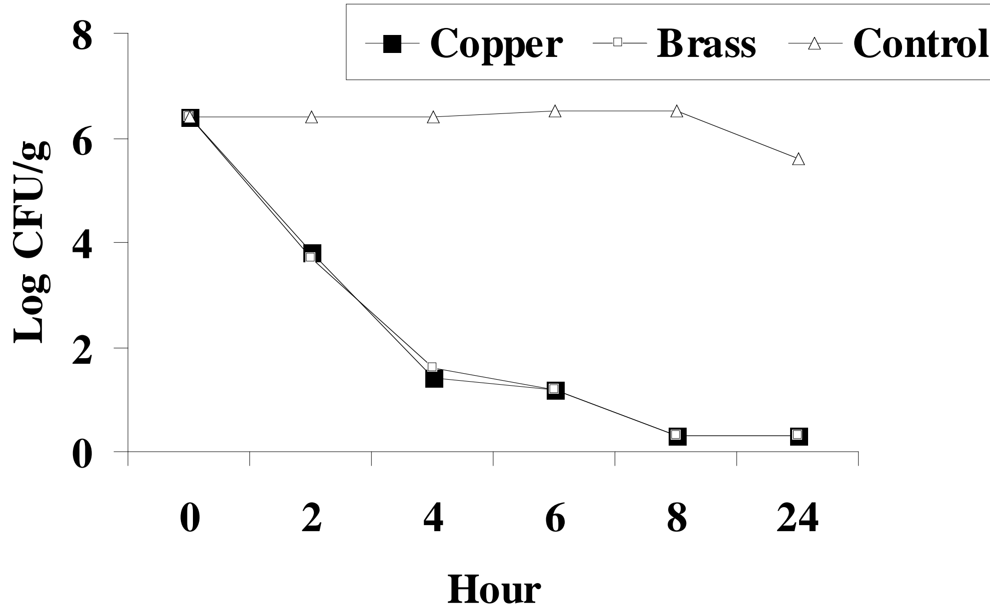

Figure 3.2. *Listeria monocytogens* in MW soft agar poured on the surface of copper and brass alloys that were incubated at 25º C

At a pH of 5 or 6, *Listeria monocytogenes* counts on the surface of copper and brass were reduced to non-detectable levels within 2 hours and the control. *Listeria monocytogenes* counts were at 4 logs at a pH of 5 and 5 logs at a pH of 6 throughout the 24 hours (Figures 3.6 and 3.7).

Listeria monocytogenes counts reached non-detectable levels when they were grown on the surface of copper within 2 hours at a pH of 7, 8 or 9, but when grown on the surface of brass, *Listeria monocytogenes* counts reached non-detectable levels in 4 hours at pH 7, 8 or 9 (Figures 3.8, 3.9 and 3.10).

3.3.3 Evaluate the Effect of Different Nutrients on the Antimicrobial Effects of Copper Ions Against *Listeria monocytogenes* on the Surface of Copper or Brass Alloys

A chemically defined minimal medium for *Listeria monocytogenes* (Modified Welshimer's soft agar, MW) was used, which contains all of the growth factors required for

Listeria monocytogenes, such as carbon source, amino acids, and vitamins (Premaratne et al., 1991).

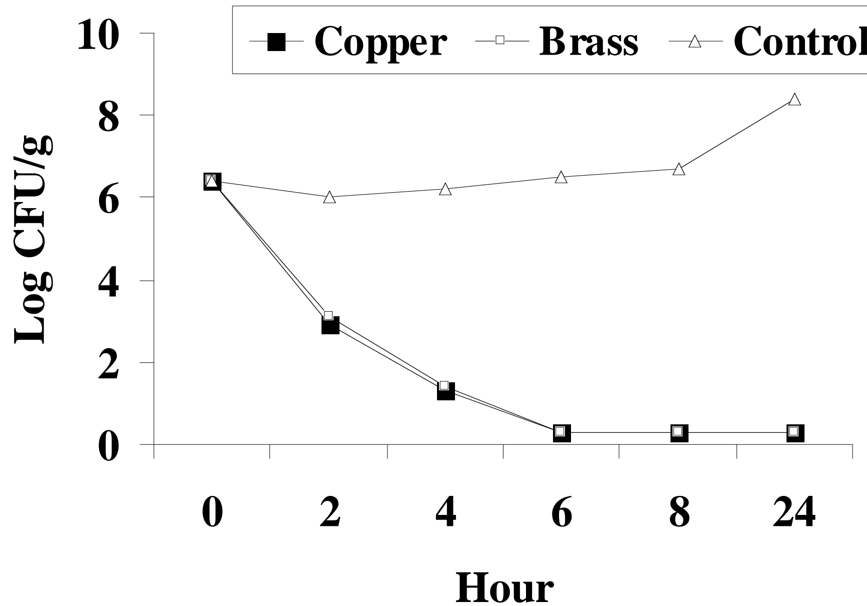

Figure 3.3. *Listeria monocytogens* in MW soft agar poured on the surface of copper and brass alloys that were incubated at 37º C

In this study some modifications, in the required sources, were made by increasing, decreasing, or replacing the required compounds in MW soft agar to examine if the availability of nutrients made *Listeria monocytogenes* more sensitive or resistant to the antimicrobial effect of copper ions.

Table 3.1 shows the effects of different concentrations of sugars in MW soft agar (cellobiose, fructose, mannose, and glucose as a control) on the antimicrobial activity of copper ions against *Listeria monocytogenes*.

At 24 hours *Listeria monocytogenes* grown on copper had bacterial counts of 2.4 log CFU/ml in MW soft agar with glucose, 2.9 log CFU/ml in MW soft agar with fructose, 4.1 log

CFU/ml in MW soft agar with mannose, and 4.3 log CFU/ml in MW soft agar with cellobiose. By 48 hours, *Listeria monocytoenes* counts were at non-detectable levels in all MWB sugar treatments on the surface of copper.

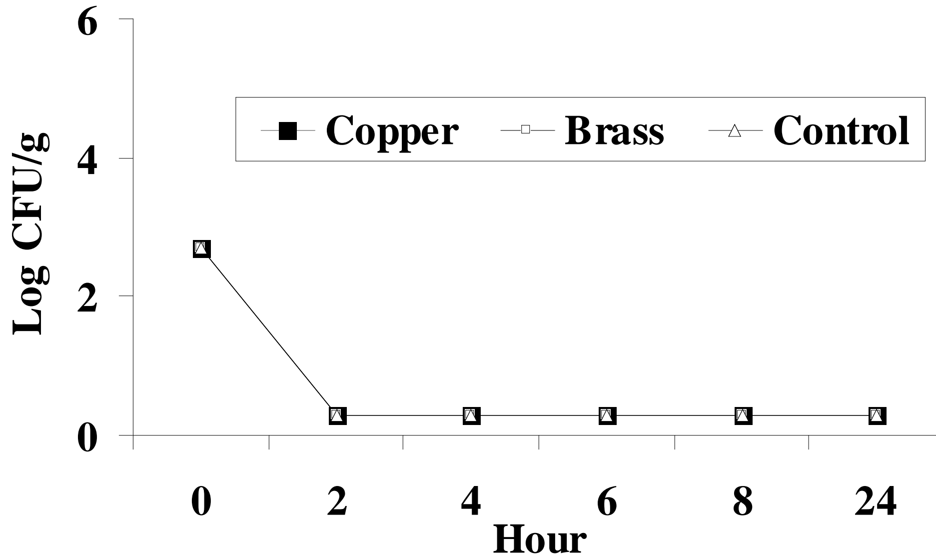

Figure 3.4. *Listeria monocytogens* in MW soft agar with a pH of 3 poured on the surface of copper and brass alloys that were incubated at 4° C

Conversely, by 48 hours, *Listeria monocytogenes* on the surface of brass was reduced to non-detectable levels in MW soft agar with glucose, 2.1 log CFU/ml in MW soft agar with mannose, 2.6 log CFU/ml in MW soft agar with cellobiose, and 2.9 log CFU/ml in MW soft agar with fructose.

Table 3.2 shows the effects of different concentrations of glucose in MW soft agar. The results showed that if the concentration of glucose in MW soft agar 10g was reduced to1g or increased to 20g, the antimicrobial activity of the copper and brass against *Listeria monocytogenes* was significantly reduced by 1.2 and 1.8 log CFU/g respectively.

Table 3.3 shows the effect of amino acid concentration in MW soft agar on the antimicrobial activity of copper ions against *Listeria monocytogenes*. After 24 hours of storage

with regular MW soft agar (control), *Listeria mocytogenes* counts had significantly decreased to 1.62 log CFU/g on the surface of brass and 3 log CFU/g on the surface of copper.

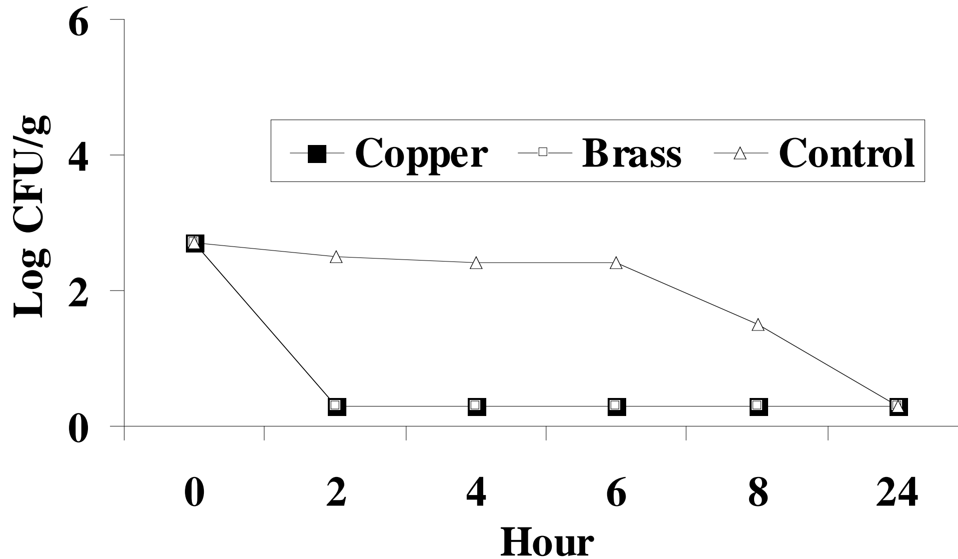

Figure 3.5. *Listeria monocytogens* in MW soft agar with a pH of 4 poured on the surface of copper and brass alloys that were incubated at 4º C

Furthermore, 2 to 3 log reductions of *Listeria monocytogenes* were found on the surface of copper or brass with MW soft agar containing one-tenth of the amino acid concentration, while with five-fold increased amino acid concentration, copper showed a 1.5 log CFU/g reduction (Table 3.3).

When five-fold of amino acid as added to MW soft agar, there were no significant difference in *Listeria monocytogenes* counts between brass and the regular MW soft agar. However, when tryptone was replaced with amino acids in MW soft agar, both copper and brass reduced *Listeria monocytogenes* counts to non-detectable levels.

After 48 hours, *Listeria monocytogenes* counts on the surface of copper and brass were reduced to non-detectable levels when amino acids were replaced with tryptone in MW soft agar.

However, with one-tenth and five-fold of the amino acid concentrations in MW soft agar, the antimicrobial activity of copper and brass against *Listeria monocyogens* was reduced (Table 3.3).

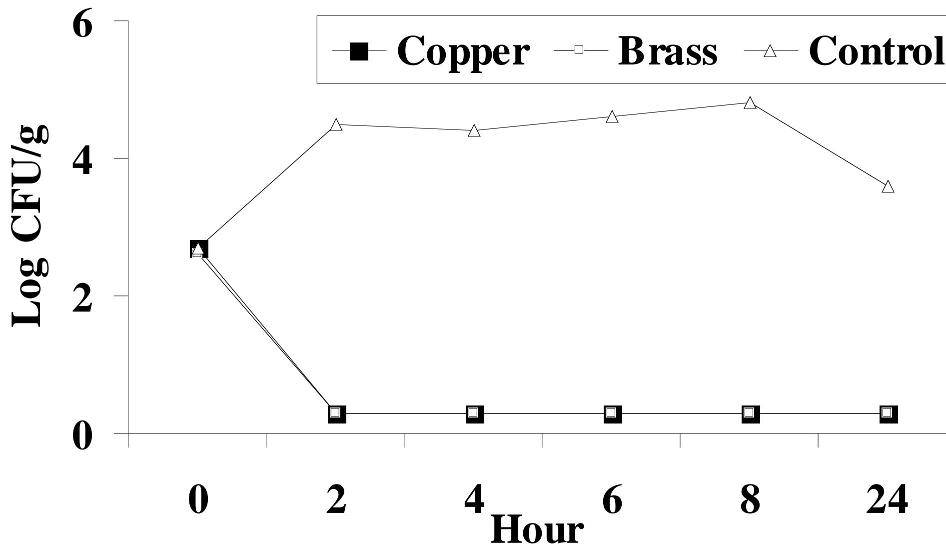

Figure 3.6. *Listeria monocytogens* in MW soft agar with a pH of 5 poured on the surface of copper and brass alloys that were incubated at 4º C

Table 3.4 shows the effects on the antimicrobial activity of copper or brass against *Listeria monocytogenes* with different concentrations of phosphate in MW soft agar (reducing by one-half and increasing two-fold). After 24 hours of storage with twice the normal phosphate concentration in MW soft agar, *Listeria monocytogenes* counts on the surface of both metals were reduced to non-detectable level.

After 48 hours, the *Listeria monocytogenes* counts were reduced to non-detectable levels in all treatments on the surface of the copper alloys. On the surface of brass, the *Listeria monocytogenes* counts were only reduced by 2 logs from controls at half the usual phosphate concentration in MW soft agar, and to non-detectable levels in twice the usual phosphate concentration in MW soft agar.

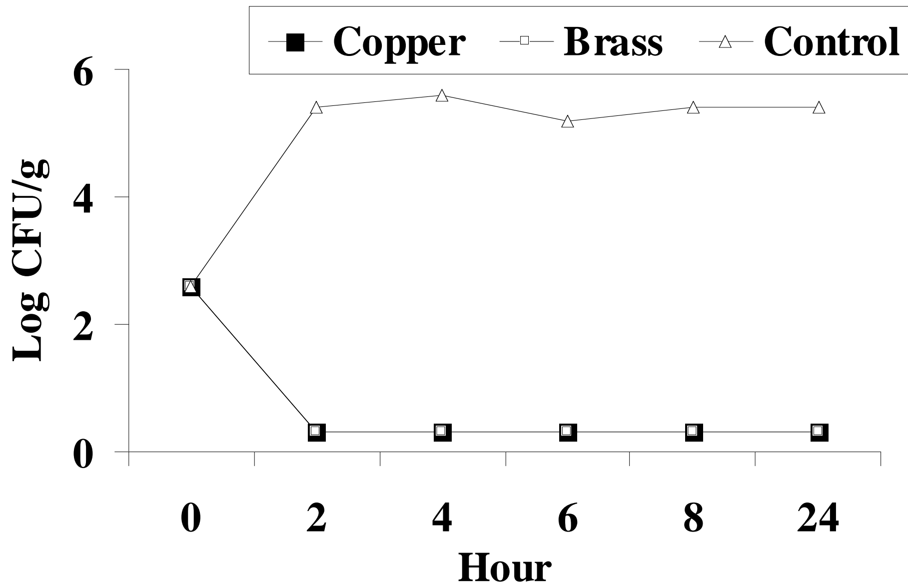

Figure 3.7. ***Listeria monocytogens*** **in MW soft agar with a pH of 6 poured on the surface of copper and brass alloys that were incubated at 4º C**

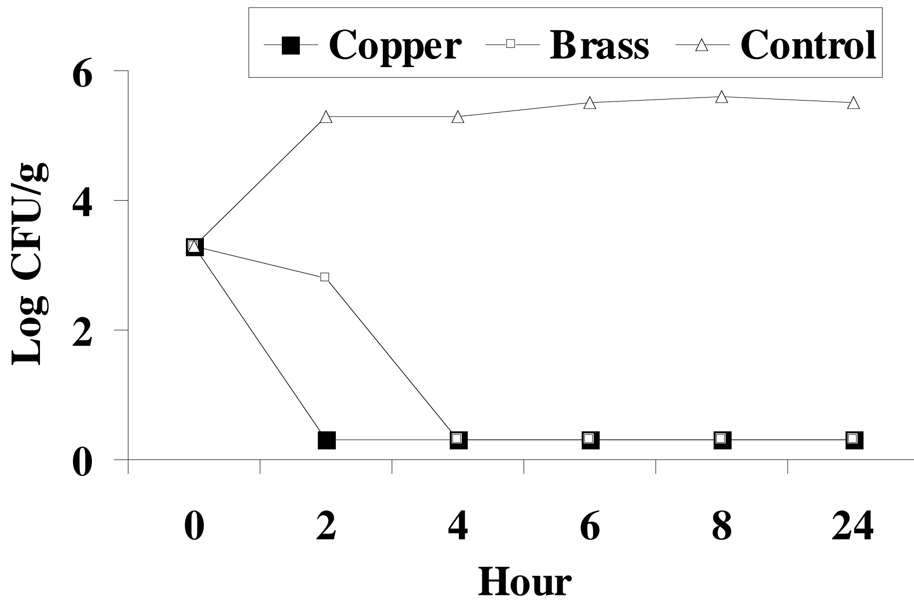

Figure 3.8. ***Listeria monocytogens*** **in MW soft agar with a pH of 7 poured on the surface of copper and brass alloys that were incubated at 4º C**

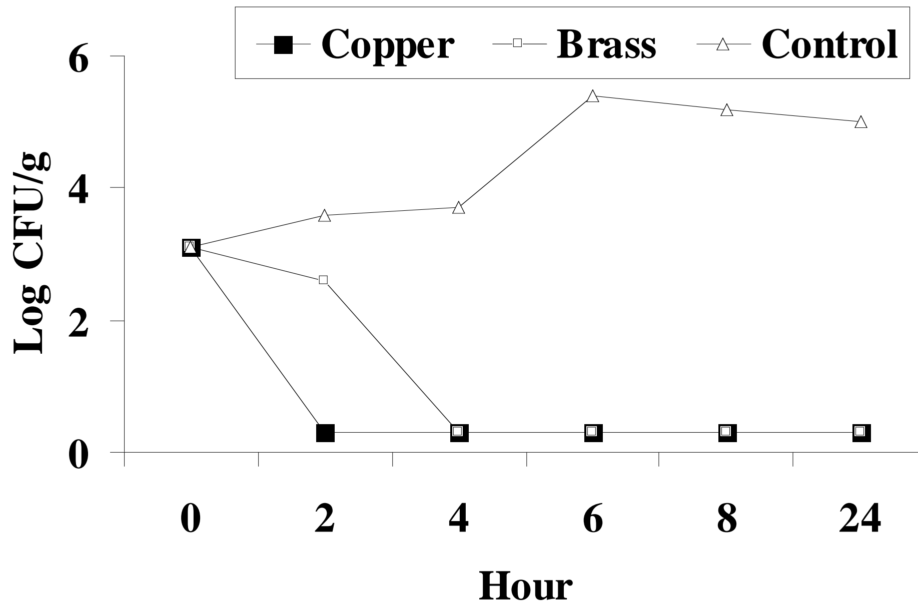

Figure 3.9. ***Listeria monocytogens*** **in MW soft agar with a pH of 8 poured on the surface of copper and brass alloys that were incubated at 4º C**

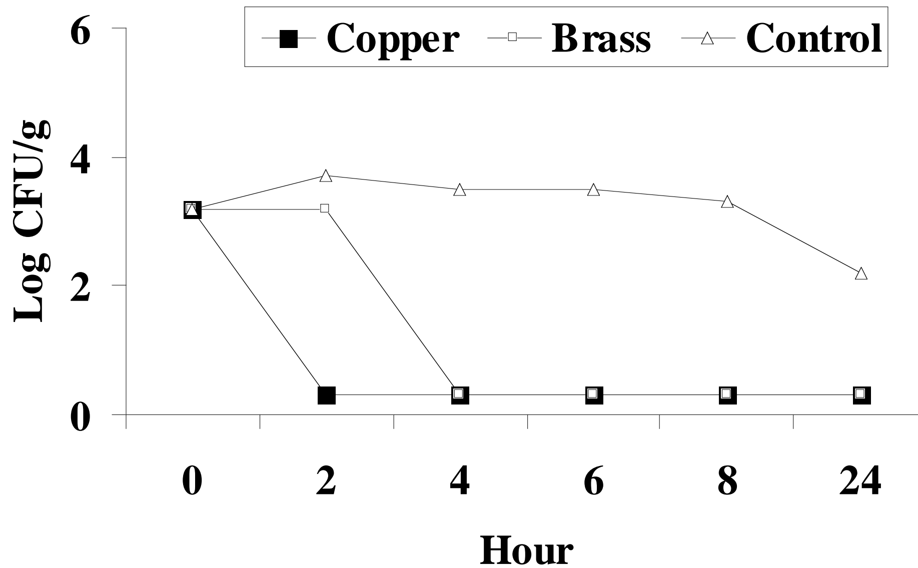

Figure 3.10. ***Listeria monocytogens*** **in MW soft agar with a pH of 9 poured on the surface of copper and brass alloys that were incubated at 4º C**

Table 3.1. ***Listeria monocytogenes*** **grown at 37ºC for 48h in MW soft agar containing different sugars on the surface of copper or brass alloys.**

		Log CFU/g [b]	
	Treatment [c]	24 Hour	48 Hour
Glucose	Control	6.30 ± 0.70 A B	6.85 ± 0.29 A
	Copper	2.35 ± 0.29 D	0.00 ± 0.00 D
	Brass	1.75 ± 0.07 E	0.00 ± 0.00 D
Cellobiose	Control	6.35 ± 0.10 B	6.70 ± 0.24 A
	Copper	4.30 ± 0.14 C	0.00 ± 0.00 D
	Brass	6.05 ± 0.07 B	2.60 ± 0.13 C
Fructose	Control	6.85 ± 0.07 A	5.60 ± 0.27 B
	Copper	2.85 ± 0.21 D	0.00 ± 0.00 D
	Brass	5.45 ± 0.29 B	2.10 ± 0.14 C
Mannose	Control	6.95 ± 0.07 A	6.70 ± 0.23 A
	Copper	4.05 ± 0.10 C	0.00 ± 0.00 D
	Brass	6.15 ± 0.21 B	2.10 ± 0.21 C

[a] Initial inoculation at time zero was 6.32 Log CFU/g.

[b] All analyses were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($p \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different sugars were substituted for glucose (Glucose (control) Cellobiose, Fructose and Mannose) in MW soft agar (10g/L), inoculated with *Listeria monocytogenes* and poured over the surface of Petri dishes (control) copper and brass alloys.

Table 3.2. ***Listeria monocytogenes*** **grown at 37°C for 48h in MW soft agar containing different concentration of glucose on the surface of copper or brass alloys**

	Treatment [b]	Log CFU/g [a]		
		0 Hour	24 Hour	48 Hour
1g Glucose	Control	5.70 ± 0.20 A	6.70 ± 0.40 A	7.50 ± 0.32 B
	Copper	5.70 ± 0.20 A	2.95 ± 0.10 F	1.15 ± 0.12 E
	Brass	5.70 ± 0.20 A	4.60 ± 0.20 D	3.05 ± 0.10 C
10g Glucose	Control	5.50 ± 0.25 B	5.90 ± 0.09 B	7.00 ± 0.09 A
	Copper	5.50 ± 0.25 B	1.95 ± 0.67 E	0.00 ± 0.00 F
	Brass	5.50 ± 0.25 B	1.60 ± 0.14 E	0.00 ± 0.00 F
20g Glucose	Control	5.35 ± 0.10 A B	5.40 ± 0.21 C	7.90 ± 0.21 A
	Copper	5.35 ± 0.10 A B	3.70 ± 0.07 G	1.80 ± 0.27 C
	Brass	5.35 ± 0.10 A B	3.65 ± 0.10 G	2.95 ± 0.10 D

[a] All analysis were based on three separate experiments with each mean $\pm$ standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[b] Different concentrations of glucose (1g/L, 10g/L (control) and 20/Lg) in MW soft agar inoculated with *Listeria monocytogenes* were poured on the surface of Petri dishes (control) copper and brass alloys.

Table 3.3. *Listeria monocytogenes* grown at 37°C for 48h in MW soft agar containing different concentration of amino acid on the surface of copper or brass alloys

	Treatment [b]	Log CFU/g [a]		
		0 Hour	24 Hour	48 Hour
1.2 g Amino Acid	Control	6.30 ± 0.05 A	6.70 ± 0.20 A	6.85 ± 0.29 A
	Copper	6.30 ± 0.05 A	2.45 ± 0.10 F	0.00 ± 0.00 F
	Brass	6.30 ± 0.05 A	1.65 ± 0.18 G	0.00 ± 0.00 F
Tryptone	Control	5.95 ± 0.19 B C	3.30 ± 0.20 E	6.25 ± 0.41 A B
	Copper	5.95 ± 0.19 B C	0.00 ± 0.00 H	0.00 ± 0.00 F
	Brass	5.95 ± 0.19 B C	0.00 ± 0.00 H	0.00 ± 0.00 F
0.12 g Amino Acid	Control	6.15 ± 0.07 A B	6.25 ± 0.05 B	7.05 ± 0.30 A
	Copper	6.15 ± 0.07 A B	4.25 ± 0.13 D	4.50 ± 0.21 E
	Brass	6.15 ± 0.07 A B	5.25 ± 0.14 C	5.25 ± 0.49 CDE
6.0 g Amino Acid	Control	5.85 ± 0.10 C	6.95 ± 0.07 A	7.00 ± 0.39 A
	Copper	5.85 ± 0.10 C	5.25 ± 0.23 C	5.30 ± 0.19 C D
	Brass	5.85 ± 0.10 C	6.15 ± 0.05 B	6.10 ± 0.20 B C

[a] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within each vertical column (sampling hour) followed by different letters are significantly different ($p \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[b] 0.12 g/L, 1.2 g/L (control) and 6.0 g/L of amino acids (L-leucine, L-isoleucine, L-arginine, l-methionine, L-cysteine, L-glutamine and L-valine) or tryptone(17g/L) replaced in MW soft agar were inoculated with *Listeria monocytogenes* on the surface of Petri dishes (control) copper and brass alloys.

Table 3.4. ***Listeria monocytogenes*** **grown at 37°C for 48h in MW soft agar containing different concentration of phosphate on the surface of copper or brass alloys**

	Treatment [b]	Log CFU/g [a]		
		0 Hour	24 Hour	48 Hour
37.52 g Phospate	Control	6.25 ± 0.13 A	6.65 ± 0.14 B	6.85 ± 0.22 A
	Copper	6.25 ± 0.13 A	2.35 ± 0.21 E	0.00 ± 0.00 D
	Brass	6.25 ± 0.13 A	1.75 ± 0.14 F	0.00 ± 0.00 D
18.76 g Phosphate	Control	6.15 ± 0.10 A	7.15 ± 0.30 A	6.40 ± 0.40 A
	Copper	6.15 ± 0.10 A	4.15 ± 0.19 D	0.00 ± 0.00 D
	Brass	6.15 ± 0.10 A	5.40 ± 0.20 C	4.80 ± 0.10 B
75.04 g Phosphate	Control	6.15 ± 0.17 A	6.85 ± 0.10 A	3.40 ± 0.20 C
	Copper	6.15 ± 0.17 A	0.00 ± 0.00 G	0.00 ± 0.00 D
	Brass	6.15 ± 0.17 A	0.00 ± 0.00 G	0.00 ± 0.00 D

[a] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[b] Different concentrations of phosphate (Sodium phosphate dibasic and Potassium phosphate monobasic) with 37.52 g/L (control), 18.76 g/L and 75.04 g/L in MW soft agar inoculated with *Listeria monocytogenes* on the surface of Petri dishes (control) copper and brass alloys.

3.4 DISCUSSION

Listeria monocytogenes is an important issue to the meat processing industry since it can grow on ready-to-eat meat products at refrigerator temperatures (FDA, 1999). This organism is commonly found in many environments and most contamination of cooked ready-to-eat foods appears to originate from the food processing plant environment. Copper and copper-based alloys have long been known for their bacteriostatic properties, but their importance in possibly controlling foodborne bacteria in the food processing environment has not been evaluated. We have found that *Listeria monocytogenes* counts reached non-detectable levels when grown in a nutrient rich media on the surface of copper or brass. These results suggest that copper and brass might be able to control *Listeria monocytogenes* in the food processing environment.

Our results indicated that the antimicrobial activity of copper ions against *Listeria monocytogenes* was affected by temperature. The *Listeria monocytogenes* counts at 4°C, on the surface of copper and brass reduced to non-detectable levels within 72 hours. At 25°C, *Listeria monocytogenes* counts had reached non-detectable levels in 8 hours when grown on the surface of copper or brass. At 37ºC *Listeria monocytogenes* counts on the surface of the copper or brass were reduced to non-detectable levels within 6 hours of incubation. Similar results were obtained with *E. coli* O157:H7 on the surface of copper and brass incubated at 4 or 20ºC. The highly toxic *E. coli* O157:H7 strain of bacteria survived for less than 6 hours at 20ºC and less than 16 hours at 4ºC on copper or brass surfaces than on stainless steel. The *E. coli* O157:H7 was detected on the stainless steel surface for up to 60 days at 4ºC and for up to 34 days at 20ºC (Keevil et al., 2000). These results could be explained by the amount of copper ions that are released into the surrounding environment; as the temperature increases so does the amount of copper ions released from the copper or brass (see Chapter 6).

Several studies investigated the antimicrobial activity of zinc against oral bacteria in dentistry devices.

Pizzo and others (2005) investigated the effect of two zinc oxide eugenol used in endodontic sealers against *Enterococcus faecalis*. *Enterococcus faecalis* was placed on the zinc sealers for 20 minutes, 24 hours and 7 days at 37ºC. Zinc sealers showed complete inhabitation of bacteria growth. Also another study was done by Surjawijaja and other (2004) evaluated the effect of Mueller-Hinton agar containing different concentrations of zinc sulfate (0.2, 0.4, 0.8, 1.4 and 2 mg/ml) against *Salmonella typhi*, *Escherichia coli, Enterobacter, Shigella* and *Vibrio cholerae.* All enteric pathogens tested were inhibited by zinc sulfate 0.2 mg/ml. *S. typhi* was inhibited by zinc sulfate of 0.8 mg/ml. *V. cholerae* were inhibited at concentrations between 1.4 and 2.0 mg/ml. *Shigella flexneri* demonstrated a similar pattern of inhibition to *S. typhi.* Furthermore, stainless steel surfaces coated with paints containing a silver and zinc were inoculated with vegetative cells of three *Bacillus* species (*B. anthracis* Sterne, *B. cereus* T, and *B. subtilis* 168) at 25°C and 80% relative humidity, the zinc coating produced approximately 3 log inactivation of vegetative cells within 5 to 24 (Galeano et al., 2003). Therefore, since brass we used in this study made up with 60% copper base, 30% zinc and 1-2% lead, the present of zinc the brass alloys could explain also why we found great inhibition of *Listeria monocytogenes* growth inoculated with MW soft agar and stored at different temperatures on the surface of brass alloys.

Our study investigated the antimicrobial effect of copper ions against *Listeria monocytogenes* in a nutrient rich media while other investigators studied the antimicrobial effects of copper ions against pathogenic microorganisms in water systems (Grosserode,1993; Junge-Mathys and Mathys, 1994). *Legionellae* are common in freshwater marine environments and

common in water systems, and did not survive in the drinking water when the water was treated with ozone and metal ionization that included copper and silver (Grosserode,1993; Junge-Mathys and Mathys, 1994). Moreover, Rogers and others (1994) found that *Legionella pneumophila* could survive and grow in the biofilms, even at temperatures of up to 50°C, on plastic surfaces but not copper. These authors concluded that copper surfaces were the best material for inhibiting *Legionella pneumophila* while plastic surfaces had no antimicrobial activity on *Legionella pneumophila*. Indeed, Schulze-Robbecke and others (1992) showed that there were less mycobacteria on copper surfaces compared to plastic in water treatment plants, domestic water supply systems, and aquaria. Our results showed that copper metal containing 99.4% copper ions and brass metal containing 60% copper ions were effective in destroying *Listeria monocytogenes* in a nutrient rich media.

Kim and Frank (1995) found that when the glucose in MWB was increased to 20 g or decreased to 1 g that biofilm formation by *Listeria monocytogenes* was increased. This could explain why we found that the antimicrobial activity of copper ions was decreased against *Listeria monocytogenes* found when the glucose content of MWB was increased to 20 g or decreased to 1 g compared to the control MWB with 10 g of glucose. Furthermore, when *Listeria monocytogenes* was grown on the surface of copper or brass in MWB containing cellobiose, fructose, or mannose, the antimicrobial activity of copper metals were reduced against this pathogen as compared to the control MWB containing glucose. The reason for these results could be due to the increased production of biofilm by *Listeria monocytogenes* when grown in MWB containing cellobiose, fructose, or mannose as the sole sugar source (Kim and Frank, 1995). We also found that the antimicrobial activity of copper ions against *Listeria monocytogenes* was decreased when phosphate in MWB was decreased. This could be attributed

to the increased production of biofilm by *Listeria monocytogenes*. Kim and Frank (1995) found that after 24 hours the biofilm formation by *Listeria monocytogenes* increased when the phosphate in MWB was decreased.

Cowart and Foster (1985) showed that *Listeria monocytogenes* appears to be sensitive to extreme nutrient starvation and high phosphate levels (Cowart and Foster, 1985). This could explain why at high phosphate levels, *Listeria monocytogenes* was more sensitive to the antimicrobial effects of copper ions.

In addition, we found that at a high or low concentration of amino acids in MWB that the effectiveness of the copper ions against *Listeria monocytogenes* was reduced. Jones and others (1995) reported that a mixture of amino acids prolonged the lag phase and decreased the doubling time with an increase in the final yield. The mechanism of reducing the antimicrobial activity of copper with availability of amino acid is not clear. However, during the lag phase of growth, the bacteria are adapting to their environment and if the lag phase is extended this may allow *Listeria monocytogenes* to adapt to the copper ions and become more resistant to the antimicrobial effects.

3.5 CONCLUSION

Our study evaluated the antimicrobial effects of copper ions against *Listeria monocytogenes* on the surface of copper and brass, as well as the effects of storage time, temperature, pH, and variation of the nutrients required in MW soft agar. The results showed that the antimicrobial effects of the copper ions against *Listeria monocytogenes* were slower at 4˚C than 25 or 37ºC.The results also showed that the pH of MW soft agar had little or no effect on the antimicrobial activity of copper ions against *Listeria monocytogenes*. However, the availably of nutrients did affect the antimicrobial effect of copper ions against *Listeria monocytogenes*.

Higher concentrations of glucose, different sugars, phosphate and amino acid reduced the antimicrobial effect of copper ions. Therefore, copper and brass could possibly be used in processing plant environments to control *Listeria monocytogenes.*

3.6 REFERENCES

Bryan, F. L. 1999. Procedures to Investigate Foodborne Illness Fifth Edition. International Association for Food Protection. Des Moines, IA.

Cowart, R. E., and B. G. Foster. 1985. Differential effects of iron on the growth of *Listeria monocytogenes*Listeria monocytogenes: minimum requirements and mechanism of acquisition. *J. Infect. Dis*. 151:721-730.

FDA/CFSAN. 2003. Foodborne Pathogenic Microorganisms and natural Toxins Handbook: The "Bad Bug Book." Center for Food Safety and Applied Nutrition, Food and Drug Administration, College park, MD. www.cfsan.fda.gov/~mow/intro.html.

FDA Food Code. 1999. U.S. Public Health Service, U.S. Dept. of Health and Human Services. Pub. No. PB99-115925. Washington, D.C.

Food Safety and Inspection Service (FSIS). 2001. Controlling Listeria Monocytogenes in Small and Very Small Meat and Poultry Plants. United States Department of Agriculture, Washington, DC. 20250-3700.

Galeano, B., E. Kroff, and W. Nicholson. 2003. Inactivition of vegetative cells, but not spore, of *Bacillus anthracis, B. cereus*, and *B. subtilis* on Stainless Steel Surfaces Coated with an antimicrobial Silver and Zinc contaning Zelolite Formilation. *J. App. Enviro. Micro*. 69(7):4329-4331.

Grosserode, M. (1993). Continuous Hyperchlorination for Control of Nosocomial *Legionella pneumophila* Pneumonia: A Ten-Year Follow-Up of Efficacy, Environmental Effects, and Costs. In: Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella:* current status and emerging perspectives. Washington, DC, *American Society for Microbiology*. 226–229.

Hitchins, A.D. 1998. *Listeria monocytogenes*. Ch. 10. In *Food and Drug Administration Bacteriological Analytical Manual*, 8th ed. (revision A), (CD-ROM version). R.L. Merker (Ed.). AOAC International, Gaithersburg, MD.

Jones, C. E., G. Shama, P. W. Andrew, I. S. Roberts, and D. Jones. 1995. Comparative study of the growth of *L. monocytogenes*L. monocytogenes in defined media and demonstration of growth in continuous culture. *J. Appl. Bacteriol*. 78:66-70.

Junge-Mathys E., and W. Mathys. 1994. Die Legionellose—ein Beispiel fŸr umweltbedingte Infektionen. [Legionellosis—An Example of Environmentally Caused Infections.] *Intensiv*. 2: 29–33.

Keevil, C. W., J. T. Walker, and A. Maule. 2000, Copper Surfaces Inhibit *Escherichia coli* O157, *Seminario Cobre y Salud,* Nov. 20, 2000, CEPAL/Comision Chilena del Cobre/ICA, Santiago, Chile).

Kim, K. Y., and J. F. Frank. 1995. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. *J. food Prot*. 58:24-28.

Neunlist M. R., M. Ralazamahaleo, J. Cappelier, V. Besnard , M. Federighi , and F. Leroi. 2005. Effect of salting and cold-smoking process on the culturability, viability, and virulence of *Listeria monocytogenes* strain Scott A. *J Food Prot*. 68(1):85-91.

Pizzo, G., G. Giammanco, E. Cumbo, G. Nicolosi and G. Gallina. 2005. In Vitro Antibacterial Activity of Endodontic sealers. *J. Dent*.

Premaratne, R. J., W. J. Lin, and E. A. Johnson. 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 57:3046-3048.

Rogers, J. A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994. The Influence of Temperature and Plumbing Material Selection on Biofilm Formation and Growth of *Legionella pneumophilia* in Potable Water Systems Containing Complex Microbial Flora, *Appl. Environ. Microbiol.* 60:1585–1592.

Schmidt, R. H. and G. E. Rodrick. 2003. Food Safety Handbook. John Wily & Sons, Inc. Hoboken, New Jersey.

Schulze-Robbecke, R., B. Janning. And R. Fischeder. 1992. Occurrence of Mycobacteria in Biofilm Samples. *Tuber Lung Dis*. 73:141–144.

Surjawidjaja, J., A. Hidayat, and M. Lesmana. 2004. Growth Inhabitation of Enteric Pathogen by Zinc Sulfate: an in Vitro Study. *Med. Princ. Pract.* 13(5):286-289.

Ward, D., D. Bernard, R.Collette, D.Kraemer, K. Hart, R. Price, and S. Otwell. 1997. Hazards Found in Seafoods In HACCP*:* Hazard Analysis and Critical Control Point Training Curriculum, 2nd ed, 173-188.

CHAPTER 4

ANTIMICROBIAL EFFECTS OF CONCRETE COATING CONTAINING COPPER IONS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AT DIFFERENT TEMPERATURES AND NUTRIENTS

4.1 INTRODUCTION

Listeria monocytogenes survives well, even under difficult conditions. Vegetables and fruits can become contaminated by this pathogen through the soil or from fertilizer. Animals and humans can harbor the bacteria without appearing ill, and can contaminate foods such as meats and dairy products. Infected individuals can shed the bacteria in the stool for several months (Health Canada, 1996). Unlike the majority of other harmful bacteria, *Listeria monocytogenes* can slowly grow on foods stored at refrigerator temperature. Therefore, ready-to-eat foods can maintain its growth. Freezer temperature appears to have very slight damaging effect on it. *Listeria monocytogenes* can also be spread during food preparation by contact with contaminated hands or counter tops (CDC, 2001).

Outbreaks of listeriosis are associated with ready-to-eat foods such as hot dogs, luncheon meats, cold cuts, fermented or dry sausage, and other deli-style meats, as well as poultry. In homes, *Listeria monocytogenes* is killed if ready-to-eat foods are reheated to steaming hot (Hof and Rocourt, 1992).

Recently, a multi-state outbreak of *Listeria monocytogenes* led to infections in United States with several deaths and miscarriages attributed to the outbreak, which was associated with eating sliceable turkey deli meat. Confirmed cases of listeriosis were reported in Pennsylvania, New York, New Jersey, Delaware, Maryland, Connecticut, Massachusetts, and Michigan (CDC, 2002).

Controlling the spread of *Listera monocytogenes* in food processing facilities has been a challenge for the entire food industry, from the smallest to the largest food processors. Floor drains in food processing facilities are a particularly important niche for its existence and can be a control point of contamination for the processing plant environment and food products (Farber

and Peterkin, 1991). In addition, *Listeria monocytogenes* grows at refrigeration temperature, forming protective biofilms, and thrives in moist environments such as floor drains in food processing plants. According to many research studies, the contamination of floor drains by *Listeria* is a serious challenge (Farber and Peterkin, 1991). When entrapped in a biofilm, *Listeria monocytogenes* shows unusual resistance to disinfectants and other treatments available to control pathogens on environmental surfaces (Pagotto et al., 2001).

Biofilms may be found on the surfaces of food and beverage processing and packaging equipment, such as in product lines, fillers, hoppers, and stuffers. They also appear on plastic cutting boards, stainless steel and plastic conveyor systems, mixers, grinders, slicers, or any surface in continuous contact with food products.

Research has shown that widespread bacteria biofilms may well develop in areas where roughness, crevices, and varying depths occur (Tompkin, 2002). Rough surfaces that contain the biofilms are more heavily colonized than the interior places. A potential reason for this special colonization may be the inability of detergents and sanitizers to reach these areas (Tompkin, 2002).

Controlling biofilms is not an easy mission. *Listeria monocytogenes* in a biofilm obtain excessive resistance to antimicrobial agents. Since most of the time cells in biofilms are more resistant to sanitizers, chemicals, and heat, they are much more difficult to kill as compared to when they are in single state. Moreover, swabbing of equipment surfaces to test for residual microbial contamination may not be efficient enough to dislodge the biofilms with the embedded microorganisms (Tompkin et al., 1999).

This study evaluated how effective concrete coatings containing copper ions are in reducing or destroying *Listeria monocytogenes* grown in biofilms at different temperatures and nutrient levels in modified Welshimer's broth.

4.2 MATERIAL AND METHODS

4.2.1 Culture Growth Conditions

The *Listeria monocytogenes* strain (V7 serotype ½ a) used during this study was obtained from the Centers for Disease Control, Atlanta GA., USA and was originally isolated from the blood of an infected individual. The bacterial culture was grown for 16 hours in Brain Heart Infusion Broth (BHI) (Difco, Detroit, Mich., U.S.A.) at 37°C. The pure cultures were stored at -70°C and sub-cultured twice in BHI broth at 37°C for 16 hours before being used.

4.2.2 Preparation of Media

To determine if *Listeria monocytogenes* cells were injured by the copper ions, Oxford agar w/ antibiotic supplement was used. To simulate biofilm production, modified Welshimer's (MW) soft agar was specially formulated for generating bacterial growth similar to that, which occurs in biofilms. Modified Welshimer soft agar was prepared as follows:

Na_2HPO_4 (30.96 g) and KH_2PO_4 (6.56 g) were dissolved in 500 ml distilled water then autoclaved, $MgSO_4$ (0.41 g) was dissolved into distilled water then autoclaved, glucose (10 g) was dissolved into distilled water then filter sterilized, the amino acid (L-leucine, L-isoleucine , L-arginine, L-methonine and L-valine) 0.1 g each were dissolved into 100 ml distilled water then filter sterilized, L-cysteine and L-glutamine (0.1 and 0.5 g, respectively) were dissolved into distilled water then filter sterilized, ferric citrate (0.088 g) was dissolved into distilled water then filter sterilized, vitamins (Riboflavin 0.5 mg, biotin 0.5 mg, thiamine 1.0 mg and thioctic acid 0.005 mg) were dissolved into distilled water separately and then filter sterilized. The above

chemicals were mixed together on the day of the experiments and the volume was brought up to 1000 ml with distilled water. Modified Welshimer's Broth (MWB) pH was 6.6 (Premaratne et al., 1991).

4.2.3 Preparation of Samples

Concrete was spread over the surface of Petri dishes and allowed to air dry for 24 hours. It was then placed under a laminar flood with a UV light to sterilize the surface of the concrete for 24 hours. Copper sulfate at concentrations of 5, 10, 15, 20, and 25 mg/L, was dissolved in sterile dH_2O, and then stirred into polyurethane. Copper oxide at concentrations of 20, 40, 60, 80 and 100% were also added to the polyurethane. One ml of each mixture was spread over the surface of the concrete and allowed to dry at room temperature. It was then placed under a UV light in a laminar flow hood for 24 hours to sterilize the surface. The bacterial cultures were grown for 16 hours in BHI broth then serially diluted to 10^7 and 1 ml of the cultures was added to 9 ml of MWB and then poured over the concrete surfaces. To prevent drying of the MWB, each 50-cm diameter Petri dish was placed in a 120-cm diameter Petri dish containing 25 ml of sterile dH_2O.

4.2.4 Bacterial Analysis

Bacterial counts were determined by collecting the MWB and placing the liquid into sterile test tubes and vortexing for 1 minute. The liquids were decimally diluted in PBS and the dilutions were plated onto Oxford media for *Listeria monocytogenes*. The plates were incubated under aerobic conditions at 37°C for 24 hours and the CFU/ml was determined. At the end of each experiment, 1 ml of all treatments, at non-detectable levels, was inoculated into 9 ml of BHI broth and incubated for 48 hours at 37°C to verify that all the bacteria were killed. The results in each BHI tube were negative and no *Listeria monocytogenes* were found.

4.2.5 Evaluate the Effect of Temperatures on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* on the Surface of Concrete Coated with Polyurethane Containing Copper Ions

This study evaluated the effect of temperature (4, 25, or 37°C) on the reduction of *Listeria monocytogenes* when grown in MWB. The bacterial counts were determined for 0, 2, 4, 6, and 8 days at 4°C and for 0, 1, 2, and 3 days at 25 and 37°C.

4.2.6 Evaluate the Effect of Different Nutrients on the Antimicrobial Effects Against *Listeria monocytogenes* on the Surface of Concrete Coated with Polyurethane Containing Copper Ions

The levels of components in the MWB were varied to determine the effect of growth nutrients have on the antimicrobial activity of copper ions against *Listeria monocytogenes*. Glucose was evaluated at concentrations of 1.0, 10, and 20 g/L. The glucose in MWB was also replaced with cellobiose, fructose, or mannose at a concentration of 10 g/L. Phosphate levels were either reduced one-fold or increased five-fold in the MWB. The levels of amino acids (L-leucine, L-isoleucine, L-arginine, L-methonine, L-cysteine, L-glutamine and L-valine) were either decreased by one-tenth or increased five-fold in MWB. Amino acids were also replaced in MWB with 17.0 g/L of Tryptone (Kim and Frank, 1995). The samples were stored at 37°C and bacterial counts were determined every 24 hours for 2 days.

4.2.7 Statistical Analysis

The effects of temperatures and nutrient levels on the antimicrobial activity of copper ions against *Listeria monocytogenes* in MWB on the surface of concrete coating containing copper ions were analyzed by statistical comparisons of all pairs using Student's t-test following one-way analysis of variance (ANOVA) (SAS Version 9.1.2, SAS Institute Inc., Cary, N.C., USA). Statistical significance occurs for $p<0.05$. All experiments were repeated 3 times.

4.3 RESULTS

4.3.1 Evaluate the Effect of Temperatures on the Antimicrobial Activity of Copper Ions Against *Listeria monocytogenes* on the Surface of Concrete Coated with Polyurethane Containing Copper Ions

Figure 4.1 shows the antimicrobial effects of copper ions against *Listeria monocytogenes* in MWB poured on the surface of concrete coated with polyurethane containing different concentrations of copper oxide at 4°C. It was observed that during the first 2 hours, a 1 to 2 log reduction was found for all concentrations of copper oxide. However, by day 8 the *Listeria monocytogenes* counts were significantly decreased by 4.5 log with 80 and 100% copper oxide compared to hour 0. Samples with 20 and 40 % copper oxide were reduced 2.5 log and the 60% copper oxide samples were reduced by 3 log after 8 days of incubation at 4°C compared to the control *Listeria monocytogenes*. In addition, the polyurethane, coated on concrete, had about a 2 log reductions in *Listeria monocytogenes* compared to the controls by day 8.

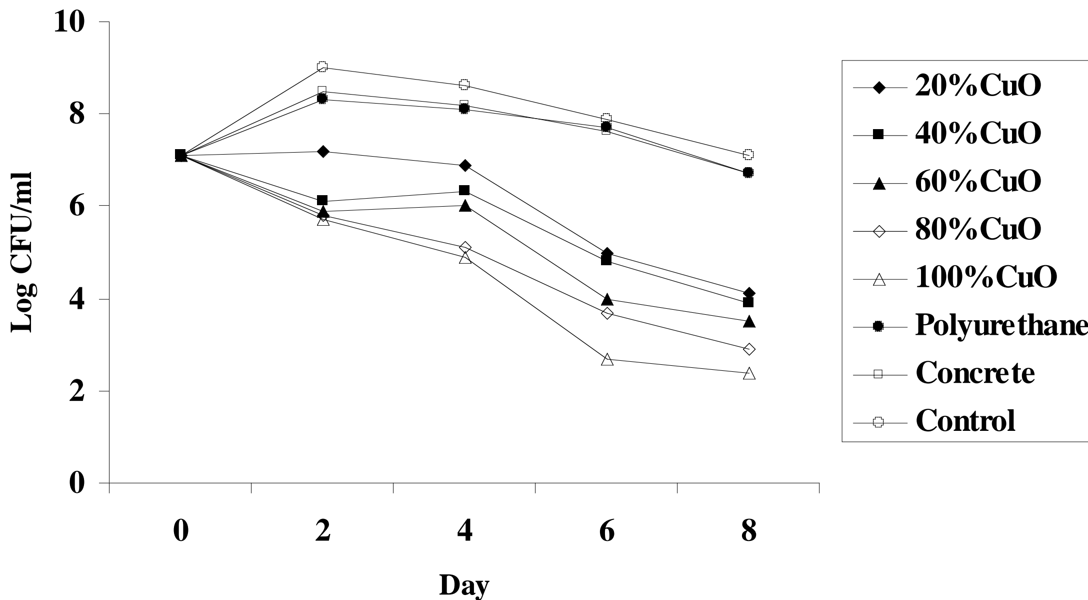

Figure 4.1. ***Listeria monocytogenes*** **in MWB on the surface of concrete coated with polyurethane containing copper oxide (CuO) that were incubated at 4°C**

Figure 4.2 shows the effects of growing *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing different concentrations of copper oxide at 25°C. All copper oxide concentrations reduced *Listeria monocytogenes* counts to non-detectable levels by day 4. Furthermore, only1 log reduction was observed for *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane compared to the control.

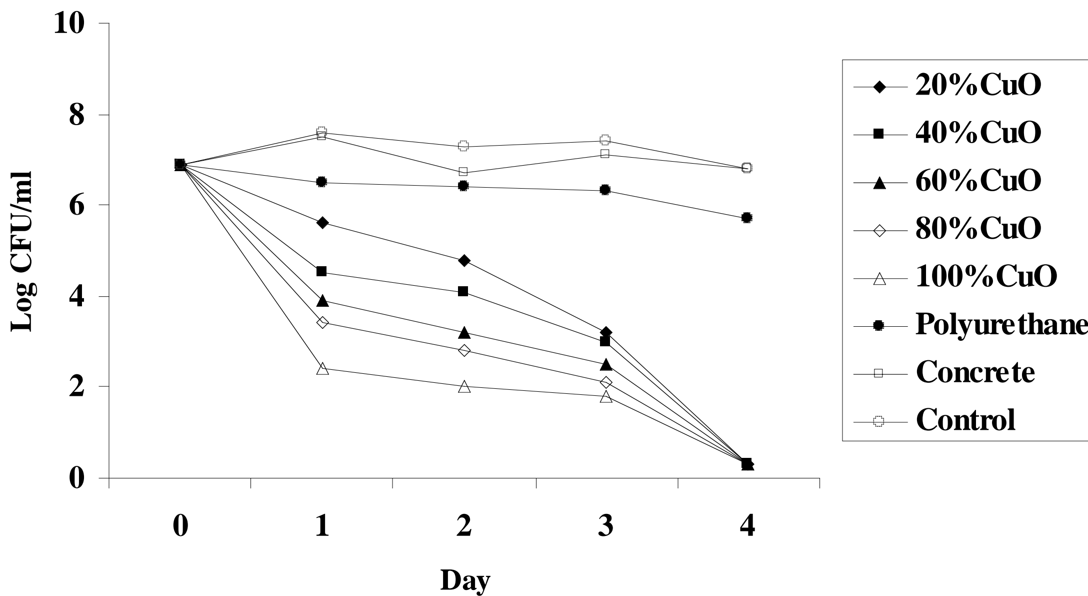

Figure 4.2. *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing copper oxide (CuO) that were incubated at 25°C

Figure 4.3 shows the effects of growing *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing different concentrations of copper oxide at 37°C. By day 3, *Listeria monocytogenes* counts were reduced to non-detectable levels in MWB on the surface of concrete coated with polyurethane with all copper oxide concentration when compared to the control.

Figure 4.4 shows the effects of growing *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing different concentrations of copper sulfate at 4°C. *Listeria monocytogenes* counts were reduced by 3.5 log on the surface of concrete coated with polyurethane containing 25 mg of copper sulfate and 3 log on the surface of concrete coated with polyurethane containing 20 mg copper sulfate compared to controls after 8 days.

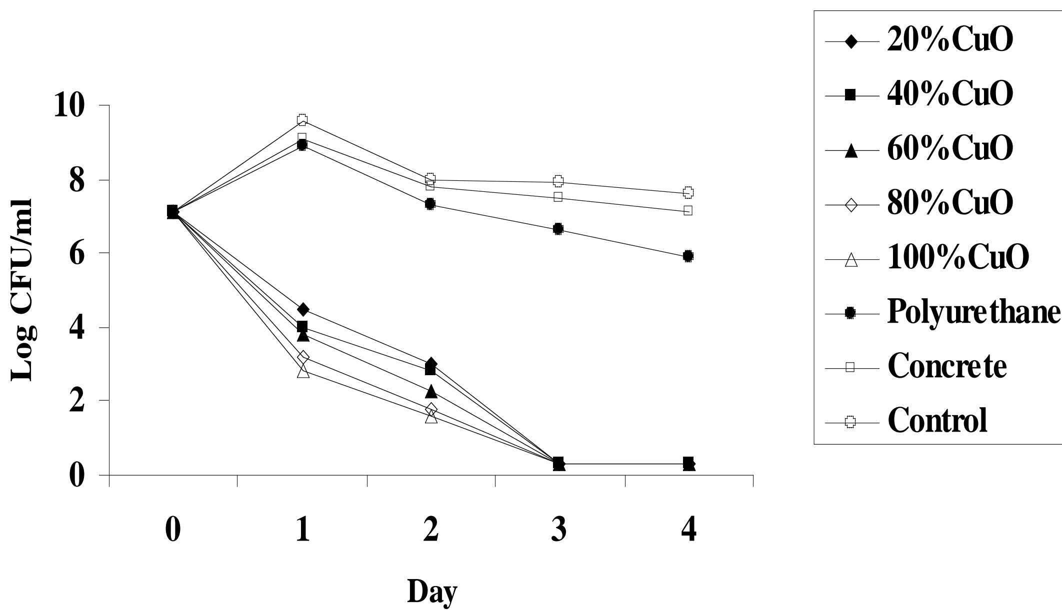

Figure 4.3. ***Listeria monocytogenes*** **in MWB on the surface of concrete coated with polyurethane containing copper oxide (CuO)that were incubated at 37°C**

Figure 4.5 shows the effects of growing *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing different concentrations of copper sulfate at 25°C. The *Listeria monocytogenes* counts, on the surface of concrete coated with polyurethane containing 15, 20 or 25 mg of copper sulfate, dropped to non-detectable levels by day 4. This was when samples were incubated at 25°C, while 5 log reductions were noticed in *Listeria*

monocytogenes counts with concrete coated using polyurethane containing 5 and 10 mg of copper sulfate.

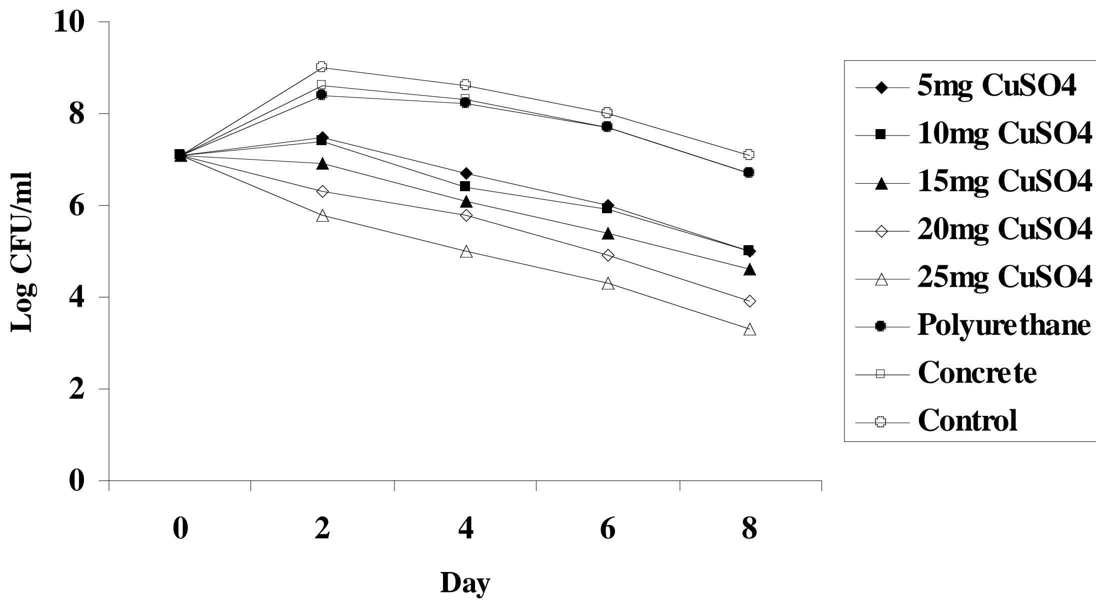

Figure 4.4. *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$) that were incubated at 4°C

Figure 4.6 shows the effects of growing *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing different concentrations of copper sulfate at 37°C. Concrete coated with polyurethane containing 5, 10, 15, 20 or 25 mg copper sulfate was reduced to non-detectable levels by day 3. At 25 or 37 °C, the *Listeria monocytogenes* counts on the surface of concrete, coated with polyurethane containing copper oxide or copper sulfate, had dropped to non-detectable levels. Concrete samples stored at 4°C could possibly be reduced to non-detectable levels if the length of time was increased.

4.3.2 Evaluate the Effect of Different Nutrients on the Antimicrobial Effects Against *Listeria monocytogenes* on the Surface of Concrete Coated with Polyurethane Containing Copper Ions

It is very important to know the ability of *Listeria monocytogenes* to grow and survive in various environments and to determine the nutritional requirements for growth. Kim and Frank (1995) found that the adherence, growth, and production of biofilm by *Listeria monocytogenes* was dependant on the availability of nutrients in the growth medium.

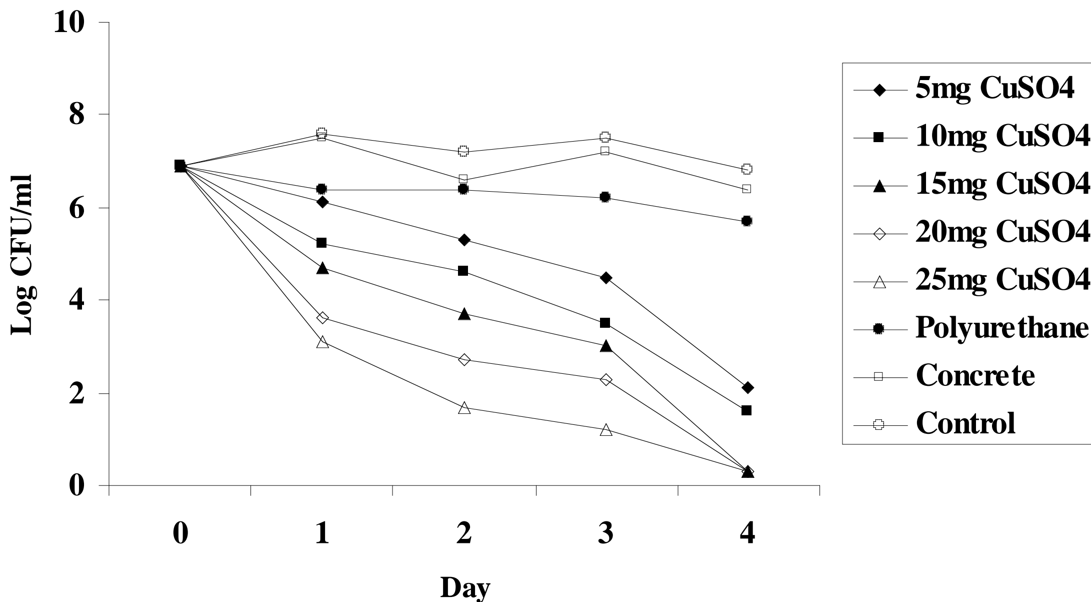

Figure 4.5. ***Listeria monocytogenes*** **in MWB on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$) that were incubated at 25ºC**

Therefore chemically defined minimal medium for *Listeria monocytogenes* (Modified Welshimer's Broth, MWB) was used, which contained all the growth factors required for *Listeria monocytogenes*, such as carbon source, amino acids, and vitamins (Premaratne et al., 1991). In this study, some modification in the required sources was done by increasing,

decreasing, or replacing the required compounds to examine if the availability of nutrients makes *Listeria monocytogenes* more sensitive or resistant to the antimicrobial effect of copper ions.

Table 4.1 shows the effect of antimicrobial activity of concrete coated with polyurethane containing different concentrations of copper oxide against *Listeria monocytogenes* grown in MWB containing different sugars. The initial inoculation for all samples was 7.13 log CFU/ml. After 24 hours of incubation at 37 °C, concrete coated with polyurethane, contaning100% copper oxide, had significantly decreased in *Listeria monocytogenes* counts for all sugars tested by 7 log, and with concrete coated with polyurethane containing 80% copper *Listeria monocytogenes* counts decreased in oxide by 5.5 logs compared to the controls. However, the reduction in *Listeria monocytogenes* counts decreased with a decrease in the concentrations of the copper oxide.

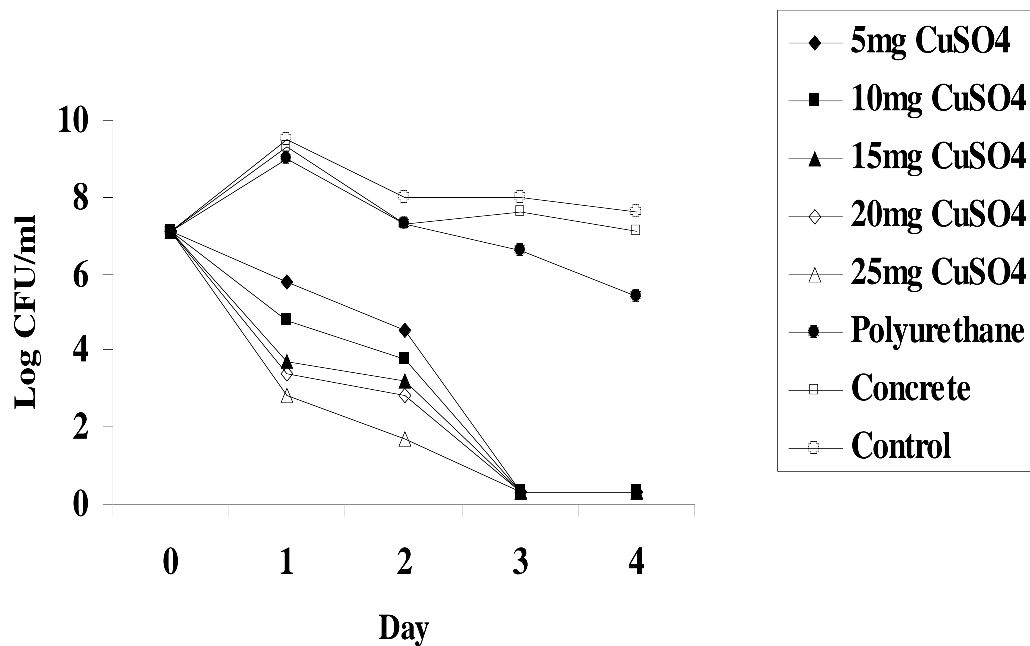

Figure 4.6. *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$) that were incubated at 37°C

Furthermore, the results showed a significant reduction of 5 to 6 log in *Listeria monocytogenes* counts, in the all copper oxide concentrations used in polyurethane coated concrete, when fructose sugar was the carbon source in MWB. Table 4.1 also showed that after 48 hours of incubation, *Listeria monocytogenes* counts were reduced to non-detectable levels, with concrete coated with polyurethane containing 20, 40, 60, 80 and 100% copper oxide, with fructose or mannose in MWB. However, with cellobiose, the *Listeria monocytogenes* counts were 2.27 and 2.00 log CFU/ml on concrete coated with polyurethane containing 20 or 40% copper oxide in MWB, respectively.

Table 4.2 shows the effect of antimicrobial activity of concrete, coated with polyurethane containing different concentrations of copper oxide, against *Listeria monocytogenes* grown in MWB containing different concentration of glucose. After 24 hours of incubations there were no significant differences in *Listeria monocytogenes* counts in MWB containing 1 or 10g of glucose on the surface of concrete coated with polyurethane containing 100% copper oxide. The bacterial counts were reduced by 6 log compared to the non-treated controls.

The *Listeria monocytogenes* counts were reduced by 5 logs with concrete coated with polyurethane containing 60, 80 or 100% copper oxide. With concrete coated with polyurethane, containing 20 or 40% of copper oxide, the *Listeria monocytogenes* counts were reduced by 3 logs. In other words, the reduction increased when the copper oxide concentration increased. Also, MWB with 20g glucose, with all concentrations of polyurethane containing copper oxide on the surface of concrete, reduced *Listeria monocytognes* counts by about 5 logs.

After 48 hours of storage, *Listeria monocytogenes* counts in MWB containing 1 or 20 g of glucose, on the surface of polyurethane containing 100% copper oxide coated on concrete, reached non-detectable levels (Table 4.2).

Table 4.1. ***Listeria monocytogenes*** **grown at 37ºC for 48h in MWB containing different sugars on the surface of concrete coated with polyurethane containing copper oxide (CuO)**

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
Glucose	Control	9.58 ± 0.05 A	8.20 ± 0.14 A
	Concrete	9.38 ± 0.09 A B	7.95 ± 0.06 A B D
	Polyurethane	9.10 ± 0.08 B	7.38 ± 0.17 E
	20% CuO	4.68 ± 0.17 G	3.18 ± 0.17 G
	40% CuO	4.23 ± 0.20 H	2.90 ± 0.08 G
	60% CuO	3.80 ± 0.08 I	2.30 ± 0.06 H
	80% CuO	3.28 ± 0.09 J K	2.00 ± 0.09 H
	100% CuO	2.85 ± 0.06 L	1.83 ± 0.09 I
Cellobiose	Control	8.18 ± 0.17 C	8.15 ± 0.13 A B
	Concrete	7.85 ± 0.06 C D	7.68 ± 0.15 D E
	Polyurethane	6.73 ± 0.18 F	6.58 ± 0.29 F
	20% CuO	4.88 ± 0.17 G	2.28 ± 0.17 H
	40% CuO	3.40 ± 0.24 J	2.00 ± 0.21 H
	60% CuO	2.83 ± 0.09 L M	0.00 ± 0.00 J
	80% CuO	2.10 ± 0.07 N O	0.00 ± 0.00 J
	100% CuO	2.00 ± 0.08 O P	0.00 ± 0.00 J
Fructose	Control	8.13 ± 0.12 C	8.13 ± 0.19 A B
	Concrete	7.68 ±0.38 D	7.80 ± 0.08 D
	Polyurethane	6.90 ± 0.08 E F	6.68 ± 0.15 F
	20% CuO	2.45 ± 0.13 M N	0.00 ± 0.00 J
	40% CuO	2.05 ± 0.06 O P	0.00 ± 0.00 J
	60% CuO	2.08 ± 0.15 N O P	0.00 ± 0.00 J
	80% CuO	1.73 ± 0.09 O P	0.00 ± 0.00 J
	100% CuO	1.25 ± 0.13 Q	0.00 ± 0.00 J
Mannose	Control	7.93 ± 0.05 C D	8.03 ± 0.12 A B
	Concrete	7.65 ± 0.17 D	7.83 ± 0.17 B D
	Polyurethane	7.15 ± 0.16 E	6.80 ± 0.14 F
	20% CuO	4.63 ± 0.12 G	0.00 ± 0.00 J
	40% CuO	3.55 ± 0.13 I J	0.00 ± 0.00 J
	60% CuO	3.18 ± 0.19 J K L	0.00 ± 0.00 J
	80% CuO	2.93 ± 0.09 K L	0.00 ± 0.00 J
	100% CuO	1.70 ± 0.08 P	0.00 ± 0.00 J

[a] Initial inoculation at time zero was 7.13 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different sugars substituted for glucose (Glucose (Control), Cellobiose, Fructose and Mannose) 10g/L in MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper oxide (CuO) coated on the surface of concrete.

Table 4.2. *Listeria monocytogenes* grown at 37ºC for 48h in MWB containing different concentration of glucose on the surface of concrete coated with polyurethane containing copper oxide (CuO)

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
1g Glucose	Control	8.23 ± 0.12 E	8.78 ± 0.15 A
	Concrete	7.90 ± 0.08 D	8.15 ± 0.13 B
	Polyurethane	6.85 ± 0.13 F	6.93 ± 0.09 D
	20% CuO	3.83 ± 0.17 J K	2.78 ± 0.15 F G
	40% CuO	3.63 ± 0.09 K L	2.43 ± 0.09 G
	60% CuO	3.65 ± 0.14 K L	1.90 ± 0.08 H
	80% CuO	3.18 ± 0.09 M N	1.38 ± 0.15 J
	100% CuO	2.90 ± 0.08 N	0.00 ± 0.00 K
10g Glucose	Control	9.60 ± 0.14 A	8.28 ± 0.15 B
	Concrete	9.45 ± 0.45 A B	7.48 ± 0.15 C
	Polyurethane	9.08 ± 0.09 C	7.28 ± 0.17 C D
	20% CuO	4.65 ± 0.13 G	3.15 ± 0.13 E
	40% CuO	4.18 ± 0.12 H I	2.93 ± 0.09 E F
	60% CuO	3.90 ± 0.08 I J K	2.50 ± 0.21 G
	80% CuO	3.40 ± 0.18 L M	1.83 ± 0.17 H
	100% CuO	2.93 ± 0.09 N	1.75 ± 0.13 H I
20g Glucose	Control	9.48 ± 0.05 A B	8.65 ± 0.17 A
	Concrete	9.23 ± 0.09 B C	8.15 ± 0.13 B
	Polyurethane	9.05 ± 0.06 C	7.05 ± 0.17 D
	20% CuO	4.85 ± 0.13 G	2.93 ± 0.09 E F
	40% CuO	4.68 ± 0.16 G	2.75 ± 0.13 F G
	60% CuO	4.33 ± 0.15 H	2.48 ± 0.15 G
	80% CuO	4.13 ± 0.12 H I J	1.40 ± 0.18 I J
	100% CuO	3.90 ± 0.08 I J K	0.00 ± 0.00 K I

[a] Initial inoculation at time zero was 7.02 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different concentrations of glucose (1g, 10g (control) and 20g) in MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper oxide (CuO) coated on the surface of concrete.

In addition, *Listeria monocytogenes* counts in 10g of glucose showed a significant reduction at the all copper oxide concentration. With all other glucose concentrations, the *Listeria monocytogenes* counts decreased when the copper oxide concentration increased in the

polyurethane coated on concrete. Table 4.3 shows the effect of antimicrobial activity of polyurethane coated concrete containing different concentrations of copper oxide, against *Listeria monocytogenes* grown in MWB containing different concentrations of amino acids, as well as replacing amino acid with tryptone.

A 2 to 3 log reduction of *Listeria monocytogenes* counts was found on the surface of concrete coated with polyurethane containing 20 or 40% copper oxide with one-tenth of the amino acid, and MWB with five times the amino acid respectively after 24 hours of incubation. In addition, the regular MWB concrete coated with polyurethane containing 60, 80 or 100% copper oxide reduced *Listeria monocytogenes* counts by about 6.5 log and with concrete coated with polyurethane containing 20 or 40% copper oxide, *Listeria monocytogenes* counts were reduced by about 4.5 log. However, after 48 hours with one-tenth or five times the amino acid, the *Listeria monocytogenes* counts on the surface of concrete, coated with polyurethane containing 80 or 100% copper oxide, were reduced by 5 log compared to non treated control (Table 4.3).

After 24 hours of storage, MWB with tryptone on the surface of polyurethane, containing 100% copper oxide coated on the surface of concrete, reduced *Listeria monocytogenes* counts to to1.82 log CFU/ml. There were no significant differences in *Listeria monocytogenes* counts on polyurethane containing 20% or 40% copper oxide on the surface of concrete when trypton replaced the amino acids in MWB, which reduced *Listeria monocytogenes* counts by 4 log compared to the control. Furthermore, after 48 hours, significant reduction in *Listeria monocytogenes* counts (4 log) occurred in MWB containing tryptone on the surface of concrete coated with polyurethane containing 40 or 60% copper oxide.

Table 4.3. *Listeria monocytogenes* grown at 37°C for 48h in MWB containing different concentration of amino acid on the surface of concrete coated with polyurethane containing copper oxide (CuO)

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
1.2 g/L Amino Acid	Control	9.50 ± 0.08 A	8.13 ± 0.19 A
	Concrete	9.40 ± 0.14 A B	7.85 ± 0.13 A B
	Polyurethane	9.08 ± 0.09 B C	7.23 ± 0.43 C D
	20% CuO	4.68 ± 0.17 M N	3.18 ± 0.17 J K
	40% CuO	4.15 ± 0.13 O P	2.93 ± 0.09 J K L
	60% CuO	3.93 ± 0.09 P Q	2.48 ± 0.15 L M
	80% CuO	3.35 ± 0.17 R	1.90 ± 0.08 N
	100% CuO	2.93 ± 0.12 S	1.70 ± 0.08 N
Tryptone	Control	8.45 ± 0.11 D E	7.75 ± 0.06 A B C
	Concrete	8.15 ± 0.13 E F	7.55 ± 0.06 B C D
	Polyurethane	7.85 ± 0.17 F	7.13 ± 0.19 D
	20% CuO	4.65 ± 0.13 M N	3.15 ± 0.13 J K
	40% CuO	4.35 ± 0.19 N O	2.93 ± 0.09 J K L
	60% CuO	3.78 ± 0.15 Q	2.18 ± 0.54 M N
	80% CuO	2.85 ± 0.13 S	0.00 ± 0.00 O
	100% CuO	1.83 ± 0.09 T	0.00 ± 0.00 O
0.1 2 g/LAmino Acid	Control	8.75 ± 0.13 C D	8.18 ± 0.17 A
	Concrete	8.28 ± 0.17 E	8.15 ± 0.13 A
	Polyurethane	7.33 ± 0.12 G	7.23 ± 0.09 C D
	20% CuO	5.30 ± 0.08 I J	4.38 ± .022 E
	40% CuO	5.15 ± 0.13 I J K	4.10 ± 0.14 E F G
	60% CuO	4.83 ± 0.12 K L M	3.83 ± 0.12 F G H
	80% CuO	4.15 ± 0.13 O P	3.25 ± 0.11 I J
	100% CuO	3.93 ± 0.09 O P	2.65 ± 0.13 K L M
6.0 g/L Amino Acid	Control	9.08 ± 0.09 M N	8.18 ± 0.15 A
	Concrete	8.33 ± 0.12 E	7.90 ± 0.08 A B
	Polyurethane	7.80 ± 0.14 F	7.15 ± 0.13 D
	20% CuO	5.75 ± 0.13 H	4.30 ± 0.24 E F
	40% CuO	5.48 ± 0.18 H I	4.08 ± 0.17 E F G
	60% CuO	5.08 ± 0.09 J K L	3.75 ± 0.20 G H I
	80% CuO	4.75 ± 0.13 L M	3.40 ± 0.29 H I J
	100% CuO	4.65 ± 0.18 M N	2.60 ± 0.21 L M

[a] Initial inoculation at time zero was 7.02 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] 1.2 g/L (control), 0.12 g/L and 6.0 g/L of amino acids (L-eucine, L-isoleucine, L-arginine, L-methionine, L-cysteine, L-glutamine and L-valine) or trypton(17g/L) replaced in MWB inoculated with *Listeria monocytogenes* on the surface of polyurethane containing copper oxide (CuO) coated on the surface of concrete.

Also, *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane containing 80 or 100% copper oxide were reduced to non-detectable levels when tryptone was replaced for amino acids in MWB.

Table 4.4 shows the effect of antimicrobial activity of polyurethane coated concrete, containing different concentrations of copper oxide, against *Listeria monocytogenes* grown in MWB containing different concentrations of phosphate. After 24 hours of storage with one-half and twice the phosphate, *Listeria monocytogenes* counts on the surface of polyurethane containing 100% copper oxide coated on concrete were reduced by 5.5 logs.

However, with one-half the phosphate, *Listeria monocytogenes* counts were reduced on the surface of polyurethane, containing 20% copper oxide coated on concrete, by 4 log.

In addition, in regular MWB, *Listeria monocytogenes* counts were reduced by 6 log on the surface of polyurethane containing 100% copper oxide coating on concrete. After 48 hours, the *Listerai monocytogenes* counts were reduced to non-detectable levels at both phosphate levels, in MWB, on the surface of concrete coated with polyurethane containing 80 and 100% copper oxide. The counts of *Listeria monocytogenes* were reduced when the concentrations of copper oxide increased on the surface of polyurethane containing copper ions.

Table 4.5 shows the effect of antimicrobial activity, of concrete coated with polyurethane containing different concentrations of copper sulfate, against *Listeria monocytogenes* grown in MWB containing different sugars**.** The initial inoculation for all sugars was 7.02 log CFU/ml. After 24 hours of incubation at 37°C, *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane, containing 20 and 25 mg of copper sulfate, reduced by 7 log with fructose or mannose in MWB.

Table 4.4. *Listeria monocytogenes* grown at 37ºC for 48h in MWB containing different concentration of phosphate on the surface of concrete coated with polyurethane containing copper oxide (CuO)

	Treatment [c]	Log CFU/ml [b]	
		24 Hour	48 Hour
37.52 g Phosphate	Control	9.93 ± 0.09 A	8.20 ± 0.18 A
	Concrete	9.35 ± 0.10 A B	7.75 ± 0.19 A B
	Polyurethane	9.10 ± 0.14 B C	7.30 ± 0.12 B C
	20% CuO	4.58 ± 0.15 G	3.20 ± 0.15 F
	40% CuO	4.23 ± 0.22 G H	2.90 ± 0.08 F G
	60% CuO	3.83 ± 0.12 I	2.45 ± 0.13 G H
	80% CuO	3.38 ± 0.15 J	1.93 ± 0.09 I K
	100% CuO	2.93 ± 0.09 K L	1.68 ± 0.06 K L
18.76 g Phosphate	Control	8.83 ± 0.15 C D	7.55 ± 0.17 B C
	Concrete	8.30 ± 0.11 E F	7.23 ± 0.12 C
	Polyurethane	8.15 ± 0.13 F	7.15 ± 0.13 C
	20% CuO	4.33 ± 0.22 G	3.20 ± 0.21 F
	40% CuO	3.93 ± 0.09 H I	2.28 ± 0.17 H I
	60% CuO	3.38 ± 0.17 J	1.35 ± 0.13 L
	80% CuO	2.93 ± 0.08 K L	0.00 ± 0.00 M
	100% CuO	1.93 ± 0.10 M N	0.00 ± 0.00 M
75.04 g Phosphate	Control	8.83 ± 0.18 D E	7.23 ± 0.12 C
	Concrete	8.23 ± 0.15 E F	6.93 ± 0.09 E
	Polyurethane	7.93 ± 0.09 F	6.45 ± 0.26 E
	20% CuO	3.23 ± 0.22 J K	2.33 ± 0.20 H I
	40% CuO	2.68 ± 0.17 L	2.15 ± 0.56 H I
	60% CuO	2.23 ± 0.20 M	1.55 ± 0.13 K L
	80% CuO	1.93 ± 0.09 M N	0.00 ± 0.00 M
	100% CuO	1.68 ± 0.17 N	0.00 ± 0.00 M

[a] Initial inoculation at time zero was 7.02 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different concentrations of phosphate (Sodium phosphate dibasic and Potassium phosphate monobasic) with 37.52 g/L (control), 18.76 g/L and 75.04 g/L used on to MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper oxide (CuO) on the surface of concrete.

Table 4.5. ***Listeria monocytogenes*** **grown at 37ºC for 48h in MWB containing different sugars on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$)**

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
Glucose	Control	9.60 ± 0.09 A	8.30 ± 0.18 A
	Concrete	9.45 ± 0.06 A B	8.10 ± 0.19 A B
	Polyurethane	9.18 ± 0.15 B	7.40 ± 0.16 D
	5 mg $CuSO_4$	6.05 ± 0.17 F	4.70 ± 0.18 G
	10 mg $CuSO_4$	4.93 ± 0.09 G	3.93 ± 0.09 H
	15 mg $CuSO_4$	3.78 ± 0.15 H	3.48 ± 0.27 I
	20 mg $CuSO_4$	3.50 ± 0.08 H I	2.90 ± 0.14 J
	25 mg $CuSO_4$	2.93 ± 0.09 J K	1.80 ± 0.08 K L M
Cellobiose	Control	8.25 ± 0.17 C	8.10 ± 0.08 A B
	Concrete	7.95 ± 0.13 C D	7.70 ± 0.18 C D
	Polyurethane	6.83 ± 0.09 E	6.30 ± 0.14 F
	5 mg $CuSO_4$	3.63 ± 0.12 H I	2.93 ± 0.09 J
	10 mg $CuSO_4$	3.23 ± 0.09 I J	2.13 ± 0.31 K
	15 mg $CuSO_4$	3.03 ± 0.12 J K	0.00 ± 0.00 N
	20 mg $CuSO_4$	2.70 ± 0.21 K	0.00 ± 0.00 N
	25 mg $CuSO_4$	2.20 ± 0.26 M	0.00 ± 0.00 N
Fructose	Control	8.20 ± 0.18 C	8.30 ± 0.26 A
	Concrete	7.95 ± 0.13 C D	7.78 ± 0.05 B D
	Polyurethane	7.00 ± 0.14 E	6.70 ± 0.18 E
	5 mg $CuSO_4$	2.65 ± 0.15 K L	1.95 ± 0.13 K L
	10 mg $CuSO_4$	2.25 ± 0.23 L M	1.55 ± 0.19 M
	15 mg $CuSO_4$	1.90 ± 0.08 M N	0.00 ± 0.00 N
	20 mg $CuSO_4$	1.63 ± 0.09 N O	0.00 ± 0.00 N
	25 mg $CuSO_4$	1.38 ± 0.09 O	0.00 ± 0.00 N
Mannose	Control	8.05 ± 0.17 D	8.05 ± 0.19 A B C
	Concrete	7.70 ± 0.18 C D	7.90 ± 0.08 B C
	Polyurethane	7.18 ± 0.15 E	6.85 ± 0.13 E
	5 mg $CuSO_4$	3.75 ± 0.23 H	2.55 ± 0.17 J
	10 mg $CuSO_4$	3.48 ± 0.27 H I	1.60 ± 0.08 L M
	15 mg $CuSO_4$	3.45 ± 0.13 H I	0.00 ± 0.00 N
	20 mg $CuSO_4$	1.93 ± 0.09 M N	0.00 ± 0.00 N
	25 mg $CuSO_4$	1.75 ± 0.13 N O	0.00 ± 0.00 N

[a] Initial inoculation at time zero was 7.0 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different sugars substituted for glucose (Glucose (control), Cellobiose, Fructose and Mannose) 10 g/L in MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper sulfate ($CuSO_4$) coated on the surface of concrete.

With cellobiose in MWB on the surface of concrete coated with polyurethane containing 5, 10 or 15 mg of copper sulfate, *Listeria monocytogenes* counts were reduced by 4 log. Furthermore, the reduction of *Listeria monocytogenes* decreased on the surface of concrete coated with polyurethane containing low concentrations of copper sulfate (5, 10 mg).

The results showed a significant reduction in *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane containing 5, 10 and 15 mg of copper sulfate with fructose sugar in MWB, as compared to the control; these reductions were 5 to 6 logs. Table 4.5 also shows that after 48 hours of incubation, *Listeria monocytogenes* counts, on the surface of polyurethane coated concrete containing 15, 20 or 25 mg copper sulfate, were reduced to non-detectable levels with cellobiose, fructose or mannose in MWB.As the concentration of copper sulfate increased in the polyurethane coated concrete, the reduction of *Listeria monocytogenes* counts also increased with inoculation into the MWB with different sugars tested.

Table 4.6 shows the antimicrobial activity of concrete coated with polyurethane containing different concentrations of copper sulfate against *Listeria monocytogenes* grown in MWB containing different concentration of glucose. After 24 hours of incubation, there were significant differences in *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane containing all copper sulfate concentrations with all glucose concentrations in MWB. *Listeria monocytogenes* counts were reduced by 6.5 logs on the surface of concrete coated polyurethane containing 25 mg copper sulfate with 10 g glucose in MWB, whereas MWB the 20g glucose reduced *Listeria monocytogenes* counts by 5 log.

After 48 hours of storage *Listeria monocytogenes* counts reached to non-detectable levels on the surface of concrete coated with polyurethane containing 25 mg copper sulfate with 1 or 20 g glucose in MWB, compared to the control.

Table 4.6. *Listeria monocytogenes* grown at 37°C for 48h in MWB containing different concentration of glucose on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$)

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
1g Glucose	Control	8.08 ± 0.09 B C D	8.58 ± 0.09 A
	Concrete	7.80 ± 0.14 C D	8.10 ± 0.08 B
	Polyurethane	6.85 ± 0.13 D E	6.95 ± 0.13 D E
	5 mg $CuSO_4$	4.28 ± 0.17 G H	3.20 ± 0.18 H I
	10 mg $CuSO_4$	4.13 ± 0.09 G H I	2.65 ± 0.17 J K
	15 mg $CuSO_4$	3.93 ± 0.19 G H I	2.25 ± 0.23 K L
	20 mg $CuSO_4$	3.83 ± 0.09 G H I	1.53 ± 0.05 M
	25 mg $CuSO_4$	3.38 ± 0.15 H I	0.00 ± 0.00 N
10g Glucose	Control	9.58 ± 0.05 A	8.20 ± 0.14 A B
	Concrete	9.35 ± 0.06 A B	7.48 ± 0.28 C
	Polyurethane	9.15 ± 0.11 A B C	7.35 ± 0.13 C D
	5 mg $CuSO_4$	5.95 ± 0.13 E F	4.65 ± 0.13 F
	10 mg $CuSO_4$	4.98 ± 0.17 G F	3.80 ± 0.21 G
	15 mg $CuSO_4$	3.78 ± 0.25 G H I	3.30 ± 0.18 H I
	20 mg $CuSO_4$	3.45 ± 0.20 H I	2.93 ± 0.09 I J
	25 mg $CuSO_4$	2.83 ± 0.09 I	1.85 ± 0.17 L M
20 g Glucose	Control	9.43 ± 0.29 A B	8.60 ± 0.14 A
	Concrete	9.15 ± 0.10 A B C	8.15 ± 0.13 B
	Polyurethane	9.08 ± 0.09 A B C	6.93 ± 0.12 E
	5 mg $CuSO_4$	4.13 ± 0.50 G H I	3.35 ± 0.17 H
	10 mg $CuSO_4$	5.13 ± 0.09 G F	2.93 ± 0.09 I J
	15 mg $CuSO_4$	4.93 ± 0.21 G F	2.35 ± 0.23 K
	20 mg $CuSO_4$	4.45 ± 0.13 G H	1.78 ± 0.15 M
	25 mg $CuSO_4$	4.15 ± 0.17 G H I	0.00 ± 0.00 N

[a] Initial inoculation at time zero was 7.0 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of three determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different concentrations of glucose (1g /L, 10g/L (control) and 20g/L) in MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper sulfate ($CuSO_4$) coated on the surface of concrete.

Also, *Listeria monocytogenes* counts, on the surface of concrete coated with polyurethane containing 25 mg copper sulfate with 10 g glucose in MWB, showed a significant reduction (1.85 log CFU/ml). As the concentration of copper sulfate increased in the polyurethane coated

concrete, the reduction of *Listeria monocytogenes* counts also increased upon inoculation into the MWB with different concentrations of glucose (Table 4.6).

Table 4.7 shows the effect of antimicrobial activity of concrete coated with polyurethane containing different concentrations of copper sulfate against *Listeria monocytogenes* grown in MWB containing different concentration of amino acids as well as replacing amino acid with tryptone. After 24 hours of storage with MWB containing tryptone on the surface of polyurethane containing 25 mg, the concrete coated with copper sulfate led to the observation that *Listeria mocytogenes* count had decreased significantly (2.37 log CFU/ml).

Whereas the polyurethane coated concrete containing 5 or 10 mg of copper sulfate, lowered the *Listeria monocytogenes* counts by 4 log when tryptone was replaced for the amino acids in MWB.

After 48 hours table 4.7 shows that *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane containing 20 or 25 mg of copper sulfate were reduced to non-detectable levels when tryptone was replaced for amino acids in MWB. A significant reduction in *Listeria monocytogenes* counts were also found with other copper sulfate concentrations in concrete coated with polyurethane (4 log). Conversely, with one-tenth and five times the amino acid in MWB, the antimicrobial activity of copper was reduced against *Listeria monocytogenes*.

Table 4.8 shows the effect of the antimicrobial activity of concrete coated with polyurethane containing different concentrations of copper sulfate against *Listeria monocytogenes* grown in MWB containing different concentration of phosphate. After 24 hours of storage at each level of phosphate, *Listeria monocytogenes* counts on the surface of concrete coated with polyurethane containing 25 mg copper sulfate were reduced by 6 logs.

Table 4.7. ***Listeria monocytogenes*** **grown at 37°C for 48h in MWB containing different concentration of amino acid on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$)**

	Treatment [c]	Log CFU/ml [b] 24 Hour	48 Hour
1.2 g Amino Acid	Control	9.48 ± 0.12 A	8.13 ± 0.12 A B
	Concrete	9.30 ± 0.08 A	7.70 ± 0.11 D E
	Polyurethane	9.08 ± 0.09 A	7.28 ± 0.17 F
	5 mg $CuSO_4$	5.83 ± 0.17 F	4.43 ± 0.17 G
	10 mg $CuSO_4$	4.90 ± 0.08 I J	3.80 ± 0.21 H I
	15 mg $CuSO_4$	3.88 ± 0.15 L M	3.23 ± 0.12 J
	20 mg $CuSO_4$	3.48 ± 0.09 M	2.85 ± 0.13 K L M
	25 mg $CuSO_4$	2.90 ± 0.08 N	1.68 ± 0.17 N
Tryptone	Control	8.55 ± 0.18 D	7.73 ± 0.09 C D E
	Concrete	8.15 ± 0.13 D	7.45 ± 0.06 E F
	Polyurethane	7.68 0.17 E	7.18 ± 0.17 F
	5 mg $CuSO_4$	4.93 ± 0.09 H I J	3.13 ± 0.19 J K
	10 mg $CuSO_4$	4.63 ± 0.19 J K	2.55 ± 0.13 M
	15 mg $CuSO_4$	3.98 ± 0.35 L	1.53 ± 0.09 N
	20 mg $CuSO_4$	2.93 ± 0.09 N	0.00 ± 0.00 O
	25 mg $CuSO_4$	2.38 ± 0.09 O	0.00 ± 0.00 O
0.12 g Amino Acid	Control	8.68 ± 0.17 B C	8.25 ± 0.19 A
	Concrete	8.25 ± 0.13 C D	8.15 ± 0.18 A B
	Polyurethane	7.25 ± 0.16 E	7.15 ± 0.13 F
	5 mg $CuSO_4$	5.78 ± 0.19 F	4.28 ± 0.17 G
	10 mg $CuSO_4$	5.38 ± 0.15 F G H	4.10 ± 0.08 G H
	15 mg $CuSO_4$	4.90 ± 0.08 I J	3.65 ± 0.13 I
	20 mg $CuSO_4$	4.18 ± 0.15 K L	3.20 ± 0.18 J K
	25 mg $CuSO_4$	3.90 ± 0.06 L M	2.93 ± 0.09 J K L
6.0 g Amino Acid	Control	9.05 ± 0.09 A B	8.08 ± 0.05 A B C
	Concrete	8.25 ± 0.26 C D	7.88 ± 0.09 B C D
	Polyurethane	7.55 ± 0.20 E	7.13 ± 0.21 F
	5 mg $CuSO_4$	5.13 ± 0.43 G H I	4.38 ± 0.15 G
	10 mg $CuSO_4$	5.43 ± 0.26 F G	4.13 ± 0.09 G H
	15 mg $CuSO_4$	5.25 ± 0.23 G H I	3.68 ± 0.14 I
	20 mg $CuSO_4$	5.13 ± 0.07 G H I	3.08 ± 0.06 J K L
	25 mg $CuSO_4$	4.83 ± 0.09 I J	2.75 ± 0.13 L M

[a] Initial inoculation at time zero was 7.02 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of four determinations. Means within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] 1.2 g/L (control), 0.12 g/L and 6.0 g/L of amino acids (L-leucine, L-Isoleucine, L-arginine, L-methionine, L-cysteine, L-glutamine and L-valine) or tryptone (17g/L) replaced in MWB were inoculated with *Listeria monocytogenes* on the surface of polyurethane containing copper sulfate (CuSO4)coated on the surface of concrete.

After 48 hours, the *Listeria monocytogenes* counts were reduced to non-detectable levels on the surface of concrete coated with polyurethane containing 20 or 25 mg copper sulfate with one-half and twice the phosphate in MWB.

Table 4.8. *Listeria monocytogenes* grown at 37ºC for 48h in MWB containing different concentration of phosphate on the surface of concrete coated with polyurethane containing copper sulfate ($CuSO_4$)

		Log CFU/ml [b]	
	Treatment [c]	24 Hour	48 Hour
37.52 g Phosphate	Control	9.50 0.08 A	8.10 ± 0.08 A
	Concrete	9.33 ± 0.05 A B	7.85 ± 0.13 A B
	Polyurethane	9.08 ± 0.17 B	7.23 ± 0.09 D E
	5 mg $CuSO_4$	5.93 ± 0.09 G	4.68 ± 0.17 G
	10 mg $CuSO_4$	4.95 ± 0.11 H	3.90 ± 0.09 H
	15 mg $CuSO_4$	3.88 ± 0.17 I	3.30 ± 0.18 I
	20 mg $CuSO_4$	3.55 ± 0.13 J	2.90 ± 0.08 J
	25 mg $CuSO_4$	2.90 ± 0.08 K	1.85 ± 0.13 L
18.76 g Phosphate	Control	8.73 ± 0.26 C	7.55 ± 0.06 B C
	Concrete	8.43 ± 0.09 C D E	7.33 ± 0.15 C D
	Polyurethane	8.25 ± 0.13 D E	7.18 ± 0.19 D E
	5 mg $CuSO_4$	5.03 ± 0.05 I J	3.25 ± 0.17 I
	10 mg $CuSO_4$	4.88 ± 0.09 H	2.43 ± 0.09 K
	15 mg $CuSO_4$	3.88 ± 0.23 I	1.53 ± 0.12 M
	20 mg $CuSO_4$	2.85 ± 0.13 K	0.00 ± 0.00 N
	25 mg $CuSO_4$	1.93 ± 0.021 M	0.00 ± 0.00 N
75.04 g Phosphate	Control	8.55 ± 0.06 C D	7.20 ± 0.08 D E
	Concrete	8.18 ± 0.05 E F	6.93 ± 0.09 E
	Polyurethane	7.93 ± 0.12 F	6.38 ± 0.15 F
	5 mg $CuSO_4$	3.58 ± 0.17 I J	2.70 ± 0.18 J K
	10 mg $CuSO_4$	2.90 ± 0.08 K	2.45 ± 0.13 K
	15 mg $CuSO_4$	2.43 ± 0.09 L	1.93 ± 0.09 L
	20 mg $CuSO_4$	1.90 ± 0.08 M	0.00 ± 0.00 N
	25 mg $CuSO_4$	1.55 ± 0.13 N	0.00 ± 0.00 N

[a] Initial inoculation at time zero was 7.02 Log CFU/ml.

[b] All analysis were based on three separate experiments with each mean ± standard deviation being average of four determinations. Means within concentration level within each vertical column (sampling hour) followed by different letters are significantly different ($P \leq 0.05$) from each other. Statistical comparisons of all pairs were analyzed using Student's t-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

[c] Different concentrations of phosphate (Sodium phosphate dibasic and Potassium phosphate monobasic) with 37.52 g/L (control), 18 g/L and 75.04 g/L were used on to MWB inoculated with *Listeria monocytogenes* on polyurethane containing copper sulfate ($CuSO_4$)on the surface of concrete.

4.4 DISCUSSION

One of the main concerns of the food industry is that *Listeria monocytogenes* can grow at refrigerator temperatures and is resistant to various environmental conditions, allowing it to survive longer under adverse conditions. Post-cooking microbial contamination of ready-to-eat meats with *Listeria monocytogenes* is a serious threat that many meat processors are trying to combat. *Listeria monocytogenes* has been found in the environment of ready-to-eat food processing plants and this could be the source for contamination of these products.

The highest incidence in the processing plant of *Listeria monocytogenes* is on wet damp surfaces such as conveyor belts, refrigerators, floors, and drains (Tompkin, 2002). During 1990 and 1991 environmental samples were collected and tested for *Listeria monocytogenes* from twelve plants processing a variety of ready-to-eat products (Tompkin et al., 1992). Out of 18,000 environmental samples collected 936 samples tested positive for *Listeria monocytogenes* (Tompkin et al., 1992). It was determined that the presence of *Listeria monocytogenes* was dependant on the unique ecology that was characteristic of each plant and the prevalence of this pathogen increased during the summer months in the processing environment. Our research has found that copper ions in coatings used on concrete might be useful for controlling or eliminating this emerging pathogen with in the ready-to-eat meat processing environment. We have shown that *Listeria monocytogenes* grown on the surface of concrete coated with polyurethane containing copper sulfate or copper oxide was reduced to non-detectable levels.

The results of this experiment indicated that temperature had an effect on the reduction of *Listeria monocytogenes* in MWB on the surface of concrete coated with polyurethane containing copper oxide or copper sulfate. We found that at refrigerator temperatures the antimicrobial activity of copper ions was reduced against *Listeria monocytogenes*. A study conducted by

Keevil and others (2000) found that *E. coli* O157:H7 was inhibited after a few hours when placed on copper surfaces, but survived for many days on stainless steel. The copper alloys they tested included copper, brass, bronze, copper-nickels, and nickel silvers. The results confirm that the antibacterial effect was present in all the tested copper alloys and increased with the copper content of the alloy. Furthermore they found that the inhibition was faster at higher temperatures. The bacteria did not grow on almost any of the copper alloy surfaces after being exposed to a temperature of 20°C from 1 to 6 hours, however, it took a longer time at 4°C to reduced the bacteria population for up to 16 hours.

One possible explanation for the effect of temperature on the antimicrobial activity of copper ions could be due to the amount of copper ions that are released into MWB; as the temperature increases so does the amount of copper ions released from the copper or brass (see Chapter 6). In addition, we found that at 25 and 37°C the population of *Listeria monocytogenes* had dropped faster. This could be attributed to the release of copper ions, at a faster rate, into MWB when stored at evaluated temperatures (see Chapter 6).

The most widely used copper compound is copper sulfate that is approved for use as a pesticide, germicide, feed additive, and soil additive. Limited research is available on the antimicrobial effects of copper metals and copper sulfate against *Listeria monocytogenes*. Most work on the antimicrobial effect of copper sulfate has concentrated on human periodontal pathogenic bacteria (Peters et al., 1986; Belcastro et al., 1994; Grytten et al., 1987; Maltx and Emilson, 1988). Larrondo and Calvo, 1990, studied the effects of copper sulfate against *Bacillus subtilis*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Candida albicans* and *Aspergillus niger*. Their results showed that *Esherichia coli* showed greater sensitivity to copper sulfate (150 mg/L) then the other bacteria

tested. Our study found that at 37°C and 25°C the most effective concentration of copper sulfate in the polyurethane coating on concrete was 15 mg, at this concentration *Listeria monocytogenes* was reduced to non-detectable levels. Furthermore, by increasing the concentration of copper oxide on the surface of concrete decreased the ability of *Listeria monocytogenes* to survive at 4°C.

The ability of copper sulfate or copper oxide to reduce *Listeria monocytogenes* to non-detectable levels was dependent on the nutrients used in the MWB media. This might be due to the production of biofilm by *Listieria monocytogenes* when grown in MWB with different nutrients, since biofilm has been shown to protect bacteria from sanitizers and antimicrobial agents. Kim and Frank (1995) investigated the effect of nutrients on the development of biofilm by *Listeria monocytogenes* on stainless steel using MWB with varied levels of components for 12 days of incubation. Their results showed that glucose levels between 1 and 20g/L in MWB produced lower levels of biofilm compared to the control 10g/L after 2 days. This might explain why we found by increasing (20g/L) or decreasing (1g/L) the amount of glucose in MWB increased the antimicrobial activity of copper sulfate and copper oxide against *Listeria monocytogenes* after 2 days.

Furthermore, some nutrients had positive effects on the antimicrobial activity of copper ions against *Listeria monocytogenes*. We found that when the glucose was replaced in MWB with tryptone, cellobiose, fructose, or mannose the antimicrobial activity of copper oxide and copper sulfate in polyurethane coated on concrete was increased against *Listeria monocytogenes*.

4.5 CONCLUSION

These studies evaluated the ability of *Listeria monocytogenes* to survive on the surface of concrete coated with polyurethane containing different concentrations of copper oxide or copper

sulfate as well as the effect of storage time, temperature, and variation of the nutrients required. The results show that storage at 4˚C reduced the antimicrobial activity of copper oxide against *Listeria monocytogenes.* However, with the same growth conditions, but at storage temperatures of 25 or 37˚C, *Listeria monocytogenes* was destroyed by day 4 and day 3, respectively. The results showed that the different concentrations and replacement of the required nutrients reduced the antimicrobial activity of copper sulfate and copper oxide against this pathogen. Higher and lower concentrations of glucose in MWB reduced the antimicrobial effect of copper ions against *Listeria monocytogenes*. Other nutrients increased the antimicrobial activity of copper ions against *Listeria monocytogenes* such as when glucose in MWB was replaced with cellobiose, fructose, mannose or tryptone. We have shown concrete coated with polyurethane that contained copper oxide or copper sulfate was able to control *Listeria monocytogenes* in a nutrient reach media and could possibly be used in a processing plant environment to control this pathogen.

4.6 REFERENCES

Belcastro, S., N. Staffolani, M. Pugliese, and F. D'Alo. 1994. An in-vitro study of the antimicrobial activity of copper and zinc salts on pure and mixed microbial cultures. Minerva. Stomatol. 43:393-396.

Centers for Disease Control and Prevention. 2002. Preliminary FoodNet data on the incidence of foodborne illnesses - selected sites, United States, 2001. Morbidity and Mortality Weekly Report 51(15), 325-9. Retrieved January 22, 2003

Center for Food Safety and Applied Nutrition. 2001. How to safely handle refrigerated ready-to-eat foods and avoid listeriosis. Retrieved January 22, 2003

Farber, J.M., and P. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55:476-511.

Grytten, J., T. Tollefsen and J. Afseth. 1987. The effect of a combination of copper and hexetidine on plaque formation and the amount of copper retained by dental plaque bacteria. Acta. Odontol. Scan. 45:429-433.

Health Canada. 1996. Incidence over time: Listeriosis, both sexes combined (incl. not specified), all ages (incl. not specified), Canada, 1987-1999. Notifiable Diseases On-Line. Retrieved January 22, 2003.

Hof, H., and J.Rocourt. 1992. Is any strain of *Listeria monocytogenes* detected in food a health risk? *Int. J. Food Microbiol*. 16:173-182.

Kim, K. Y., and J. F. Frank. 1995. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. *J. food Prot*. 58:24-28.

Keevil, C. W., J. T. Walker, and A. Maule. 2000, Copper Surfaces Inhibit *Escherichia coli* O157, *Seminario Cobre y Salud,* Nov. 20, 2000, CEPAL/Comision Chilena del Cobre/ICA, Santiago, Chile).

Larrondo, J. V. and M. A. Calvo. 1990. Influence of the addition of mineral salts on the inhibitory activity of strains of the Arthrinium genus. Microbios. 63:17-20.

Maltx, M. and C. G. Emilson. 1988. Effect of copper fluoride and copper sulfate on dental plaque, Streptococcus mutants and caries in hamsters. Scand. J. Dent. Res. 96:390-392.

Pagotto, F., E. Daley, J. Farber, and D.Warburton. 2001. MF-HPB 30. Isolation of *Listeria monocytogenes* from all food and environmental samples. In: Compendium of Analytical Methods. (2).

Peters, M., V. Vanden Berghe and A. Meheus. 1986. Antimicrobial activity of seven metallic compounds against penicillinase producing and non-penicillinase producing strains of *Neisseria gonorrhoease*. Genitourin Med. 62:163-165.

Premaratne, R. J., W. J. Lin, and E. A. Johnson. 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes. Appl. Environ. Microbiol*. 57:3046-3048.

Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the Food-Processing Environment. *J. Food Prot.* 64:709-725.

Tompkin R.B., V. N. Scott, D. T. Bernard, W. H. Sveum, and K. S. Gombas. 1992. Guidelines to prevent post-processing contamination from *Listeria monocytogenes*. *Dairy, Food and Environ. Sanit.* 19:551-562.

CHAPTER 5

ANTIMICROBIAL EFFECT OF COPPER IONS AGAINST BACTERIA IN THE ENVIRONMENT OF CRAWFISH PROCESSING PLANTS

5.1 INTRODUCTION

The ability of *Listeria monocytogenes* to form biofilms increases makes this organism dangerous. The unusual growth and survival properties add to the complexity of controlling *Listeria monocytogenes* from contaminating contact surfaces and food processing equipment. *Listeria monocytogenes* can grow in cool as well as moist environments, such as those found in any food process facilities. The incomplete removal of meat and fat from equipment, and improper sanitation can allow biofilms to develop. The most common reservoirs are drains and hoses that lay or drag across the floors in processing facilities (Fatemi and Frank, 1999).

Contamination of cooked products occurs most commonly at processing facilities when products are contaminated by food contact surfaces. This can occur between cooking, handling, and packaging steps. In addition, contamination can occur through other sources, such as an employee's apron or shoes, equipment, or the floor (Beresford, 2001). The risk of finding *Listeria monocytogenes* in ready-to-eat products increases when the contact surfaces are contaminated. *Listeria monocytogenes* attaches to contact surfaces in food-processing plants and develops into a bacterial biofilm by producing a glycocalyx that allows this pathogen to adhere to the contact surfaces. In addition, biofilms are highly resistant to sanitizers (Carpentier and Cerf, 1993).

Crawfish is a delicacy enjoyed for years in Louisiana. From November through June, they can be consumed in boiled and seasoned state, or are a main ingredient in an array of tasty dishes from appetizers to entrees. Seafood processing plants in Louisiana have the capacity to process this product year around in precooked frozen form. The processing of the crawfish begins with harvesting farm raised crawfish under controlled water conditions to ensure a quality product. The crawfish are then washed and inspected prior to boiling. Crawfish are boiled whole

to reach an internal temperature of 180ºF. All crawfish is then hand peeled in a sanitary environment and packers check the crawfish meat to assure cleanliness and quality. Finally, the crawfish meat is vacuum packaged bagged and iced for fresh shipment, or bagged and quick-frozen for future orders.

The crawfish industry is facing increasing demands for preventing the occurrence of microbial coatings such as biofilms with in the processing environment. A critical point is to document the presence of bacteria in biofilm on processing equipment (Mosteller and Bishop, 1993). Therefore, an indirect way to detect foodborne biofilms on visually clean equipment surfaces is vital in the processing plants along with the elimination of biofilm-bound bacteria on processing equipment, which is also a difficult task. If the biofilm is established, it can not be removed by daily sanitation unless additional actions are taken (Gibson et al., 1999).

Manufacturers have practiced a variety of methods such as mechanical treatment as well as additional disinfection. The results showed that a reduction in bacterial load could be achieved; however, no single method or chemical has been able to eliminate the microorganisms (Mafu et al., 1990). Furthermore, in order to minimize biofilm-bound bacteria on processing equipment, the critical locations should be identified and paid full attention to during sanitation. Finally, the right selection and usage of cleaners and disinfectants as well as sufficient sanitation programs must be incorporated (Van Den ElzenVAN and Snijders, 1993).

This study evaluated the effectiveness of copper in reducing bacterial counts at different locations in two crawfish processing plants located in southwest Louisiana during the crawfish season (March- May, 2005).

5.2 MATERIAL AND METHODS

5.2.1 Preparation of Media

Listeria species counts were determined by using oxford agar w/ antibiotic supplement (25 ml). To determine other microorganism, which could be growing along with *Listeria* species, total plate count agar and pseudomonas F agar were used. UVM and BHI broth were also used to confirm the growth of the bacteria.

5.2.2 Preparation of Samples

Copper alloys were cut into 4" disks (Thomas Industrial Network, User Services, 5 Penn Plaza, New York, NY 10001), sterilized by autoclaving, and placed at 12 various locations at each processing plant. Three samples were placed in the live room, two on the floor, and one in the drain. Three samples were placed in the walk-in cooler; on the floor, on the shelf, and in the drain. Three more samples were placed in the peeling room; on the floor, under the peeling table, and in the drain. Finally, the last three samples were placed in the packaging area; on the floor, under the sink, and in the drain. Every sample had a control at each location.

5.2.3 Bacterial Analysis

Bacterial counts were determined by swabbing all locations, in both plants, every two weeks for two months (March- May, 2005). The swabs were placed into BHI and than plated onto the oxford, total plate count agar and pseudomonas F agar. The plates were incubated under aerobic conditions at 37°C for 24 hours and the CFU/g was determined. The Pseudomonas plates were count on UV light stand and only colonies that fluorescence were counted.

5.2.4 Statistical Analysis

The effect of copper ions on the reduction of bacterial counts for each location in both plants was analyzed by determining the mean of each location.

5.3 RESULTS AND DISCUSSION

Listeria monocytogenes is a foodborne pathogen that causes an estimated of 2,500 infections and 500 deaths annually in the US (CDC, 2000). *Listeria monocytogenes* contamination of cooked ready-to-eat (RTE) foods has been linked to food processing plant environment. Although a recent USDA instruction encouraged RTE meat plants to apply environmental testing programs, the associations between the presence of *Listeria monocytogenes* in different environmental sites and finished product contamination has not been obviously recognized using quantitative criteria (Schmidt and Rodrick, 2003). In addition, *Listeria monocytogenes* widespread occurrence in nature, its ability to grow at refrigeration temperatures and form protective biofilms, allows this pathogen to thrive in moist environments such as floors and drains in the food processing plants. The drains contaminted with *Listeria* are especially challenging because when entrapped in a biofilm *Listeria* becomes protected against disinfectants and treatments that are usually used to control this pathogen (FSIS, 1990).

Table 5.1 shows the counts of total microorganism on the surface of copper metals, concrete, and steel drains placed in different locations at two crawfish processing plants located in Lousiana. By the end of week 2, the copper surfaces had lower total bacterial counts compared to the controls in plant A. Also, the bacterial counts on the surface of copper were lower compared to the controls in plant B. Furthermore, by the end of week 2, both plants had stopped processing crawfish and were cleaned with sanitizers, therefore, this could explain why the total counts were lower during the second week compared to the other weeks. Crawfish were being processed during week 4 at both plants, and a 0.50 to 1.88 log reduction for total bacterial counts had occurred, for most of the copper surfaces compared to the controls except for the copper drains in the cooler, live room, and packaging room in plant A (Table 5.1). By week 6 and 8, all copper surfaces in

plant B had reduced total bacterial counts by about 1 log compared to the control surfaces (Table 5.1).

The microflora on a final fish product is composed of the original flora and the microflora of the processing environment. The processing steps from the point of harvesting to the end of production have the potential to add to the microflora on the final seafood product. Therefore, the presence or absence of bacteria on seafood and fish product is a gathering of the harvest environment, hygienic conditions, and practices associated with equipment and personnel in the processing atmosphere (McLauchlin, 1993). The results of this experiment showed that usage of copper surfaces in the processing plant environments reduced the number of the total bacteria that were found in both plants. This indicates that utilization of copper in the environments of seafood plants could reduce the microflora on the finished seafood products.

The most common storage practice for preserving seafood and fish is chilling. During the chilling process, the temperature of the seafood and fish is reduced below the optimal temperature for growth of most foodborne pathogens. However, some microflora of seafood can grow and survive slowly during aerobic ice storage such as *Pseudomonas* species and *Shewanella putrefaciens* (Gram et al., 1987). This study showed that the microflora counts were reduced on the surfaces of copper even at refrigerator temperatures, therefore, the growth and survival of these microorganisms could be controlled with copper surfaces placed in the cooler.

Table 5.2 shows the total count of *Pseudomonas* species found in the environmental locations of two crawfish processing plants located in Louisiana. Week 0 showed high counts of total *Pseudomonas* species in both plants. *Pseudomonas* species counts were high on the surface of these samples because both plants were in the peak season of crawfish processing.

Table 5.1. Total counts of microorganisms for plant A and B at different locations

		Log CFU/cm									
		Week 0		Week 2		Week 4		Week 6		Week 8	
Location	Sample	A	B	A	B	A	B	A	B	A	B
Live Room	Copper surface	3.25	5.02	1.15	2.13	4.14	3.63	1.71	2.42	3.64	3.69
	Concrete	3.25	5.56	2.45	4.21	4.69	4.69	4.69	4.69	4.69	4.69
	Copper drain	5.78	5.21	1.32	2.21	3.06	3.38	0.00	3.45	3.59	3.69
	Steel drain	5.12	6.43	3.63	4.23	4.69	4.63	4.69	4.99	4.72	4.74
Cooler	Copper surface	1.53	4.83	0.00	4.21	1.31	3.44	1.17	0.00	1.79	3.01
	Concrete	2.33	4.84	2.31	4.53	2.13	4.72	4.48	3.43	2.35	4.55
	Copper drain	3.81	5.52	2.31	4.32	4.47	3.47	3.30	3.47	3.01	3.41
	Steel drain	5.23	5.42	4.63	4.35	4.67	4.63	4.67	4.66	4.67	4.57
Peel room	Copper surface	3.15	2.95	0.00	3.25	2.99	3.53	1.85	2.45	4.59	3.56
	Concrete	1.15	2.95	0.00	4.65	3.21	4.66	4.69	4.66	5.76	4.77
	Copper drain [a]	------	5.65	------	0.00	------	3.29	-----	3.37	------	3.77
	Steel drain [a]	------	5.76	------	2.73	------	4.56	------	4.59	------	4.89
Packaging room	Copper surface	1.92	3.11	0.00	0.00	3.92	3.69	4.69	3.69	4.97	3.45
	Concrete	2.55	3.54	1.86	3.99	4.69	4.69	4.90	4.69	5.11	4.98
	Copper drain	1.7 3	4.72	2.43	0.00	4.69	3.29	3.00	3.24	4.51	3.51
	Steel drain	2.62	4.90	4.22	1.24	4.85	4.56	4.26	4.22	5.66	4.99

[a] Plant A had no drain in the peel room, so no copper drains were placed into this location and no counts were measured.

By week 2, both processing plants had stopped processing and were cleaned with sanitizers. However, reduction of total *Pseudomonas* species counts on the surface of copper was observed for the different locations in plant A. Processing plant B on the other hand, showed reduced *Pseudomonas* species counts in the copper samples, in most locations, as compared to the control samples except for the cooler. By weeks 6 and 8, all copper surfaces for plant B had reduced *Pseudomonas* species counts. The reduction occurred by about 1 log compared to the control surfaces (Table 5.2).

Pseudomonas species has the ability to grow at refrigerator temperatures and produce heat-stable extracellular enzymes such as lipases, proteases, and lecithinases which can promote spoilage of seafood and fish (Champagne et al., 1994). The microbial flora connected with freshly harvested seafood and fish is mainly due to the environment in which the fish are caught that can correspond to spoilage (Shewan, 1977). The microflora that are normally found in the seafood and fish processing environment, are psychrotrophic or psychrophilic Gram-negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Alcaligenes, Shewanella,* and *Flavobacterium. Aeromonas* species In addition, several types of *C. botulinum* are found in the marine environment (Dodds 1993). Results of this experiment showed that copper samples placed in the crawfish processing environments reduced the counts of *Pseudomonas* species This suggests that the utilization of copper in seafood and fish processing plant environment might reduce the spoilage bacteria on the surface of seafood.

Table 5.3 shows the total count of *Listeria* species found in the environmental locations of two crawfish processing plants located in Louisiana. During the initial visit, *Listeria* species were detected in both plants on most surfaces and drains except for plant A.

Table 5.2. Total counts of *Pseudomonas* species for plant A and B at different locations

		Log CFU/cm									
		Week 0		Week 2		Week 4		Week 6		Week 8	
Location	Sample	A	B	A	B	A	B	A	B	A	B
Live Room	Copper surface	3.62	4.71	1.22	2.33	3.62	3.64	1.71	2.42	3.63	3.69
	Concrete	3.53	4.75	1.44	4.55	4.69	4.71	4.61	4.79	4.64	4.69
	Copper drain	5.82	5.42	1.32	2.42	2.77	1.77	0.00	3.45	4.69	3.68
	Steel drain	5.82	5.92	3.51	4.56	4.87	4.64	4.69	4.67	4.97	4.74
Cooler	Copper surface	1.95	3.42	0.65	4.05	1.38	3.55	1.20	0.00	1.83	1.76
	Concrete	2.31	3.45	2.97	4.65	3.87	4.60	4.44	4.63	4.52	4.56
	Copper drain	4.01	5.42	2.31	4.61	4.41	3.41	3.41	3.47	4.21	3.03
	Steel drain	5.61	5.44	4.64	4.65	4.69	4.74	4.98	4.71	4.99	4.57
Peel room	Copper surface	1.82	3.76	0.00	1.12	2.26	3.66	0.00	2.45	4.54	3.58
	Concrete	3.45	3.78	0.88	3.99	3.11	4.69	4.20	4.68	4.95	4.72
	Copper drain [a]	------	5.53	------	0.00	------	3.69	-----	3.56	-----	3.95
	Steel drain [a]	------	5.66	------	4.62	------	4.58	------	4.59	-----	4.67
Packaging room	Copper surface	4.35	4.25	1.45	1.85	4.12	3.58	4.69	3.58	4.34	3.54
	Concrete	2.92	4.56	0.00	4.56	4.69	4.66	4.69	4.69	5.19	4.68
	Copper drain	2.11	4.62	2.22	0.00	4.69	3.54	3.30	3.29	4.54	3.51
	Steel drain	2.63	4.74	3.81	1.93	4.80	4.62	4.23	4.63	5.21	4.65

[a] Plant A had no drain in the peel room, so no copper drains were placed into this location and no counts were measured.

No *Listeria* species were detected in the peel room. Also, in plant B, no *Listeria* species were detected in the packaging room. At the end of week 2, *Listeria* species were found on the surface of the copper samples (Surface and drains) in either of the two plants. However, *Listeria* species were found in the control samples in the live room and the cooler in each plant, and in the packaging room drain in plant B, even though, during week 2 both plants had stopped processing crawfish and were cleaned with sanitizers. By the end of week 4, both processing plants had lower *Listeria* species counts on the surface of all copper samples as compared to the control surfaces. Finally, at the end of week 8, *Listeria* species were absent in all copper samples in plants A and B. However, the pathogen was detected on the steel drain in the live room, and the concrete surface in the packaging room for plant A control. In addition, *Listeria* species were found in plant B in control samples in the live and peel room drains but were not detected in other locations (Table 5.2).

A nine-month study was carried out in 11 locations to evaluate the degree of contamination on work surfaces, equipment, personnel, and slaughterhouses. The study evaluated the presence of *Salmonella* species*, Listeria* species, and *Yersinia* species Results indicated that slaughter floors, cold room floors, and worktables can harbor these pathogens (Schuchat, 1992). Since *Listeria* species can be found on floors, drains, and cold rooms, the results of this study indicated that copper surfaces and drains could provide a measure to reduce *Listeria* species in the seafood and fish processing plant environment.

Thimothe and others (2002) monitored the presence of *Listeria species* and *Listeria monocytogenes* in the environment of two crawfish processing plants during the crawfish season. Samples included raw crawfish, cooked crawfish, and environmental sponge samples that were collected during the middle and end of processing.

Table 5.3. Total counts of *Listeria* species for plant A and B at different locations

		Log CFU/cm									
		Week 0		Week 2		Week 4		Week 6		Week 8	
Location	Sample	A	B	A	B	A	B	A	B	A	B
Live Room	Copper surface	0.00	1.21	0.00	0.00	3.69	0.00	0.00	0.00	0.00	0.00
	Concrete	0.00	0.65	0.00	3.45	4.01	4.69	0.00	4.36	0.00	0.00
	Copper drain	0.00	3.61	0.00	0.00	2.32	1.10	0.00	0.00	0.00	0.00
	Steel drain	0.00	3.87	3.12	2.91	4.69	4.52	3.38	4.69	3.44	2.10
Cooler	Copper surface	0.00	1.15	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.00
	Concrete	0.00	2.11	1.35	2.11	1.67	4.72	1.15	0.00	0.00	0.00
	Copper drain	0.00	2.11	0.00	0.00	2.72	1.54	0.00	0.00	0.00	0.00
	Steel drain	2.34	0.00	3.32	4.53	3.44	3.20	3.51	3.73	0.00	0.00
Peel room	Copper surface	0. 00	0.00	0.00	0.00	0.00	0.00	1.80	1.30	0.00	0.00
	Concrete	0.00	0.00	0.00	0.00	1.17	4.66	0.00	1.98	0.00	0.00
	Copper drain [a]	------	2.31	------	0.00	------	0.00	-----	1.43	-----	0.00
	Steel drain [a]	------	2.42	------	0.00	------	3.80	------	4.02	-----	1.20
Packaging room	Copper surface	0.00	0.00	0.00	0.00	1.82	0.00	1.00	0.00	0.00	0.00
	Concrete	0.00	0.00	0.00	0.00	3.80	4.69	0.00	4.10	2.07	0.00
	Copper drain	0.00	0.00	0.00	0.00	1.60	1.20	0.00	0.00	0.00	0.00
	Steel drain	0.00	0.00	0.00	2.87	2.98	3.25	0.00	3.25	0.00	0.00

[a] Plant A had no drain in the peel room, so no copper drains were placed into this location and no counts were measured.

Listeria monocytogenes were found in three raw crawfish samples and one environmental sample. In addition, *Listeria* species were detected in the raw crawfish, the environmental samples that were collected from drains, and on the employee contact surfaces, however, none of the finished product samples contained *Listeria* species or *Listeria monocytogenes*.

Lappi and others (2004) also found *Listeria* species and *Listeria monocytogenes* in two crawfish processing plants. They isolated the *Listeria species* in raw or whole crawfish and in the environmental materials; however, during the crawfish season, no *Listeria* species or *Listeria monocytogenes* were detected in the finished product. In addition, they showed that *Listeria species* in the raw crawfish varied from year to year. Our results showed that copper surfaces and copper drains could possibly reduce the counts of *Listeria* species in the processing plants, thereby reducing any possible contamination of the finished product.

For plant B, the copper surfaces located in the cooler (5ºC) did not have a reduction in the bacterial counts until week 4, whereas bacterial counts on the surface of the copper located in the live room were reduced by the end of week 2 as compared to the control surfaces. A similar trend was observed during the in-vitro studies (chapter 3 and 4) where the antimicrobial effects of copper ions against *Listeria monocytogenes* were reduced at refrigerator temperatures. Furthermore, this study has shown that the reduction of antimicrobial effects of copper ions at refrigerator temperatures is due to the slower release of copper ions from the metal surfaces (Chapter 6).

5.4 CONCLUSION

This study evaluated the performance of copper alloy, used as surfaces and drains, against *Pseudomonas* and *Listeria* species present in the crawfish processing plant environments. The results showed that copper surfaces and drains have some effect in the overall reduction of the total

microorganism and *Pseudomonas* species. In addition, surfaces and drains made from copper reduced *Listeria* species counts to non-detectable levels in seafood processing plants. The results of this experiment indicated that copper alloys could be used in the environment of seafood and fish processing plants to control *Listeria* species and other bacteria.

5.5 REFERENCES

Beresford, M.R., P. W.Andrew, and G.Shama . 2001. *Listeria monocytogenes* adheres to many materials found in food-processing environments. *J Applied Microlgy*. 90:1000-1005.

Carpentier, B., and O. Cerf . 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *J Applied Bacteriology*. 75:499-511.

Centers for Disease Control and Prevention. 2002. CDC Surveillance Summaries, March 17, 2000. MMWR 2000;49(No. SS-1).

Champagne, C. P., R. R. Laing, D. Roy, and A. A. Mafu**.** 1994. Psychrotrophs in dairy products: their effects and their control. *Crit. Rev. Food Sci. Nutr*. 34:1-30

Dodds K. L. 1993. *Clostridium botulinum* in the environment. In: Hauschild AHW, Dodds KL, editors. *Clostridium botulinum*: ecology and control in foods. New York: M Dekker. 21-52.

Fatemi, P., and J. Frank . 1999. Inactivation of *Listeria monocytogenes/Pseudomonas* Biofilm by Peracid Sanitizers. *J Food Prot*. 62(7):761-765.

FSIS. 1990. FSIS Directive 10,240.1 Rev.1. Microbiological Monitoring program: Sampling, Testing Procedures and Actions for *Listeria Monocytogenes* and *Salmonella*. FSIS, USDA, Washington, DC (Available from FSIS information Office.)

Gibson ,H., J. Taylor, K. Hall, and J. Holah . 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *J. Applied Micrology*. 87:41-48.

Gram L, and H. Huss. 2000. Fresh and processed fish and shellfish. In: Lund BM, Baird-Parker TC, Gould GW, editors. The microbiological safety and quality of food. Gaithersburg (MD): Aspen. p 472-506.

Huss, H. H., P. K. Ben Embarek, and V. From Jeppesen. 1995. Control of biological hazards in cold smoked salmon production. *Food Control*. 6 (6):335-40.

Lappi, V., J. Thimothe, J. Walker, J. Bell, K. Gall, M. Moody, and M. Wiedmann. 2004. Impact of intervention strategies on *Listeria* contamination pattern in crawfish processing plants: A longitudinal study. *J. Food Prot.* 67(6):1163-1169

Mafu, A.A., D.Roy, J. Goulet, and P. Magny, P. 1990. Attachment of *Listeria monocytogenes* to Stainless Steel, Glass, Polypropylene, and Rubber Surfaces After Short Contact Times. *J. Food Prot.* 53(9):742-746.

McLauchlin, J**.** , 1993. Listeriosis and *Listeria monocytogenes. Environ. Policy and Practice*. 3: 201-214.

Mosteller, T.M., and J. R.Bishop. 1993. Sanitizer Efficacy Against Attached Bacteria in a Milk Biofilm. *J. Food Prot*. 56(1):34-41.

Schmidt, R. H., and G. E. Rodrick. 2003. Food safety handbook. John Wiley & Sons, Inc. Hoboken, New Jersey.

Thimothe, J., J. Walker, V. Suvanich, K. Gall, M. Moody, and M. Wiedmann. 2002. Detection of *Listeria* in crawfish processing plants and in raw, whole crawfish and processed crawfish (*Procamarus species*). *J. Food Prot*. 65(11):1735-1739.

Schuchat, A. 1992. The Role of Food in Sporadic Listeriosis: A Case-Control Study of Dietary Risk Factors. *Journal of the American Medical Association*.

Shewan, J. M. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. Proceedings of the conference on handling, processing, and marketing of tropical fish. London: Tropical Products Institute. 51-60.

Van Den ElzenVAN, A.M.G., and J. M.Snijders.1993. Critical points in meat production lines regarding the introduction of *Listeria monocytogenes*. *The Veterinary Quarterly*.15(4):143-145.

CHAPTER 6

THE AMOUNT OF COPPER IONS RELEASE INTO MODIFIED WELSHIMER'S BROTH OR ABSORBED BY COOKED AND RAW SHRIMP

6.1 INTRODUCTION

Copper is a reddish metal that occurs naturally in rocks, soil, water, sediment, and air. It constitutes about 50 parts copper per million parts soil. Copper also occurs naturally in plants and animals (Rose, 1969). It is an important element for living organisms including humans and animals. Copper is known for two properties; malleability and ductility. These two properties make copper an "easy to shape metal". Some of the applications include coining, electrical wiring, and some water pipes. Copper is also found in many mixtures of metals, alloys, such as brass and bronze (Copper development center, 2003) which leads to the formation of various compounds. Some examples of such compounds include naturally occurring minerals as well as manufactured chemicals. The most commonly used compound of copper is copper sulfate. Many copper compounds can be recognized by their blue-green color, which is a result of the oxidization of the metal.

Nowadays, the antimicrobial activity of copper has been extended to include fungicides, pesticides, antifouling paints, antimicrobial medicines, oral hygiene products, hygienic medical devices, antiseptics, and a congregation of other useful applications (Klevay, 2000). Copper is an essential element for human health. These elements, along with amino and fatty acids as well as vitamins, are required for normal metabolic processes. However, as the body cannot synthesize copper, the human diet must supply regular amounts for absorption (Harrison et. al., 2000). The adult body contains between 1.4 and 2.1mg of copper per kilogram of body weight. Copper combines with certain proteins to produce enzymes that act as catalysts to help a number of body functions, some also help provide energy required by biochemical reactions. Others are concerned with the transformation of melanin for pigmentation of the skin and help to form cross-links in collagen and elastin fibers, which helps to keep up with the repair of the

connective tissues (Rock et. al., 2000). This is especially important for the heart and arteries. Research shows that copper deficiency is one of the principal factors that contribute to an increased risk of developing coronary heart disease (Araya, 2003). Food and Agricultural Administration (FAA) recommended that the population mean intake of copper should not exceed 12 mg/day for adult males and 10 mg/day for adult females (Rayssiguier et. al., 1993). A lot of the copper we ingest comes from non-food sources, such as copper cooking utensils, copper water pipes, and copper kettles, used for food processing and storage (Shils et. al.,1999). Copper deficiency may result in body weakness, digestive disturbances, and impaired respiration**.** In addition, all medicinal iron preparations contain traces of copper. Infants, especially those who are born premature, may develop copper deficiency, which usually presents itself in the form of chronic diarrhea, and later develops into anemia. Also copper deficiency has been reported in protein energy malnutrition (Saari and Sahuschke, 1999).

This study evaluated how much copper ions were released into raw and cooked shrimp placed on the surface of copper and brass alloys which were stored at 4 and 37ºC .

6.2 MATERIAL AND METHODS

6.2.1 Preparation of Shrimp

Copper and brass alloys were cut into 3” disks, sterilized by autoclaving then shrimp samples were placed onto the surface of the copper, brass and Petri dishes (control). The samples were incubated at 4 and 37ºC for 2, 10, 20, 40, 60 minutes and 24 hours.

6.2.2 Preparation of Copper and Brass with MWB

Copper and brass alloys were cut into 3” disks (Thomas Industrial Network, User Services, 5 Penn Plaza, New York, NY 10001), sterilized by autoclaving and placed into the stomacher bag that contained Modified Welshimer’s Broth (MWB), then incubated at 4, 25 and 37ºC for 4 days.

6.2.3 Shrimps and MWB Analysis

After incubation, the shrimp samples and MWB were place into a stomacher bag with 5ml of sterile water and stomached for 60 seconds. To determine how much copper ions were released into the shrimp samples the inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used (AOAC, 1995). In addition, Microwave Assisted Acid digestion method was used by utilizing the Digestion Vessel Assembly (Microwave Assisted Digestion of Siliceous and organically Based Matrices EpA Method 3052, Dec 1996).

6.3 RESULTS AND DISCUSSION

Copper is an essential micronutrient for microorganism and individuals. People who have deficiency in their diet they can be prone to anemia, growth retardation, impaired reproductive performance with low birth weight infants, bone disorders with osteoporosis, cardiovascular defects (Pizarro et al, 2001).

The normal daily copper stability is maintained at 1.3 mg in children between 6-10 years of age, and 2 to 5.8 mg in adults (Underwood, 1971). FDA suggested Daily Intake for copper is 2 mg for adults. Also US National Research Council recommends a copper intake of 50 μg/kg/d to maintain health (Klevay et al., 1980).

Furthermore, the World Health Organization (WHO) and the Food and Agricultural Administration (FAA) recommended that the population mean intake of copper should not exceed 12 mg/day for adult males and 10 mg/day for adult females (Olivares, 1998).

Table 6.1 shows that at 4°C, the amount of copper ions released from copper or brass alloys by the end of 24 hours, in both raw and cooked shrimp, were less than 4 ppm. These values are below the risk of toxic levels that were recommended by the health and food agencies.

However, the cooked shrimp that was placed on the surface of brass alloy had absorbed 13.00 ppm of copper ions by the end of 24 hours, which exceeds the toxic levels.

Table 6.1: The amount of copper Ions released into raw and cooked shrimp placed onto the surface of copper and brass alloys stored at 4ºC

		Copper ions (ppm)	
	Time	Raw	Cooked
Copper	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	< 4.00
	60 min	< 4.00	< 4.00
	24 h	< 4.00	< 4.00
Brass	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	< 4.00
	60 min	< 4.00	< 4.00
	24 h	< 4.00	13.00
Control	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	< 4.00
	60 min	< 4.00	< 4.00
	24 h	< 4.00	< 4.00

Table 6.2 shows the amount of copper ions from copper and brass alloys transferred to raw and cooked shrimp stored at 37 ºC. The amount of copper absorbed by the raw shrimp, by the end of 60 minutes, for both metals, was less than 4 ppm, which is below the toxic levels recommended. However, by extending the incubation time to 24 hours, both raw and cooked shrimp exceeded the toxic levels to 38.35 ppm and 55.70 ppm on the surface of copper or brass, respectively.

Furthermore, the amount of copper ions released from the copper alloy to the cooked shrimp stored at 37ºC by the end of 60 minutes was <4.00 ppm which indicated that these samples were still at safe levels. Conversely, the amount of copper ions absorbed by the cooked

shrimp at 24 hours was at 29.10 ppm. The amount of copper ions absorbed by the cooked shrimp on the surface of brass alloys at 40 minutes was 4.87 ppm, and at 60 minutes was 4.11. However, by increasing the time of incubation, the amount of copper ions absorbed by the cooked shrimp, after 24 hours, had increased to 33.45 ppm.

Table 6.2: The amount of copper Ions released into raw and cooked shrimp placed onto the surface of copper and brass alloys stored at 37°C

		Copper ions (ppm)	
	Time	Raw	Cooked
Copper	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	< 4.00
	60 min	< 4.00	< 4.00
	24 h	38.35	29.11
Brass	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	4.87
	60 min	< 4.00	4.11
	24 h	55.71	33.45
Control	2 min	< 4.00	< 4.00
	10 min	< 4.00	< 4.00
	20 min	< 4.00	< 4.00
	40 min	< 4.00	< 4.00
	60 min	< 4.00	< 4.00
	24 h	< 4.00	< 4.00

During the normal processing of seafood and fish, the exposure of these products to the contact surfaces should not exceed 30 min. Results of this experiment showed that shrimp samples exposed to copper or brass for 60 min did not exceed toxic levels.

Table 6.3 shows that at the end of 24 hours, the amount of copper ions released into MWB from copper was 107 ppm and from brass 8.59 ppm at 4°C. Brass released less copper ions because it contains about 60% copper ions compared to copper metal, which contains

approximately 99.3% copper ions. Furthermore, the amount of copper ions released into MWB from copper and brass alloys increased when it was stored at 25 or 37°C.

Table 6.3: The amount of copper ions released into MWB from copper and brass alloys stored for 24h

	Copper ions (ppm)		
	4ºC	25ºC	37ºC
Copper	107	157	166
Brass	8.59	15.91	10.91
Control	0.039	0.075	0.067

Therefore, when the temperatures increased, the ions were release more rapidly. This could explain why the antimicrobial activity of the copper ions was reduced against *Listeria monocytogenes* at 4°C. This pathogen was inhibited faster when inoculated on the surface of copper and brass stored at 37 °C (Chapter 3 and 4).

6.4 CONCLUSION

This study evaluated the amount of copper ions absorbed by raw and cooked shrimp on the surface of copper and brass alloys, at different storage times and temperatures. The results showed that storage of raw or cooked shrimp at 4˚C on the surface of copper and brass did not allow the absorption of copper ions to exceed the toxic level by the end of 24 hours. However, when brass was stored at 4˚C, under the same conditions, it exceeded the toxic level by 1 ppm after 24 hours of storage. The amount of copper ions absorbed, decreased as the temperature and storage times decreased. The shrimp samples that were exposed to surfaces made of copper or brass alloys for 40 minutes or less, did not exceed the acceptable levels stored at 4 and 37˚C. However, by the end of 24 hours of incubation, at 4 and 37˚C, the raw and cooked shrimp on the

surface of copper and brass exceeded the levels of copper. The results of this experiment indicated that food processors could utilize copper or brass alloys as food contact surfaces in the processing plants to avoid the risk of *Listeria monocytogenes* outbreaks.

6.5 REFERENCES

Araya, M., M. Olivares, F. Pizarro, M.Gonzalez, H. Speisky , and R. Uauy. 2003. Copper exposure and potential biomarkers of copper metabolism. *Biometals*. 16(1):199-204.

Copper development center. 2003. http://www.copper.org.sg/publications/cca/0309/pg06_japan_research.html

Harrison M. D, C. E. Jones, M. Solioz, and C. Dameron. 2000. Intracellular copper routing: the role of copper chaperones. *Trends Biochem Sci.* 25:29-32.

Klevay L. M. 2000. Cardiovascular disease from copper deficiency — a history. *J Nutr.* 130:489S-492S.

Klevay L.M., S. J. Reck, R. A. Jacob, G. M. Logan. J. M. Jr, Munoz, and H. H. Sandstead. 1980. The human requirement for copper. I. Healthy men fed conventional, American diets. *Am J Clin Nutr*. 33(1):45-50.

Microwave Assisted Digestion of Siliceous and organically Based Matrices EPA Method 3052, Dec 1996.

Official Methods of Analysis of AOAC International. 1995. 16th ed.

Olivares M, F. Pizarro, H. Speisky, B. Lonnerdal, and R. Uauy. 1998. Copper in infant nutrition: safety of World Health Organization provisional guideline value for copper content of drinking water. *J Pediatr Gastroenterol Nutr*. 26(3):251-7.

Pizarro F, M. Olivares, M. Araya, V. Gidi V, and R. Uauy. 2001. Gastrointestinal effects associated with soluble and insoluble copper in drinking water. *Environ Health Perspect.* 109(9):949-52.

Rayssiguier Y, E. Gueux, L. Bussiere, and A.Mazur. 1993. Copper deficiency increases the susceptibility of lipoproteins and tissues to peroxidation in rats. *J Nutr.* 123:1343-1348.

Rock E., A. Mazur, and J. O'Connor. 2000. The effect of copper supplementation on red blood cell oxidizability and plasma antioxidants in Middle-aged healthy volunteers. *Free Rad Biol Med.* 28:324-329.

Rose, J. 1969. Copper in History & Distillation. Encyclopedia Brittanica, volume 6, Copper. http://www.aromaticplantproject.com/articles_archive/copper.html.

Saari J. T., and D. A. Sahuschke. 1999. Cardiovascular effects of dietary copper deficiency. *Biofactors.* 10:359-375.

Shils M. E, J. Olson, M. Shike, and A. Ross. 1999. Modern Nutrition in health and Disease, 9th ed. Baltimore, MD: Williams and Wilkins. 241-252.

Underwood EJ. 1997. Trace Elements in Human and Animal Nutrition, 3rd Ed. NY: Academic Press. USEPA. Exposure Factors Handbook.

CHAPTER 7

CONCLUSION

Listeria monocytogenes, a foodborne pathogen, is of primary concern for ready-to-eat food processors. This unique pathogen has the ability to survive and grow over a large range of temperatures including refrigerator temperature, which poses a threat for the ready-to-eat food processors. The ready-to-eat products and deli meat industry is continuously developing and creating different products. This study has given processors further information to successfully maintain and improve their principles of quality while continuing to produce a safe product.

The assessment of *Listeria monocytogenes* at different temperatures, on copper and brass alloys, showed that this foodborne pathogen was destroyed with time over. Processing plants need to demonstrate concern in sanitation on using this metal in the refrigerator since *Listeria monocytogenes* has the ability to grow at refrigerator temperature. The most effective temperatures for reduction of *Listeria monocytogenes* on the surface of copper and brass was 25 to 37ºC. It should also be observed that based on the type of nutrients and nutrient concentration, the antimicrobial activity of copper and brass against *Listeria monocytogenes* varies for both copper and brass. The type and concentrations of sugar can affect the survival of *Listeria monocytogenes* on copper and brass surfaces.

The use of concrete coated with polyurethane containing copper ions showed significant reduction in *Listeria monocytogenes* counts compared to the controls. It was observed that the concrete surface with higher concentrations of copper oxide and copper sulfate had more antimicrobial benefits against *Listeria monocytogenes* stored at refrigerator temperatures. Concrete coated with polyurethane containing copper oxide and copper sulfate was shown to have strong antimicrobial activity against *Listeria monocytogenes* in vitro, reducing this foodborne pathogen to non-detectable levels. In addition, this study observed that based on the type of nutrients and nutrient concentrations, the potential for growth of *Listeria monocytogenes*

varies for copper oxide and copper sulfate. In addition, the type and concentrations of sugar varies in the antimicrobial activity of copper ions against *Listeria monocytogenes*. Concrete coated with polyurethane, containing different concentrations of copper ions, could possibly be used on the surfaces of the processing plants for the control of *Listeria monocytogenes*.

The use of copper alloys in the crawfish processing environments showed a significant reduction in *Listeria* species counts when the copper was placed on floors, drains and shelves at room and refrigerator temperatures. In addition, *Pseudomonas'* existence in the overall count of bacteria, present on the copper surface and copper drains, had decreased in number compared to the controls.

The amount of copper ions released from copper and brass alloys into raw and cooked shrimp did not exceed the toxic level of copper after 24 hrs of storage at 4ºC. However, the amount of copper ions released and absorbed by the shrimp increased when the temperature increased. The results showed that for all temperatures tested, and for both metals, the copper ions absorption by the shrimp samples did not exceed the toxic level for up to 60 min of placement.

In conclusion, this dissertation demonstrates that there is still a need to study the reduction mechanism of *Listeria monocytogenes* in copper ions. In addition, there is still a need to study the antimicrobial activity of copper ions against different foodborne pathogens. There is also a need to develop additional methods to control or inhibit the growth of *Listeria monocytogenes* in the ready-to-eat processing environments. Moreover, more research should be conducted regarding the application of the copper and brass alloys and concrete coated with different copper ions in long term experiments with advanced copper/ brass equipment. The

findings of this study will facilitate food processors ability to control *Listeria monocytogenes* on ready-to-eat products and environments.

VITA

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