Antimicrobial Activity of N-halamine Coated Materials in Broiler Chicken House

by

Tian Ren

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 9, 2015

Keywords: N-halamine, antimicrobials, broiler chicken house

Approved by

Tung-Shi Huang, Chair, Professor of Poultry Science
Jean Weese, Professor of Poultry Science
Floyd M. Woods, Associate Professor of Horticulture

Abstract

Broiler chicken houses are usually at a high risk of pathogen contamination travelling from birds to the facility or vice versa, during production. A hygienic living environment is important for producing healthy chickens. N-halamines are ideal antimicrobial agents due to their superior antimicrobial efficiency against a broad spectrum of bacteria, nontoxicity, high stability, rechargeability and low cost. The objective of this study was to assess the antimicrobial activity and the antimicrobial longevity of N-halamine (1-chloro-2,2,5,5-tetramethyl-4-imidazoidinone (MC), a non-bleaching compound)-coated materials in broiler chicken houses. The antimicrobial test of MC illustrated that 0.04% MC solution was able to kill *Salmonella* Typhimurium and *Campylobacter jejuni* completely at 10^6 CFU/mL. Based on this finding, we investigated the storage stability of MC coated materials and their antimicrobial longevity using iodometric/thiosulfate titration and the "sandwich test" method, respectively, over a four week time period. Results showed that the antimicrobial activity of 1% MC treated samples were able to kill *Salmonella* Typhimurium and *Campylobacter jejuni* completely up to four weeks, at the concentration of 10^6 CFU/cm^2, where the active chlorine atoms in the treated coupons decreased from the initial 10^{16} atoms/cm^2 to 10^{15} atoms/cm^2. Therefore, MC is assumed to be used as a novel antimicrobial agent in chicken broiler house.

Acknowledgments

I would like to first appreciate my major professor, Dr. Tung-Shi Huang, for his guidance, support and patience during the past three years. His knowledge in the field of food microbiology and the food industry, his hardworking spirit, and his optimistic attitude, have made the completion of this thesis possible. I also would like to thank my committee members, Dr. Jean Wease in Food Science and Dr. Floyd M. Woods in Horticulture, for their expertise and precious time. I would like to extend my appreciation to my laboratory co-workers, Mingyu Qiao, Lei Zhang and Allen Gardner, for their help throughout this program. Also I would like to show my thanks to Dr. Buket Demir from Chemistry and Dr. Lin Zhang from Polymer Science, for the technical support based on their expertise. Finally I would like to thank my parents, Shuangxi Ren and Wenying Wang, for their selfless love, support and sacrifice throughout my academic studies and my whole life.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	One-way analysis of variance
BPB	Butterfield's phosphate buffer
BPW	Buffered peptone water
CDC	Disease Control and Prevention
CFU	Colony forming units
CFR	Code of Federal Regulations
C. jejuni	*Campylobacter jejuni*
DI water	Deionized water
DMSO	Dimethyl sulfoxide
FBP	Ferrous sulfate, Sodium biosulfite, and Sodium pyruvate
GMP	Good Manufacturing Practice
HA	Hydantoin acrylamide
HACCP	Hazard Analysis Critical Control Points
MC	1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone
MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nalidixic acid
OD	Optical density
PET	Polyethylene terephthalate
Quats	Quaternary ammonium compounds
SAS	Statistical Analysis System

S. Typhimurium	*Salmonella* Typhimurium
TSA	Trypticase soy agar
TSB	Trypticase soy broth
USDA	United States Department of Agriculture
USDA FSIS	United States Department of Agriculture Food Safety and Inspection Service

Chapter 1 Introduction

1.1 Background

Food safety is increasingly drawing public's attention. In the US, according to a survey from the Centers for Disease Control and Prevention(CDC, 2011) and a report from Scharff (2012), foodborne pathogens caused many diseases, death and economic loss. With the ever-increasing demand for poultry products in the US (ERS, 2015), incidences of pathogen contaminations in poultry products can significantly affect human health. Therefore, protecting the safety of poultry products is a critical issue for reducing the risk of foodborne diseases.

In the poultry industry, antimicrobials are commonly used. It has been known that many factors may attribute to the cross-contamination. Chicken broiler house is a relatively vulnerable place for the cross-contamination in the poultry industry. It may spread the pathogens through hatching, or spatial contact, such as poor hygiene barriers, contaminated water supply, insufficient HACCP (Hazard Analysis Critical Control Points), and poor GMP (Good Manufacturing Practice). Therefore, many antimicrobials are used in the poultry product or processing facilities, especially on the surface areas, in an effort to eliminate or reduce the foodborne pathogens. However, the most commonly used antimicrobials in the poultry industry have several problems that cannot be ignored, such as corrosiveness, short shelf life and are potentially harmful to humans. In addition, even if antimicrobials could sanitize the surfaces effectively, clean surface areas could still be contaminated easily by a contaminated product and thus cross-contaminate following equipment and products. Therefore, using antimicrobial coating materials is one of promising ways to solve these problems.

N-halamines are ideal antimicrobial agents due to their superior antimicrobial activity against a broad spectrum of microorganisms, nontoxicity, high stability, rechargeability and low cost (Hui and Debiemme-Chouvy, 2013; Kenawy, et al., 2007). Antimicrobial activities of various materials coated with N-halamines have been investigated, such as fabrics(Demir, et al., 2015) and paints(Kocer, Cerkez, et al., 2011).

MC, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone, is a type of monochlorinated N-halamine, possessing high stability and powerful antimicrobial activity. A study showed that MC coated fabrics was able to kill *Staphylococcus aureus* with six log reduction (complete inactivation) within 5 min contact time and four log reduction of *Escherichia. coli* O157: H7 (Demir, et al., 2015). Also, the MC coated fabrics that are stored in a dark environment retained their initial active chlorine up to six months. In summary, MC compounds and its coated polymers are potential biocidal agents that may be used in food related fields, as well as in chicken broiler house.

1.2 Purpose of study

The purpose of this study is to evaluate the antimicrobial activity of MC and its coating materials in broiler chicken house. To achieve this goal, several specific objectives are listed: 1) to investigate the bactericidal ability of MC compounds towards two main foodborne pathogens in poultry production: *Salmonella* and *C. jejuni*; 2) to investigate the active chlorine in MC coated materials; 3) to assess the antimicrobial longevity of MC coated materials.

1.3 Significance of study

Considering the incidences of foodborne diseases and the increasing demand of poultry products, it is important to protect the chicken products from being contaminated. Antimicrobials are commonly used in the poultry industry in order to keep products safe,

however, the problems associated with these agents cannot be ignored. Therefore, finding a novel antimicrobial agent without those disadvantages is important to the food industry as well as to the public's health. Assessing the antimicrobial activity of MC and its coating materials can lay the foundation for the industry to substitute the current agents with the new one. Also, test of active chlorine in coated materials makes it able to investigate the feasibility of the new agents when applied to the chicken broiler house.

Chapter 2 Literature Review

2.1 Food safety concern

With globalization of the food industry, food safety becomes a major concern. Food safety means taking measures in food production, handling, preparation, storage, distribution and other procedures to prevent hazardous effects. Hazards in food safety concerns are usually categorized as physical, chemical and biological spoilage that threaten consumers' health.

In the US, according to the survey from the Centers for Disease Control and Prevention (CDC), there are about 48 million people who get sick with 128,000 hospitalizations, and 3,000 deaths from foodborne diseases each year (CDC, 2014).

Foodborne diseases also result in significant economic loss. Scharff (2012) estimated that the total annual economic cost is 51 billion dollars including estimates of medical expenditures and productivity loss, by using the basic. Therefore, taking measures to reduce microbial contaminations in foods can reduce the economic loss dramatically. According to a CDC study, by preventing a single fatal case of *E. coli* O157 infection, it can reduce about 7 million dollars loss (CDC, 2011).

Poultry products are the highest risk food that can cause foodborne diseases. It is estimated that each year there are more than 220,000 people infected with salmonellosis and 600,00 people are infected with campylobactererosis by consuming contaminated poultry products. The costs are estimated as 712 million and 1.3 billion dollar losses, respectively (Batz, et al., 2011).

Poultry products are in an increase demand in the US during the past decades. According to a report from USDA(ERS, 2015), in 2012, Alabama State produced one billion broilers, which value approximately 3 billion dollars. Considering the highly demand for poultry products, incidences of pathogen contaminations in poultry products can significantly affect human health. Therefore, to protect the safety of poultry products becomes a critical issue for reducing the risk of the foodborne diseases.

Many factors attribute to the cross-contamination of broiler poultry production. With the growing poultry products demand, it leads to an expansion of the poultry industry. To produce foods on a large scale, good operating practice is significantly important to keep food safe, but it is not enough to ensure the safety of final products. From farm to dining table, any failure in one single step along the production chain may result in contamination of the final products. Numerous food direct and non-direct contact surfaces such as conveyer belts may have the potential for cross-contamination.

A variety of transmission vehicles in processing plant or hatchery are another major sources to spread pathogens. The vertical transmission is through hatching, passing pathogens from hens to eggs. However, this vertical transmission as a source of contamination is still debating. The horizontal way is to transmit through spatial contact, which is considered as the most common transmission for microorganisms. Broiler chicken house is a relatively vulnerable place for cross-contamination in the poultry industry. Some common risk factors have been identified. For instance, poor hygiene barriers offer pathogens and wild insects an entrance to the plant; contaminated water supply is an obvious potential source of pathogens' infection; inappropriate staff behaviors like insufficient cleaning of boots and coats result in cross-contamination (Newell and Fearnley, 2003). Therefore, to eliminate or reduce

pathogens in the plant, especially on the surface area, antimicrobial is one of the important strategy to reach this goal.

Currently, the most commonly used antimicrobials in the poultry industry are chlorine and quats, and they are effective towards several microorganisms. However, chlorine is corrosive to many facilities. Furthermore, they are soluble in water so that they can leach free chlorine into water, readily reacted with other chemicals and producing by-products, which are reported harmful to humans. In addition, both chlorine and quats have a short shelf life due to their low stability. They are easily decomposed and lease hazardous chemicals.

Moreover, even if antimicrobials sanitize the poultry processing surfaces to a high degree, the clean surface can be easily contaminated by a contaminated product and thus cross-contaminate the products during the processing.

Considering the drawbacks of current antimicrobial agents, and the increasing emerging resistant pathogenic microbial strains in the poultry industry, it is important to find a novel way to address it. Antimicrobial coating materials are promising because they can efficiently inactivate contact bacteria, and prevent cross-contaminations.

2.2 Foodborne pathogens

Salmonella and *Campylobacter jejuni* are two major foodborne pathogens, which are frequently transmitted by poultry and poultry products. It has been reported that contaminated poultry products attribute approximately 72% campylobactererosis outbreaks and 20% salmonellosis outbreaks based on data from 1998 to 2008 (Batz, et al., 2011). As mentioned above, foodborne pathogens have various vehicles to contaminate poultry and poultry products. In broiler house, insufficient sterile barrier, poor maintenance, contaminated water and improper staff behavior can lead to the spread of microorganisms. Furthermore, in the

processing plant, scalding, defeathering, and eviscerating are vulnerable points of spread. In addition, in the market or in kitchens, these two bacteria can also be spread through consuming or improper handling of raw or under-cooked poultry products. The high incidence rate of diseases and the huge cost make it urgent to control the spread of *Salmonella* and *Campylobacter* in the poultry industry.

2.2.1 *Salmonella* spp.

Salmonella is a genus of gram-negative, non-spore forming, facultative anaerobe, and rod-shaped bacterium with 2-3 μm long and 0.4-0.6 μm wide in size. They belong to the Enterobacteriaceae family (Montville and Matthews, 2008). There are more than 2,000 serotypes of *Salmonella* based on differences in somatic (O), flagellar (H), and capsular (Vi) antigens (He and Kv, 1998). According to epidemiologic classification that is based on the preferences of the host, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Heidelberg are the three most common serotypes isolated from humans (Pui, et al., 2011).

Salmonellosis is an infection caused by *Salmonella* spp. People who are infected with *Salmonella* usually develop diarrhea, fever, abdominal cramps, and vomiting. It usually lasts for four to seven days, and most people will recover from it without treatment. However, immunocompromised individuals such as the elderly, infants and individuals with chronic diseases, are easily infected with *Salmonella* and need to be hospitalized.

Salmonella are widely distributed in nature and can survive in various types of foods. Poultry and fresh produce are the main carriers of salmonellosis outbreaks, which are about 21% and 16%, respectively (Batz, et al., 2011). *Salmonella* have a wide range of growth temperature between 8°C to 45°C, and pH between 4 and 9, with water activity higher than 0.94 (Silva and Gibbs, 2012). Intestines of broilers are the most common reservoirs for *Salmonella*. According to the USDA-FSIS report, in domestic grocery store, 3.9% and 18%

of young chicken and ground chicken, respectively, were *salmonella* positive (USDA-FSIS, 2013).

Chicken broilers that are contaminated with *Salmonella* serve as reservoirs, as well as infection sources. Both birds that have recovered from salmonellosis and those that were infected but never displayed symptoms are potential carriers of this pathogen, providing consumers the risks of diseases. Contaminated livestock is also a source of antibiotic-resistant *Salmonella*, which is a severe problem of food safety and clinic treatment (He and Kv, 1998).

2.2.2 *Campylobacter jejuni*

Campylobacter was first identified in 1913 as 'Vibrio fetus' that caused abortion of sheep (McFadyean and Stockman, 1913). In the 1970s the study of *Campylobacter* developed rapidly due to the improvement of selective growth media for *Campylobacter*. Advanced isolation techniques with improved selective media made it possible to diagnose species and test its properties.

Campylobacter is a genus of Gram-negative, non-sporeforming, curved rods that are about 0.5-5 μm long and 0.2-0.5 μm wide in size (Mahon and Manuselis, 2000). Generally *Campylobacter* species are motile by a single polar flagellum or multiple flagella. *Campylobacter* is a microaerophilic bacterium and can grow better in a low oxygen environment, and the optimal condition is at the atmosphere of 5% O_2, 10% CO_2, and 85% N_2.

Presently, *Campylobacter* contains 14 species in total, which are C. jejuni, *C. coli, C. concisus, C. curvus, C. fetus, C. gracilis, C. helveticus, C. hyointestinalis, C. lari, C. mucosalis, C. rectus, C. showae, C. sputorum,* and *C. upsaliensis* (Vandamme, et al., 2010).

C. jejuni is reported as one of the major species that causes bacterial foodborne diseases in many developed countries.

The proportion of chicken broilers with *Campylobacter* varies out globally. In the US, a survey indicated that nearly 90% of flocks are colonized. *C. jejuni* infection has been reported as the most common bacterial cause of human diarrhea (Altekruse, et al., 1999). Generally, outbreak of *C. jejuni* infections has been associated with consumption of contaminated meat, undercooked or raw poultry and milk. More specifically, poultry products need to take more responsibility of *C. jejuni* infections since 72% of the campylobacterioris outbreaks are due to consuming the contaminated chicken and chicken products. *C. jejuni* has been detected on chicken skin and exposed surfaces like intestine. Intestine is the reservoir for *C. jejuni*, since *C. jejuni* grows best at 37 °C to 42 °C, which is the body temperature of chicken.

Contaminated products are a source of *Campylobacter jejuni*/*coli* (CJC) enteritis, and the contaminations of *C. jejuni* in the poultry industry have several sources. In broiler chicken houses, feces are the potential sources of spread. When chickens are slaughtered and processed, their feces may contaminate equipment, and transmit the bacteria to other birds. When workers handle birds inappropriately, they are the victims and the carriers of *C. jejuni*. Therefore, development of an effective way to inactivate *C. jejuni* on the surface of broiler houses is important to reduce the cross-contamination.

Currently, there are increasing rates of human infections caused by antimicrobial resistant strains of *Campylobacter*, which makes it more difficult for clinical treatment. Many infections caused by antimicrobial resistant strains occur in developing countries due to the unrestricted or under-restricted use of antimicrobial drugs for human or animals. For

instance, 20% of *C. jejuni* isolates from chicken in grocery stores are ciprofloxacin-resistant (Smith, et al., 1998). Therefore, non-antibiotic antimicrobial use in food animals is prudent.

2.3 Current antimicrobials

Antimicrobial agents are defined as "substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors (21CFR 170.3)." Traditional antimicrobials, such as acetic acid, parabens, and sulfites, have been in use for many years. For instance, nitrites have long been applied in smoking and curing meats to prevent the growth of microorganisms.

However, there are more concerns in current antimicrobial agents applications. Many antimicrobials are in a short shelf life, while long-term usage is desirable. Furthermore, despite the huge diversity of antimicrobial agents, antibiotic resistant bacteria are increasing (Alves, et al., 2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a serious concern in regard to food safety risk now, and there are no efficient methods to control it. In addition, no matter the efficiency of antimicrobial agents to the birds, if there is any contaminate falls down on the surface, following processing run would be cross-contaminated. Therefore, developing an effective antimicrobial-based material to instantly eliminate the microorganisms on the surface is necessary.

Currently, there are several types of antimicrobials which can be used to prevent the cross-contamination on the surfaces. They are metal-based materials, quaternary ammonium compounds, cationic polymers, antimicrobial peptides, essential oils, light-activated antimicrobials and N-halamines. All the antimicrobial agents coated materials have shown biocidal activity towards various pathogens, but also appeared problems when applied into the food industry. For instance, Metal-based materials such as silver and zinc are efficient

antimicrobials. However, the biocidal efficiency always decreases in the presence of organic matter. Also the safety of these materials is another concern (Bastarrachea, et al., 2015).

2.4 An introduction to N-halamines

N-halamines are ideal antimicrobial agents due to their superior antimicrobial efficiency against a broad spectrum of microorganisms, nontoxicity, high stability, rechargeability and low cost (Hui and Debiemme-Chouvy, 2013; Kenawy, et al., 2007). Antimicrobial activities of many N-halamines have been investigated by coating them on various material surfaces such as fabrics (Demir, et al., 2015) and paints (Kocer, Cerkez, et al., 2011). The results show that these N-halamines have excellent antimicrobial activities.

N-halamines are a group of compounds containing one or more nitrogen-halogen covalent bond(s) that can be formed reversibly with chlorine, bromine, or iodine. It has been shown that oxidized halogens in halamines can either oxidize microbial membranes or be dissociated to become free halogens in aqueous media to terminate targeted microbes (Kenawy, et al., 2007). N-halamines are valuable antimicrobial agents due to their rechargeability. N-halamines can be recharged after exposed to halogens, so they have the antimicrobial ability again. The general structure of N-halamines is shown in Figure 2-1

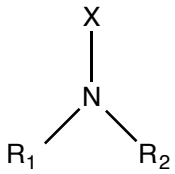
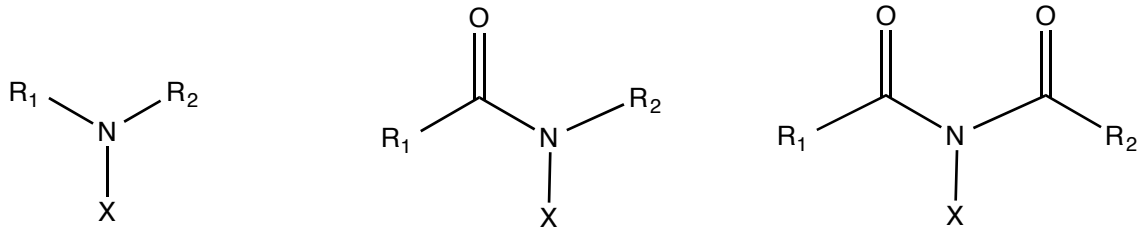


Figure 2-1. General structure of N-halamines. R_1, R_2 = H, Cl, Br, I, inorganic, organic group. X= Cl, Br, I.

N-halamines can be either organic or inorganic, depending on R_1 and R_2. If the substitution group is an inorganic group, then it is an inorganic N-halamine. Inorganic N-

halamines generally indicate chloramines, which are less effective in antimicrobial activity compared to free chlorine. However, they have lower reactivity towards organic substances compared to organic N-halamines.

The structure of N-halamines determines the properties of the chemicals. N-halamines with cyclic structures are usually stable due to the absence of alpha-hydrogen, and prevents alpha-hydrogen dehydrogenation. Imides, amides and amines are three important types of organic N-halamines. The trends of their stability and antimicrobial activity are determined by their structures, whose structures are shown in Figure 2-2. Stabilities of N-halamines increase in the order of imide> amide> amine (Qian and Sun, 2003), while their antimicrobial efficiencies are in the reverse order (Lauten, et al., 1992).



Amine Amide Imide

Figure 2-2. General Structures of amine, amide and imide N-halamines.

2.5 N-halamine polymers and coatings

N-halamines were introduced in 1968 (Kovacic, et al., 1970); and numerous N-halamines compounds have been synthesized in following years. Organic N-halamines can be divided into monomers and polymers based on molecular size, and also can be divided into cyclic and acyclic N-halamines based on the structure. As discussed above, the structure has great influence on the properties and functions of these N-halamines. Usually, cyclic monomer structures tend to be more stable, which are mostly used for coating beads for water

disinfection. For acyclic monomers, they are easy to be polymerized and formed polymers, which tend to be less toxic and easily coated on polymer surfaces (Wonley, et al., 2000).

Synthesis techniques of N-halamines have been developed rapidly; meanwhile, the application of N-halamines is also advanced. During the past decades, numerous N-halamine polymers, which are considered as combinations of polymeric materials such as fabrics and N-halamines, have been developed to inactivate microorganisms on the contact surface in several areas such as water purification systems.

Prior studies have indicated there are five major approaches to prepare polymeric N-halamines. The first approach is by homo or hetero polymerization. Kocer and co-workers (2011) have synthesized a new monomer hydantoin acrylamide (HA), which increases the number of N-H groups from one to three. Then this new monomer HA is copolymerized with a siloxane monomer (SL), and coated on fabrics by siloxane tethering groups with covalent bonds. Then the N-H group is replaced by N-Cl by soaking in household bleach. N-halamines in coated polymeric are more stable than the N-halamine in the monomeric coatings. Also, if the copolymer is positively charged, then there is no need to have a potential covalent group like siloxane. N-halamines precursors could be simply coated onto bleached materials layer by layer and then treated with household bleach (Cerkez, et al., 2012).

Numerous biocidal polymers have been made by grafting approaches. Generally, N-halamine precursor monomers are prepared first, and then they are grafted to polymer backbones, forming N-halamine derivatives. Numerous N-halamine polymers are made by coating on fabrics. For some materials without reactive sites like the hydroxyl group in cellulose, it is difficult to coat biocidal N-halamines on their surface. Therefore, it is necessary to build a ‘bridge’ to bind both the N-halamine moiety and polymers onto that bridge, thus creating an antimicrobial surface. Chen and Han (2011) have designed a grafting

technique that have been proven to have superior antimicrobial activities. The design incorporates a layer of the inert polymer surface to form a bilayer structure. Then PtBA Block and PS Block were inserted in the layer and materials, producing a link or a frame for N-halamines. Finally, N-halamines were able to attach to the blocks in order to bond to the materials. This concept is considered as universally suitable for any inert polymer to form desirable biocidal activities through cross linking agents, as long as appropriate blocking substances and antimicrobial agents are chosen. Another method of coating on inert polymeric materials like polyethylene terephthalate (PET) is reported by Liu et al (Liu, et al., 2010). The principle is to form an interpenetrating network by mixing some monomer precursors, crosslinking agents and PET to immobilize N-halamines onto an inert polymeric surface.

In addition, N-halamine containing polymers can be synthesized directly in the form of monomers like polystyrene and chlorinated, and then the N-halamine monomers are coated onto the surface of beads or non-woven fabrics (Sun, et al., 1994). Since antimicrobial activity is strongly dependent on its active surface area, coating onto beads can significantly enlarge the contact interface and thus enhance the efficiency. Those N-halamine coated beads, especially for nano size particles, are excellent in various applications such as water purification systems (Dong, et al., 2011). The coatings can also be derived directly from some natural polymers that have –NH-R group through chlorination like chitosan derived N-halamine.

2.6 Antimicrobial activities of N-halamines and their copolymers

N-halamines have displayed a powerful antimicrobial activity. N-halamines have a superior biocidal function to a broad range of microorganisms, from gram-negative to gram-positive bacteria (Li, et al., 2014), fungi, yeasts and viruses (Sun, et al., 1994). Furthermore,

microorganisms can be inactivated in a contact time as short as 5 min. For some microbes up to 9 log reduction (complete kill) can be achieved (Li, et al., 2014). In addition, many studies illustrate that most N-halamines are highly stable, especially in the dark environment. Even after disinfection, in which halogens are consumed, N-halamines can be generated with household bleach. Moreover, N-halamine polymers tend to be insoluble in water and do not leach organic compound or free halogen into water (Sun, et al., 1994).

As mentioned above, N-halamines have many advantages over other antimicrobial agents in regard to superior biocidal activity to a broad spectrum of microbes, long-term stability, rechargeability, less toxicity, less leaching to aqueous solution, and low cost. Therefore, N-halamines have been widely used as efficient disinfectants for various applications such as water purification systems (Yongjun Chen, et al., 2003), food packaging and food storage (Quintavalla and Vicini, 2002), by being successfully applied to various materials as coatings: textile, plastic, wooden, glass, metal, rubber, etc. Antimicrobial activities of these materials are also studied.

2.7 Antimicrobial modes of action

Mechanisms of antimicrobial agents in food are generally classified into three categories, by where they interfere with: cell membranes, genetic substances and protein synthesis (Davidson, et al., 2005). Different antimicrobials and targets may have various modes of action.

N-halamines exert powerful biocidal activity due to the oxidation state of halogen atoms in N-Cl groups. However, the exact mode of action is not confirmed. When microorganisms contact N-halamine coated surface, there are several possible approaches to be inactivated. First, the Cl^+ could diffuse through cell walls by the spacer of polymers, and then enter inner area between cell walls and adjacent cell membranes. Next, the Cl^+ in the N-

Cl group of N-halamine adsorbs onto the membrane surface, causing cell lysis by interacting with negatively charged phospholipids bilayers. So, bacteria are killed or inactivated when the ions and other functional constituents inside the cells are leaking out (Ikeda, et al., 1984). Furthermore, N-halamine may oxidize proteins on the membrane by reacting with –SH groups of proteins, thereby disrupting disulfide structure (Siedenbiedel and Tiller, 2012). Another hypothesis states that negative chlorine may be decomposed and diffuse into the cell through the cell membrane, disrupting the ion balance of the cell and preventing synthesis of some proteins in the cell.

2.8 MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone)

MC, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone, was firstly synthesized by Tsao decades ago (Tsao, et al., 1991). MC is a type of monochlorinated N-halamine, possessing high stability and powerful antimicrobial activity. A study shows that MC is an effective additive for hydroponic gardening, which is excellent in surface sterilization (Elder and Reid, 1993). MC coated fabrics are also shown high antimicrobial activity with six log reduction (complete kill) of *S. aureus* within 5 min contact time and four log reduction of *E. coli* O157:H7 (Demir, et al., 2015). In addition, MC coated fabrics stored in dark environments retain their initial chlorine content for six months. Furthermore, the chlorine atoms attached to MC molecules are not dissociated until contact with microorganisms, which makes it safe to humans and the environment (Demir, et al., 2015). MC compounds and MC-coated polymers are potential biocidal agents in water purification systems, food packaging and many other areas. The structure of MC is shown in Figure 2-3.

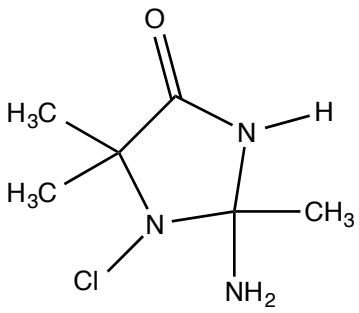


Figure 2-3. Structure of MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone)

Chapter 3 Methods and Materials

3.1. Solubility determination

The solubility of the MC for application in solutions was determined by using a modified standard protocol (NTP, 2003). The deionized water (DI), dimethyl sulfoxide (DMSO), and 95% ethanol were used as solvents. Then, 0.01 g MC powder was added into three test tubes containing 0.2 mL of each solvent, respectively, to make the concentration of 50 mg/mL. Each tube was shaken thoroughly. Where the MC dissolved into the solvents completely at this volume, there was no need to further test solubility procedures. Where the 0.01g MC was not completely dissolved into the specific solvent, the following tests were performed by increasing the volume of each solvent to 5 mL. Solutions were mixed to attempt to make the MC concentration at 20 mg/mL. If the MC was dissolved into the solutions completely at this point then no further step was taken. If the MC was not dissolved at this point, then the volume of solvents was increased to 20 mL in the solutions, in an effort to make the concentration at 0.5 mg/mL. By following this procedure, solvents were increasingly added to the solution until the MC was completely dissolved.

3.2. MC antimicrobial activity test

3.2.1 Bacteria preparation

One loop of *Campylobacter jejuni* from frozen culture was added into 10 mL of Brucella broth (Hardy Co., CA) with FBP (Ferrous sulfate, Sodium biosulfite, and Sodium pyruvate). The culture was incubated at 42°C for 48 h, in a one quart Ziploc bag filled with a microaerophilic gaseous mixture of 5% O_2, 10% CO_2 and 85% N_2. The incubation procedure was repeated for three times, which took six days to make the *C. jejuni* stable. Then, the

culture was streaked on Campy-Cefex agar and incubated in the same microaerophilic condition mentioned above. An isolated colony was selected and added into 10 mL of Brucella-FBP broth, incubated for 24 h under the same microaerophilic condition. According to previous research data in our lab, after 24 h the population of *C. jejuni* is approximately 2×10^9 CFU/mL. The bacterial suspension was diluted 50 times with buffered peptone water (BPW) to prepare a suspension of 4×10^7 CFU/mL of *C. jejuni*.

For *Salmonella* Typhimurium, one loop of frozen nalidixic acid (NA) resistant strain culture was added into 10 mL Trypticase® soy broth (TSB, BD Co., MD). The culture was incubated at 37°C for 24 h, and then transferred into another flask with 10 mL of TSB. The incubation procedure was also repeated for three times taking six days to make the *S.* Typhimurium stable. Then the culture was streaked on Trypticase® soy agar (TSA, BD Co., MD) with 100-ppm NA plate, and the plate was incubated in the same condition mentioned above. An isolated colony was picked and added into 10 mL of TSB, incubated for 12 h in the same condition. *S.* Typhimurium culture was washed with Butterfield's phosphate buffer (BPB) three times and then the $O.D._{640\ nm}$ of the bacteria suspension was adjusted to 0.5000, which indicates the *S.* Typhimurium population suspension is about 4×10^7 CFU/mL.

3.2.2 Antimicrobial test

An aliquot of 100 μL of *C. jejuni* suspension (4×10^7 CFU/mL) was added into each 4 mL water contained 0, 0.02, 0.04, 0.08, and 0.16% MC. Each concentration was duplicated. The mixture was vortexed thoroughly. Then, 200 μL of the mixture from each tube were transferred into an empty tube, at the contact time of 5, 10, 30, and 60 minutes. At each contact time, 200 μL samples were diluted decimally with BPW to 10^3. Each dilution was plated out on Campy-Cefex agar plate in triplicates. These plates were incubated in the same

microaerophilic conditions for 48 h, and then the colonies were emulated and recorded. In addition, the population of the inoculum was also determined by spread-plate method.

An aliquot of 100 μL *S.* Typhimurium (4×10^7 CFU/mL) was added into 4 mL water contained 0, 0.02, 0.04, and 0.06% of MC. All of the concentrations were duplicated. The mixture was vortexed thoroughly. Then, 200 μL of the mixture from each concentration were transferred into an empty tube, with the contact times of 5, 10, 30, and 60 min. From each contact time, 200 μL samples were removed and diluted decimally with BPP to 3 times. Each dilution was plated out on TSA with 100 ppm NA plates in triplicate. These plates were incubated at 37°C for 24 h, and then the colonies were emulated and recorded. In addition, the population of the was determined by spread-plate method.

3.3. Antimicrobial longevity test of MC coated materials

3.3.1 Sample preparation

Stainless steel, galvanized metal, aluminum, plastic, and pressure treated wood were selected as tested materials. The slides of materials were cut into the size of 2.54 × 2.54 cm^2 coupon, and 540 pieces of each material were prepared. All of the square coupons were autoclaved and completely dried before use. Then, 240 pieces from each material were spread with 0.1% MC ethanol solution and 240 pieces were spread with 1% MC ethanol solution. All MC treated coupons were air dried. After the ethanol was evaporated completely, all of MC treated coupons were placed into a dark, enclosed cabinet at room temperature. Samples were removed at the times of 0, 3, 6 d and 2, 3, 4, 5, 6, 7, 8 wk, to test the antimicrobial activity for evaluating the MC stability on various materials.

3.3.2 Antimicrobial efficacy testing

The "Sandwich test" method was used to evaluate the antimicrobial activity of MC treated materials (Worley, et al., 2005). To begin with, six stainless steel coupons from each group coated with 0%, 0.1% and 1% MC solution were taken out from cabinet, and placed into sterile petri dishes. All of the coupons had the MC-coated side facing up. The other four materials were assayed in the same way.

The *C. jejuni* inoculum (4×10^7 CFU/mL) was prepared with the same procedures mentioned above. An aliquot of 25 μL of this suspension was placed onto the center of a coupon, and covered with a second identical coupon with MC coated side face down. Then, a sterile weight was placed on the top of the second coupon to ensure complete contact of the two coupons. After 2 h contact time, the samples were placed into a 50 mL centrifuge tube which contained 5 mL 0.05 N of sodium thiosulfate to terminate the active chlorine residues for further disinfection. The tube was vortexed for 2 min in an effort to wash off all bacteria that attached to the coupons into the solution. Then, 10 fold serial dilutions were made by placing 100 μL of sample into 900 μL buffered peptone water (BPW), up to three dilutions, and each dilution was plated out the Campy-Cefex agar. The plates were incubated at 42°C microaerophilic conditions for 48 h, and the colonies on plates were counted and recorded for analysis. Each test was triplicated.

The method used to test the biocidal activity against *S.* Typhimurium was the same as to use for *C. jejuni*. The differences were the dilution buffer, media, plates, and the culture environment. For *Salmonella*, BPB buffer was used as the dilution buffer. After plating out on trypticase soy agar (TSA), plates were incubated at 37°C for 24 h. Then colonies were counted and recorded for further data analysis.

3.4 Determination of active chlorine content in MC coated coupons

A modified Iodometric/thiosulfate titration was used to determine the bond active chlorine content on the MC coated coupons. In a 125 mL flask, 0.25 g of potassium iodide was dissolved into 5 mL of 0.1 N acetic acid and 45 mL of absolute ethanol. Two coupons were added into the flask and it was immediately sealed with parafilm. They were shaken vigorously for 2 min to mix well. Then, standardized 0.00375 N of sodium thiosulfate titrant was added to the flask until the color turned from yellow to colorless and remained colorless for 1 min, which indicated the titration end point. The volume of sodium thiosulfate was recorded and the titration was repeated three times. Then, the average volume of the titrant was used for chlorine calculation according to the following equation:

$$Cl^{+}\ (atom/cm^{2}) = \frac{6.02 \times 10^{23} \times N \times V}{2 \times A}$$

Where N and V are the normality (equiv/L) and volume (L) of the sodium thiosulfate, respectively, and A is the surface area (cm^2) of the coupon.

3.5 Data analysis

One-way analysis of variance (ANOVA) tests and Dunnett's test (SAS 9.4, SAS Institute Inc., NC) were applied in this study to determine the antimicrobial activity of MC and its coated materials, at a 95% confidence level.

Chapter 4 Results and Discussions

4.1 Solubility determination

Table 4-1. Results of MC solubility test in three solvents at room temperature.

Solution	DI water*	DMSO**	95% Ethanol
Solubility	0.05%	2%	5%

*DI water: Deionized water **DMSO: Dimethyl sulfoxide

The results showed that MC solubility in DI water was the lowest among the three solvents, which was 0.05%. The solubility of MC in DMSO and 95% ethanol was 2% and 5%, respectively (Table 4-1). MC is an organic chemical with many hydrophobic groups such as the four methyl groups; therefore MC has a higher solubility in organic solvents.

4.2 Antimicrobial efficiency

Table 4-2. Reduction of *Salmonella* Typhimurium (%) treated with MC in Butterfield's phosphate buffer

MC	Contact time (min)			
Concentration	5	10	30	60
0.00%	41.72±3.66	36.56±3.23	37.20±1.29	43.44±1.51
0.02%	9.25±2.15	47.74±5.81	98.56±0.06	99.97±0.00
0.04%	46.45±5.81	95.23±0.09	99.98±0.01	100.00±0.00
0.06%	92.84±0.24	99.66±0.01	100.00±0.00	100.00±0.00

* The inoculum of *S.* Typhimurium was 3.1×10^6 CFU/mL

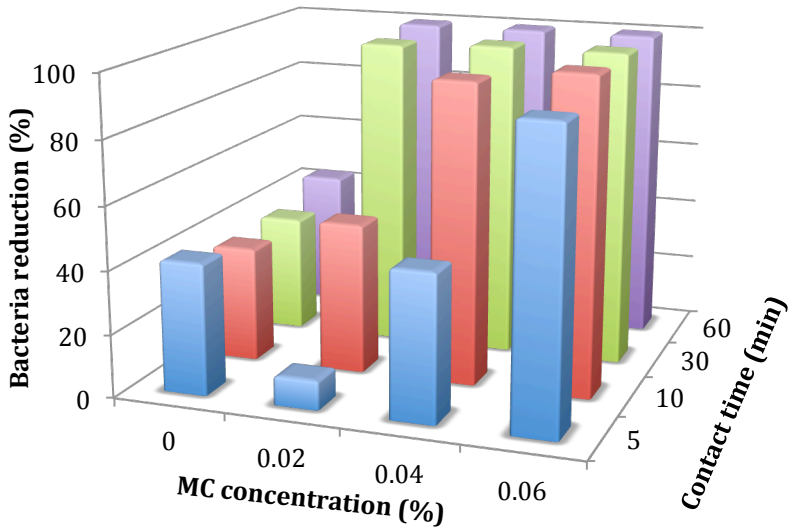


Figure 4-1. Antimicrobial activity of MC against *Salmonella* Typhimurium

Compared with the control group, samples with various concentrations of MC showed strong antibacterial activity. At each contact time, with the increase in MC concentration, the percentage of *S.* Typhimurium reduction increased (Figure 4-1). MC at 0.04% concentration inactivated approximately 50% of the *Salmonella* within five minutes contact time. At the 0.06% level, MC was able to inactivate most of the *Salmonella* within five minutes. At the 30 min contact time, the percentage of bacteria reduction increased from 37.2% to 100% (completely killed) as the concentration of MC increased from 0.00% to 0.06%. Following one hour of contact, the 0.02% MC was able to inactivate almost all of the *Salmonella* (99.97%), while at 0.04% and 0.06% MC levels, was able to inactivate 100% of the *Salmonella*.

The reduction of *Salmonella* also increased with longer contact time between the bacteria and the MC (Figure 4-1). The control group did not show any significant difference in *Salmonella* reduction at different contact times; however, for the MC treated groups, the biocidal activity increased with longer contact time. In the 0.02% MC solution, it can cause *Salmonella* reduction from 9.25% to 99.97%, with contact time from 5 min to 30 min. Similarly, the antimicrobial activity of solutions with 0.04% MC increased from 46.45% at 5

min to 100% at 30 min of contact time. At the 30 min contact time, 0.06% of MC was able to completely kill all of the inoculated bacteria.

Table 4-3. Reduction of *Salmonella* Typhimurium (%) treated with MC in Butterfield's phosphate buffer (2nd trial)

MC Concentration	Contact time (min)			
	5	10	30	60
0.00%	29.93±2.52	30.09±0.30	29.33±1.33	30.37±2.37
0.02%	24.00±0.44	36.59±6.22	94.96±0.15	99.64±0.00
0.04%	33.04±2.37	75.26±3.11	99.74±0.02	100.00±0.00
0.06%	93.36±0.12	99.40±0.03	100.00±0.00	100.00±0.00

* The inoculum of *S.* Typhimurium was 4.5×10^6 CFU/mL

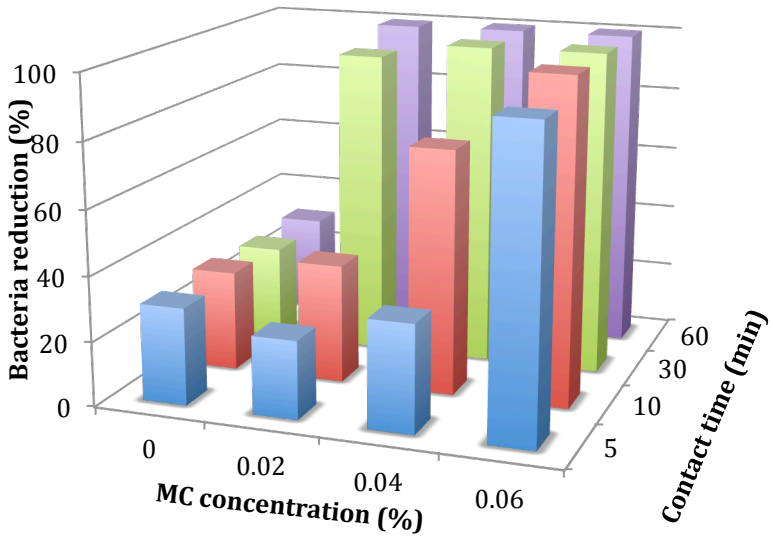


Figure 4-2. Antimicrobial activity of MC against *Salmonella* Typhimurium (2nd trial)

Table 4-3 shows the results of repeated experiment of MC's antimicrobial activity against *S.* Typhimurium. The bacterial inoculum was slightly different from the previous trial, but the trends of bacteria reduction were similar to the first experiment.

As the concentrations of MC increased, the percent reductions in bacteria increased as well (Figure 4-2). Also it shows that with the longer time, there were higher reductions in *S.* Typhimurium. Interestingly, there was a linear relationship between MC concentrations and *Salmonella* reductions at the 10 min contact time (Figure 4-3), which indicated the survival of *Salmonella* decreased proportionally with the increased MC concentration. When the MC concentration reached 0.06%, it could inactivate inoculated *Salmonella* completely in a short time.

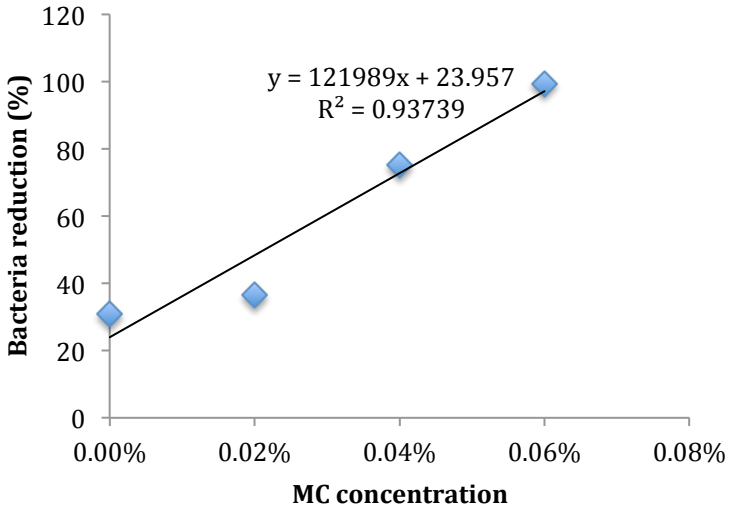


Figure 4-3. Relationship between MC concentration and *Salmonella* reduction at 10 min contact time

Table 4-4. Reduction of *Campylobacter jejuni* (%) treated with MC in buffered peptone water

MC Concentration	Contact time (min)			
	5	10	30	60
0.00%	15.40±2.30	19.50±0.46	17.47±0.23	30.57±4.14
0.02%	37.24±0.23	37.24±1.15	42.99±1.38	98.51±0.02
0.04%	49.43±1.84	98.64±0.07	100.00±0.00	100.00±0.00
0.08%	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.16%	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00

* The inoculum of *C. jejuni* was 2.9×10^6 CFU/mL

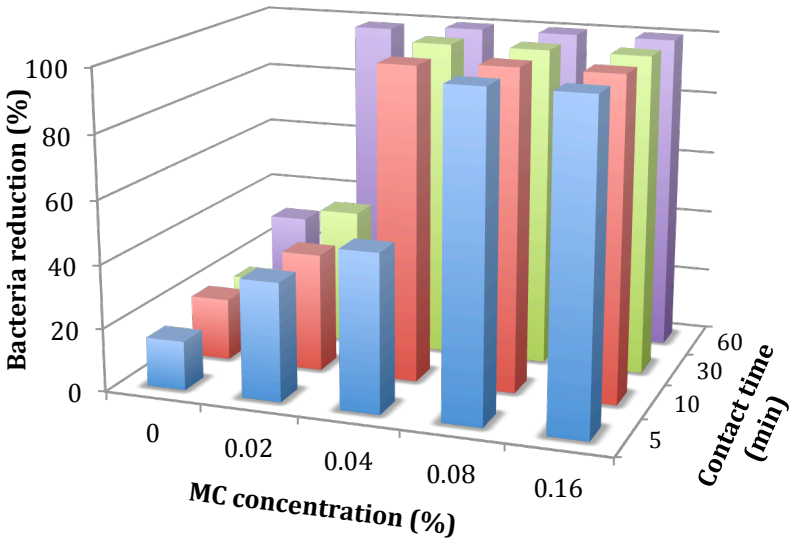


Figure 4-4. Antimicrobial activity of MC against *Campylobacter jejuni*

As in the *Salmonella* inactivation test, the percentage of inactivated *C. jejuni* increased as MC concentration increased (Figure 4-4). In the 0.02% MC treatment, five minutes contact time, 37.24% *C. jejuni* was inactivated, which was twice that of the control group. When the MC content was increased to 0.04%, the reduction of *C. jejuni* increased to 49.43% in five minutes of contact time. Likewise, 0.08% MC was able to inactivate 100% of

the *C. jejuni*. In addition, the solution with 0.04% or higher concentration of MC was able to kill all the inoculated *C. jejuni* at 30 min, while 0.02% MC was only able to inactivate 42.99% at the contact time of 30 min.

As contact time extended, the biocidal efficacy of MC increased (Figure 4-4). The solution contained 0.02% MC was only able to kill 42.99% of the *Campylobacter* within 30 min, but it almost completely inactivated all of the bacteria (98.51%) when the contact time was extended to one hour. Likewise, 0.04% MC killed 49.43% of the inoculated *C. jejuni* within 5 min, whereas there was a 98.64% reduction with a contact time of 10 min.

Table 4-5. Reduction of *Campylobacter jejuni* (%) treated with MC in buffered peptone water (2^{nd} trail)

MC	Contact time (min)			
Concentration	5	10	30	60
0.00%	19.49±1.54	15.38±5.64	20.00±0.00	22.05±1.03
0.02%	27.69±2.56	32.31±0.00	45.64±1.03	95.03±0.05
0.04%	71.28±0.00	99.28±0.00	100.00±0.00	100.00±0.00
0.08%	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.16%	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00

***** The inoculum of *C. jejuni* was 1.3×10^6 CFU/mL

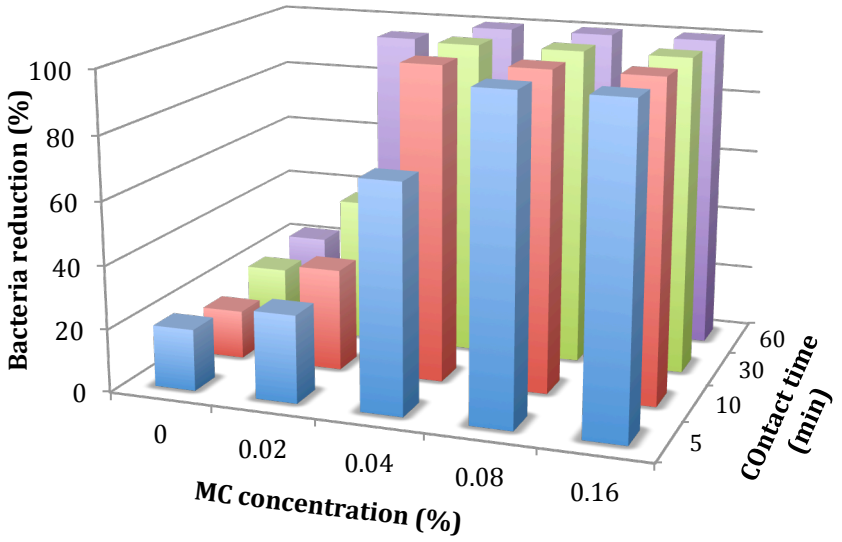


Figure 4-5. Antimicrobial activity of MC against *Campylobacter jejuni* (2nd trial)

In the second trial, the trend of antimicrobial efficacy against *C. jejuni* was similar to the first trial. The antimicrobial activity of MC against *C. jejuni* increased with both higher concentration and longer contact time (Figure 4-5). Within 5 min of contact time, reduction in *C. jejuni* increased from 19.5% in the control group to 27.69% in 0.02% MC, 71.28% in 0.04% MC, and 100% bacteria inactivated in 0.08% MC. Furthermore, at 0.02% MC level, it was only able to inactivate 27.69% of bacteria in 5 min, while the inactivation percentage increased to 45.64% and 95.03% at 30 min and 1 h of contact time, respectively.

In summary, results from these four experiments indicate that the reduction in both *S.* Typhimurium and *C. jejuni* increased with the higher concentration of MC as well as longer contact time, which is in good agreement with previous study (Demir, et al., 2015).

4.3 Active chlorine contents on MC coated materials

ntent of active chlorine in the MC-coated Materials

me	Cl^+ atom/cm^2 ($\times 10^{16}$) in 1% MC coated coupons				
	Stainless Steel	Galvanized Metal	Aluminum	Plastic	Pretreated W
D	3.76±0.04[a]	4.19±0.02[b]	2.98±0.07[b]	3.11±0.04[b]	2.79±0.0
D	3.73±0.02[a]	4.05±0.09[b]	3.22±0.05[a]	3.31±0.04[a]	2.76±0.0
D	3.63±0.19[a]	5.61±0.21[a]	2.04±0.05[c]	3.11±0.13[b]	3.14±0.0
W	3.62±0.08[a]	3.98±0.09[b]	1.97±0.07[d]	3.08±0.05[b]	2.76±0.0
W	1.94±0.02[b]	2.29±0.09[c]	1.43±0.02[d]	1.15±0.05[c]	1.36±0.0
W	1.31±0.64[c]	1.33±0.30[d]	1.20±0.16[e]	0.73±0.05[d]	0.59±0.1

rent characters in column indicate significant difference ($P < 0.05$)

Table 4-6 shows the quantities of active chlorine atoms per square centimeter on MC coated materials. Results showed that within three days from the initial application, the amount of active chlorine atoms stayed at the same level. In two weeks, there was still no significant reduction of active chlorine on the coupons from each material. The chlorine load on the different materials was varied due to the structures. The roughness of surface, and the flatness of coupons could influence the coating of MC, resulted in the various chlorine loads. Dunnett's statistic test showed that the amount of active chlorine in 3 weeks and 4 weeks were significantly reduced from those of the first two weeks. Chlorine loadings on MC coated aluminum were slightly different from other four materials. From day 6 after initial application, the chlorine contents decreased significantly over 4 weeks.

The active chlorine loss was associated with the N-Cl bond dissociation. An earlier study showed that the main reason of MC dissociation was due to the photodissociation of N-Cl bond, when it was exposed to light (Demir, et al., 2015). However, since these samples were stored in a dark cabinet, photodissociation of the chemical was not the cause. In addition, a previous study indicated that in order to provide antimicrobial activity at 10^6 CFU/sample level, 10^{17} atoms/cm^2 loadings were sufficient (Ren, et al., 2010). However, in this research, the following results showed that when the active chlorine atoms decreased to 5.85×10^{15} atoms/cm^2, the coupons were still able to 100% kill the inoculated *S.* Typhimurium and *C. jejuni* at 10^6 CFU/sample.

4.4 Antimicrobial longevity of MC-coated materials

Table 4-7. Survival *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated stainless steel in 2 h contact time (CFU/sample).

Time	*S.* Typhimurium*			*C. jejuni***		
	0***	0.1%	1%	0	0.1%	1%
0 D	2.4×10^5	0	0	2.6×10^4	0	0
3 D	2.3×10^5	0	0	4.5×10^4	1.8×10^4	0
6 D	2.3×10^5	2.1×10^5	0	2.1×10^4	1.8×10^4	0
2 W	2.4×10^5	2.9×10^5	0	2.1×10^4	1.8×10^4	0
3 W	3.5×10^5	4.2×10^5	0	3.3×10^4	5.9×10^4	0
4 W	3.6×10^5	3.4×10^5	0	4.3×10^4	4.1×10^4	0

* Inoculum of *S.* Typhimurium were 1.4×10^6, 9.3×10^5, 1.1×10^6, 1.1×10^6, 1.5×10^6, and 1.2×10^6 CFU/sample at different test times
** Inoculum of *C. jejuni* were and 1.3×10^6, 1.2×10^6, 8.0×10^5, 1.0×10^6, 1.3×10^6, and 1.1×10^6 CFU/sample at different test times
*** MC percentage treated

Table 4-8. Percentage reduction of *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated stainless steel in 2 h contact time.

Time	*S.* Typhimurium			*C. jejuni*		
	0	0.1%	1%	0	0.1%	1%
0 D	82.86±0.00	100.00±0.00	100.00±0.00	98.02±0.96	100.00±0.00	100.00±0.00
3 D	75.39±0.89	100.00±0.00	100.00±0.00	96.22±1.08	98.54±0.42	100.00±0.00
6 D	78.99±2.90	81.21±2.16	100.00±0.00	97.33±0.38	97.78±1.26	100.00±0.00
2 W	78.59±5.86	73.33±1.31	100.00±0.00	97.91±0.14	98.18±1.03	100.00±0.00
3 W	76.74±1.27	72.30±0.91	100.00±0.00	74.58±8.79	95.45±0.56	100.00±0.00
4 W	70.19±5.54	71.67±3.63	100.00±0.00	96.08±0.03	96.24±0.10	100.00±0.00

Table 4-7 and 4-8 display the antimicrobial activities of MC coated stainless steel coupons against *S.* Typhimurium and *C. jejuni* throughout four weeks. Stainless steel coupons treated with 1% MC inactivated 100% of the *S.* Typhimurium and *C. jejuni* up to four weeks of storage. This result indicated that the coated stainless steel with 1% MC performed excellent antimicrobial activity that remained stable for a long period of time.

Stainless steel with 0.1% MC coated retained its antimicrobial activity for six days against *S.* Typhimurium, and for four weeks against *C. jejuni*. Specifically, stainless steel coupons treated with 0.1% MC on the initial day and three days afterwards were able to kill all the inoculated *S.* Typhimurium in 2 h contact time. Six days after treatment, the coupon originally treated with 0.1% MC was able to kill 81.21% of *S.* Typhimurium. Afterwards, the 0.1% MC treated stainless steel showed similar antibacterial activity as the untreated control group (Table 4-8). In general, the antibacterial activity of MC treated coupons decreased over time; the antimicrobial activity of MC against *C. jejuni* was stronger than that of *S.* Typhimurium.

Table 4-9. Survival *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated galvanized metal in 2 h contact time (CFU/sample).

Time	*S.* Typhimurium*			*C. jejuni***		
	0***	0.1%	1%	0	0.1%	1%
0 D	4.7×10^3	4.4×10^1	0	0	0	0
3 D	2.8×10^3	2.5×10^3	0	0	0	0
6 D	6.8×10^3	6.1×10^3	0	0	0	0
2 W	4.6×10^3	2.4×10^5	0	0	0	0
3 W	4.1×10^3	8.4×10^4	0	0	0	0
4 W	5.9×10^3	5.4×10^4	0	0	0	0

* Inoculum of *S.* Typhimurium were 1.4×10^6, 9.3×10^5, 1.1×10^6, 1.1×10^6, 1.5×10^6, and 1.2×10^6 CFU/sample at different test times
** Inoculum of *C. jejuni* were and 1.3×10^6, 1.2×10^6, 8.0×10^5, 1.0×10^6, 1.3×10^6, and 1.1×10^6 CFU/sample at different test times
*** MC percentage treated

Table 4-10. Percentage reduction of *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated galvanized metal in 2 h contact time.

Time	*S.* Typhimurium			*C. jejuni*		
	0	0.1%	1%	0	0.1%	1%
0 D	99.67±0.08	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
3 D	99.70±0.16	99.73±0.19	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
6 D	99.39±0.16	99.45±0.05	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
2 W	99.58±0.02	77.78±4.68	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
3 W	99.73±0.01	94.39±2.49	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
4 W	99.51±0.02	99.55±0.01	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00

Table 4-9 and 4-10 show the antimicrobial effects of MC coated galvanized metal at various time periods against *S.* Typhimurium and *C. jejuni* in two hours of contact. Similar to stainless steel, galvanized metal coated with 1% MC inactivated 100% of both strains of bacteria within 2 h of contact time and the antimicrobial activity lasted up to four weeks. This result showed that galvanized metal with 1% MC was able to effectively inactivate bacteria for several weeks. Similarly, coating coupons with 0.1% MC effectively killed the two bacteria over time. Compared with the untreated group, samples treated with 0.1% MC killed inoculated *S.* Typhimurium more efficiently, on day one, three, and six (Table 4-10).

Interestingly, there was no *C. jejuni* detected in two MC treated groups, and the untreated control group. Galvanized metal is usually produced by submerging steel or iron in

molten zinc, which is done in an effort to prevent the steel or iron from rusting. Zinc has the function as an antimicrobial agent for several foodborne pathogens (Llorens, et al., 2012); therefore, the termination of *Campylobacter* on the galvanized metal coupons may have been due to the presence of the zinc coating.

MC-coated galvanized metal showed more effective antimicrobial activity against *C. jejuni* than that of *S.* Typhimurium. 0.1% MC was able to inactivate all the inoculated *C. jejuni* but cannot completely kill *S.* Typhimurium. In addition, according to Demir's (2015) study, it showed that 1 wt % MC-coated polypropylene nonwoven face-piece material had stronger biocidal efficacy on *Staphylococcus aureus* than that of *Escherichia coli* O157:H7. Therefore, MC-coated materials have different antimicrobial activity towards various types of pathogens.

Table 4-11 Survival *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated aluminum in 2 h contact time (CFU/sample).

Time	*S.* Typhimurium*			*C. jejuni***		
	0***	0.1%	1%	0	0.1%	1%
0 D	5.0×10^5	1.9×10^2	0	5.8×10^5	0	0
3 D	3.5×10^5	9.5×10^4	0	4.7×10^5	4.2×10^5	0
6 D	6.1×10^5	2.6×10^5	0	3.5×10^5	3.3×10^5	0
2 W	3.6×10^5	6.7×10^5	0	3.6×10^5	3.4×10^5	0
3 W	4.6×10^5	8.2×10^5	0	7.5×10^5	7.6×10^5	0
4 W	5.2×10^5	4.3×10^5	0	3.9×10^5	4.0×10^5	0

* Inoculum of *S.* Typhimurium were 1.4×10^6, 9.3×10^5, 1.1×10^6, 1.1×10^6, 1.5×10^6, and 1.2×10^6 CFU/sample at different test times
** Inoculum of *C. jejuni* were and 1.3×10^6, 1.2×10^6, 8.0×10^5, 1.0×10^6, 1.3×10^6, and 1.1×10^6 CFU/sample at different test times
*** MC percentage treated

Table 4-12. Percentage reduction of *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated aluminum in 2 h contact time.

Time	*S.* Typhimurium			*C. jejuni*		
	0	0.1%	1%	0	0.1%	1%
0 D	64.29±1.35	99.99±0.01	100.00±0.00	55.43±1.26	100.00±0.00	100.00±0.00
3 D	62.25±6.08	89.82±1.14	100.00±0.00	60.92±1.39	65.23±0.69	100.00±0.00
6 D	44.24±7.29	76.77±2.34	100.00±0.00	55.83±4.14	58.89±0.79	100.00±0.00
2 W	66.87±6.19	39.39±1.98	100.00±0.00	64.44±5.29	66.44±1.37	100.00±0.00
3 W	69.48±1.17	45.48±8.00	100.00±0.00	42.12±4.43	41.88±1.47	100.00±0.00
4 W	56.30±2.77	64.44±4.16	100.00±0.00	64.65±1.25	64.04±1.03	100.00±0.00

Similarly to the two materials mentioned above, aluminum with 1% MC was able to inactivate 100% of the *S.* Typhimurium and *C. jejuni* in two hours contact time, and this antimicrobial activity lasted for four weeks.

Compared with untreated aluminum, samples with 0.1% MC showed more effective antimicrobial activity towards *S.* Typhimurium within the initial six days of treatment, and against *C. jejuni* within two weeks. More specifically, 0.1% MC treated aluminum had nearly 1.5 times more efficacy in killing *Salmonella* than the control group on day six after the initial treatment. It was similar to the treatment against *C. jejuni*. On the first day of treatment, 0.1% MC coated aluminum was able to completely kill the inoculated *C. jejuni*. Then after 2 weeks of storage, it had similar antimicrobial activity with that of the untreated control group. In addition, MC coated aluminum showed similar antimicrobial activity against both *C. jejuni* and *S.* Typhimurium.

Table 4-13. Survival *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated plastic in 2 h contact time (CFU/sample).

Time	*S.* Typhimurium*			*C. jejuni***		
	0***	0.1%	1%	0	0.1%	1%
0 D	1.5×10^5	3.8×10^2	0	2.7×10^4	0	0
3 D	1.0×10^5	7.4×10^4	0	4.1×10^4	2.5×10^4	0
6 D	1.4×10^5	9.2×10^4	0	2.9×10^4	2.2×10^4	0
2 W	1.8×10^5	1.5×10^5	0	2.9×10^4	2.0×10^4	0
3 W	1.3×10^5	1.9×10^5	0	6.9×10^4	3.8×10^4	0
4 W	2.3×10^5	2.2×10^5	0	4.0×10^4	4.0×10^4	0

* Inoculum of *S.* Typhimurium were 1.4×10^6, 9.3×10^5, 1.1×10^6, 1.1×10^6, 1.5×10^6, and 1.2×10^6 CFU/sample at different test times
** Inoculum of *C. jejuni* were and 1.3×10^6, 1.2×10^6, 8.0×10^5, 1.0×10^6, 1.3×10^6, and 1.1×10^6 CFU/sample at different test times
*** MC percentage treated

Table 4-14 Percentage reduction of *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated plastic in 2 h contact time.

Time	*S.* Typhimurium			*C. jejuni*		
	0	0.1%	1%	0	0.1%	1%
0 D	89.57±5.76	99.97±0.03	100.00±0.00	97.95±1.39	100.00±0.00	100.00±0.00
3 D	89.27±0.99	92.04±1.49	100.00±0.00	96.59±1.52	97.93±0.83	100.00±0.00
6 D	87.43±3.21	91.66±0.57	100.00±0.00	96.33±1.03	97.22±1.90	100.00±0.00
2 W	83.64±1.31	86.67±0.86	100.00±0.00	97.07±1.04	97.96±1.06	100.00±0.00
3 W	91.05±0.12	87.26±1.17	100.00±0.00	94.68±0.58	97.08±1.59	100.00±0.00
4 W	80.93±2.50	81.30±0.69	100.00±0.00	94.40±0.42	96.34±0.48	100.00±0.00

Table 4-13 and 4-14 shows the effects of antimicrobial activity of MC coated plastics. As the same as previous materials, plastic coated with 1% MC was able to inactivate all the *S.* Typhimurium and *C. jejuni* in two hours contact time, and this antimicrobial activity lasted for four weeks.

In addition, plastic coated with 0.1% MC displayed a significant antimicrobial activity towards *S.* Typhimurium on days 0, 3, 6, 14, and one more week against *C. jejuni*. In general, the efficacy of aluminum coated with 0.1% MC in inactivating bacteria decreased with longer storage time.

Table 4-15 Survival *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated pressure treated woods in 2 h contact time (CFU/sample).

Time	*S.* Typhimurium*			*C. jejuni***		
	0***	0.1%	1%	0	0.1%	1%
0 D	3.7×10^4	2.6×10^3	0	0	0	0
3 D	2.6×10^4	1.8×10^4	0	0	0	0
6 D	2.9×10^4	1.9×10^4	0	0	0	0
2 W	2.3×10^4	8.0×10^3	0	0	0	0
3 W	6.6×10^3	6.9×10^3	0	0	0	0
4 W	1.2×10^4	4.9×10^3	0	0	0	0

* Inoculum of *S.* Typhimurium were 1.4×10^6, 9.3×10^5, 1.1×10^6, 1.1×10^6, 1.5×10^6, and 1.2×10^6 CFU/sample at different test times
** Inoculum of *C. jejuni* were and 1.3×10^6, 1.2×10^6, 8.0×10^5, 1.0×10^6, 1.3×10^6, and 1.1×10^6 CFU/sample at different test times
*** MC percentage treated

Table 4-16 Percentage reduction of *Salmonella* Typhimurium and *Campylobacter jejuni* after inoculated on MC coated pressure treated woods in 2 h contact time.

Time	*S.* Typhimurium			*C. jejuni*		
	0	0.1%	1%	0	0.1%	1%
0 D	97.38±0.13	99.82±0.01	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
3 D	97.16±1.11	98.04±0.47	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
6 D	97.33±0.32	98.30±0.70	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
2 W	97.93±1.30	99.27±0.25	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
3 W	99.56±0.02	99.54±0.17	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
4 W	99.02±0.04	99.59±0.08	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00

The 0.1% MC coated pressure treated woods showed higher antimicrobial activity against *S.* Typhimurium from day 0 to week 2 when compared to the control group. The 1% MC coated pressure treated woods were able to completely kill the inoculated *S.* Typhimurium up to four weeks.

From Table 4-16, the results showed that *C. jejuni* was completely killed in the control, 0.1% and 1% MC coated pressure treated woods from day 0 up to 4 weeks. Pressure treated woods are the woods which contain chemicals as preservatives to prevent microbial infections. So the chemicals from the pressure treated woods killed the inoculated *C. jejuni*.

MC-coated pressure treated woods showed more effective antimicrobial activity against *C. jejuni* than that of *S.* Typhimurium. 0.1% MC was able to inactivate all the inoculated *C. jejuni* but cannot completely kill *S.* Typhimurium. Therefore, when MC coated materials applied to the chicken broiler house as an antimicrobial agent, the concentration of MC should be 1% in order to inactivate all types of foodborne pathogens.

Chapter 6 Conclusions

This objective of this study was to evaluate the antimicrobial activity of MC, and its coated materials in broiler chicken houses. We also tested the stability of the bactericidal activity over a period of four weeks storage.

In the MC antimicrobial test, we found that at higher MC concentration, or with longer contact time, the antimicrobial activity of MC against *S.* Typhimurium and *C. jejuni* increased. Solutions containing 0.04% or higher concentration of MC were able to completely inactivate both of the inoculated *S.* Typhimurium and *C. jejuni* at 10^6 CFU/mL within a contact time of 30 min. Additionally, when we extended contact time to one hour, solutions with 0.02% or higher concentration of MC were able to kill more than 95% of both *S.* Typhimurium and *C. jejuni* inoculated.

Materials coated with 1% MC were able to inactivate all the inoculated *S.* Typhimurium and *C. jejuni* at 10^6 CFU/sample within 2 h contact time, and this antimicrobial activity lasted for at least four weeks. Materials coated with 0.1% MC killed more than 99.8% of the *S.* Typhimurium and all of the inoculated *C. jejuni* on the initial day, and then their antimicrobial activities decrease afterwards. In the first two weeks after materials were treated with MC, active chlorine content on materials did not decrease. After three weeks of treatment, the amount of active chlorine decreased significantly. Where the active chlorine atoms decrease from the initial 10^{16} atoms/cm^2 to 10^{15} atoms/cm^2 over this three-week period, the MC treated coupons were still able to completely inactivate all the inoculated microbes.

Our results indicate that MC is a promising antimicrobial agent, which can be applied into broiler chicken houses. For the purpose of inactivating both *S.* Typhimurium and *C. jejuni*, we found that 1% MC is suitable to be used to coat on various materials.

Reference

Altekruse, S. F., Stern, N. J., Fields, P. I., & Swerdlow, D. L. (1999). Campylobacter jejuni--an emerging foodborne pathogen. *Emerging Infectious Diseases, 5*(1), 28-35.

Alves, M. J., Ferreira, I. C. F. R., Martins, A., & Pintado, M. (2012). Antimicrobial activity of wild mushroom extracts against clinical isolates resistant to different antibiotics. *Journal of Applied Microbiology, 113*(2), 466-475.

Bastarrachea, L. J., Denis-Rohr, A., & Goddard, J. M. (2015). Antimicrobial Food Equipment Coatings: Applications and Challenges. *Annual Review of Food Science and Technology, 6*(1).

Batz, M. B., Hoffmann, S., & Morris, J. G. (2011). Ranking the Risks: The 10 Pathogen-Food Combinations With the Greatest Burden on Public Health. In, University of Florida, Emerging Pathogens Institute.

CDC. (2011). Estimates of Foodborne Illness in the United States. Accessed on March 30, 2015. Retrived from: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html

Cerkez, I., Kocer, H. B., Worley, S. D., Broughton, R. M., & Huang, T. S. (2012). N-halamine copolymers for biocidal coatings. *Reactive and Functional Polymers, 72*(10), 673-679.

Chen, Y., & Han, Q. (2011). Designing N-halamine based antibacterial surface on polymers: Fabrication, characterization, and biocidal functions. *Applied Surface Science, 257*(14), 6034-6039.

Chen, Y., Worley, S. D., Kim, J., Wei, C. I., Chen, T.-Y., Santiago, J. I., Williams, J. F., & Sun, G. (2003). Biocidal Poly(styrenehydantoin) Beads for Disinfection of Water. *Industrial & Engineering Chemistry Research, 42*(2), 280-284.

Davidson, P. M., Sofos, J. N., & Branen, A. L. (2005). *Antimicrobials in Food, Third Edition*: CRC Press.

Demir, B., Cerkez, I., Worley, S. D., Broughton, R. M., & Huang, T.-S. (2015). N-Halamine-Modified Antimicrobial Polypropylene Nonwoven Fabrics for Use against Airborne Bacteria. *ACS Applied Materials & Interfaces, 7*(3), 1752-1757.

Dong, A., Huang, J., Lan, S., Wang, T., Xiao, L., Wang, W., Zhao, T., Zheng, X., Liu, F., Gao, G., & Chen, Y. (2011). Synthesis of N-halamine-functionalized silica–polymer core–shell nanoparticles and their enhanced antibacterial activity. *Nanotechnology, 22*(29).

Elder, E. D., & Reid, J. C. (1993). Potential hydroponic application of an organic N-halamine disinfectant. In K. J. Hatcher (Ed.), *Georgia Water Resources Conferece*. Universtiy of Georgia.

ERS, U. (2015). Livestock & Meat Domestic Data. Accessed on March 30, 2015. Retrived from: http://www.ers.usda.gov/data-products/livestock-meat-domestic-data.aspx

He, E., & Kv, N. (1998). Microbial food borne pathogens. *Salmonella*. *The Veterinary clinics of North America. Food animal practice, 14*(1), 17-19.

Hui, F., & Debiemme-Chouvy, C. (2013). Antimicrobial N-Halamine Polymers and Coatings: A Review of Their Synthesis, Characterization, and Applications. *Biomacromolecules, 14*(3), 585-601.

Ikeda, T., Yamaguchi, H., & Tazuke, S. (1984). New polymeric biocides: synthesis and antibacterial activities of polycations with pendant biguanide groups. *Antimicrobial Agents and Chemotherapy, 26*(2), 139-144.

Kenawy, E.-R., Worley, S. D., & Broughton, R. (2007). The Chemistry and Applications of Antimicrobial Polymers: A State-of-the-Art Review. *Biomacromolecules, 8*(5), 1359-1384.

Kocer, H. B., Cerkez, I., Worley, S. D., Broughton, R. M., & Huang, T. S. (2011). N-Halamine Copolymers for Use in Antimicrobial Paints. *ACS Applied Materials & Interfaces, 3*(8), 3189-3194.

Kocer, H. B., Worley, S. D., Broughton, R. M., & Huang, T. S. (2011). A novel N-halamine acrylamide monomer and its copolymers for antimicrobial coatings. *Reactive and Functional Polymers, 71*(5), 561-568.

Kovacic, P., Lowery, M. K., & Field, K. W. (1970). Chemistry of N-bromamines and N-chloramines. *Chemical Reviews, 70*(6), 639-665.

Lauten, S. D., Sarvis, H., Wheatley, W. B., Williams, D. E., Mora, E. C., & Worley, S. D. (1992). Efficacies of Novel N-Halamine Disinfectants against Salmonella and Pseudomonas Species. *Applied and Environmental Microbiology, 58*(4), 1240-1243.

Li, C., Xue, L., Cai, Q., Bao, S., Zhao, T., Xiao, L., Gao, G., Harnoode, C., & Dong, A. (2014). Design, synthesis and biocidal effect of novel amine N-halamine microspheres based on 2,2,6,6-tetramethyl-4-piperidinol as promising antibacterial agents. *RSC Advances, 4*(88), 47853-47864.

Liu, S., Zhao, N., & Rudenja, S. (2010). Surface Interpenetrating Networks of Poly(ethylene terephthalate) and Polyamides for Effective Biocidal Properties. *Macromolecular Chemistry and Physics, 211*(3), 286-296.

Llorens, A., Lloret, E., Picouet, P. A., Trbojevich, R., & Fernandez, A. (2012). Metallic-based micro and nanocomposites in food contact materials and active food packaging. *Trends in Food Science & Technology, 24*(1), 10-20.

Mahon, C. R., & Manuselis, G. (2000). *Textbook of diagnostic microbiology second edition*. Philadephia, PA: W. B. Saunders Company.

McFadyean, J., & Stockman, S. (1913). Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion. In. London: HMSO.

Montville, T., & Matthews, K. (2008). *Food microbiology: an introduction second edition*. Washington, D. C.: ASM Press.

Newell, D. G., & Fearnley, C. (2003). Sources of Campylobacter Colonization in Broiler Chickens. *Applied and Environmental Microbiology, 69*(8), 4343-4351.

Pui, C. F., Wong, W. C., Chai, L. C., Tunung, R., Jeyaletchumi, P., Noor Hidayah, M. S., Ubong, A., Farinazleen, M. G., Cheah, Y. K., & Son, R. (2011). Review Article *Salmonella*: A foodborne pathogen. *International Food Research Journal, 18*, 465-473.

Qian, L., & Sun, G. (2003). Durable and regenerable antimicrobial textiles: Synthesis and applications of 3-methylol-2,2,5,5-tetramethyl-imidazolidin-4-one (MTMIO). *Journal of Applied Polymer Science, 89*(9), 2418-2425.

Quintavalla, S., & Vicini, L. (2002). Antimicrobial food packaing in meat industry. *Meat Science, 62*(3), 373-380.

Ren, X., Zhu, C., Kou, L., Worley, S. D., Kocer, H. B., Broughton, R. M., & Huang, T. S. (2010). Acyclic N-Halamine Polymeric Biocidal Films. *Journal of Bioactive and Compatible Polymers*, 25, 392-405.

Scharff, R. L. (2012). Economic Burden from Health Losses Due to Foodborne Illness in the United States. *Journal of Food Protection, 75*(1), 9.
Siedenbiedel, F., & Tiller, J. C. (2012). Antimicrobial Polymers in Solution and on Surfaces: Overview and Functional Principles. *Polymers, 4*(1), 46-71.
Silva, F. V. M., & Gibbs, P. A. (2012). Thermal pasteurization requirements for the inactivation of *Salmonella* in foods. *Food Research International, 45*, 695-699.
Smith, P., Stewart, & Fyfe. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology, 26*(2), 118-122.
Sun, G., Wheatley, W. B., & Worley, S. D. (1994). A new cyclic N-halamine biocidal polymer. *Industrial & Engineering Chemistry Research, 33*(1), 168-170.
Tsao, T. C., Williams, D. E., Worley, C. G., & Worley, S. D. (1991). Novel N-halamine disinfectant compounds. *Biotechnology Progress, 7*(1), 60-66.
USDA-FSIS. (2013). Progress Report on *Salmonella* and *Campylobacter* Testing of Raw Meat and Poultry Products, 1998-2013. In USDA-FSIS. Accessed on March 30, 2015. Retrived from: http://www.fsis.usda.gov/wps/wcm/connect/fsis- content /internet/main/topics/data-collection-and reports/microbiology/annual-progress-reports.
Vandamme, P., Debruyne, L., De Brandt, E., & Falsen, E. (2010). Reclassification of Bacteroides ureolyticus as Campylobacter ureolyticus comb. nov., and emended description of the genus Campylobacter. *International Journal of Systematic and Evolutionary Microbiology, 60*(9), 2016-2022.
Wonley, S. D., Sun, G., Sun, W., & Chen, T.-Y. (2000). Monomeric and polymeric cyclic amine and N-halamine compounds. In, vol. US6020491A. United States: Auburn University.
Worley, S. D., Chen, Y., Wang, J. W., Wu, R., Cho, U., Broughton, R. M., Kim, J., Wei, C. I., Williams, J. F., Chen, J., & Li, Y. (2005). Novel N-halamine siloxane monomers and polymers for preparing biocidal coatings. *Surface Coatings International Part B: Coatings Transactions, 88*(2), 93-99.