

**Applying N-halamine Antimicrobial in Poultry Production and Food Processing for
Improving Food Safety**

by

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Abstract

Food safety and food losses are drawing public attention, especially regarding meat and meat products. Using antimicrobial absorbent pads in packed meat trays and antimicrobial air filters in chicken production may provide an avenue to solve these problems. However, many antimicrobial materials investigated did not provide a significant and rapid antimicrobial action. N-halamines are generally the most effective antimicrobial materials due to their superior antimicrobial efficacy against a broad spectrum of microorganisms, as well as their nontoxicity, stability and rechargeability. In this study, N-halamine coated absorbent pads were applied into food trays loaded with fresh raw chicken and beef meat in order to reduce the foodborne pathogens and spoilage-related bacteria in the food pads and meat. Air filtration materials in chicken houses were also incorporated with N-halamine compound to inactivate the accumulated avian influenza virus on the air filters. Results showed the major spoilage bacteria in the developed absorbent pads loaded with meat were significantly reduced during 11 days of storage, without negatively affecting the quality (color, pH) of the food products. With an *in vitro* test, 0.05% MC treated cellulose materials of absorbent pads were able to reduce inoculated *E. coli* O157: H7, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Campylobacter jejuni* to under the detection limit within the predetermined contact time. In an antiviral test, the aerosolized AI viruses were completely inactivated when they went through the filters coated with the N-halamine compound. In addition, the incorporation process of these materials is simple and straightforward and does not add any significant costs to the price of the pristine materials. These results suggested that this novel approach could be applied in the food and poultry industry to eliminate the

multiplying of microorganisms in the packaging systems and the spread of airborne pathogens in the indoor environment, thus reducing the chances of poultry and human diseases.

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I. Literature review

Introduction

The demand for minimally processed, or ready-to-eat foods and the globalization of food trade with long distance transportation poses great challenges on food quality and safety (Appendini and Hotchkiss, 2002). Foodborne illnesses associated with foodborne pathogens are important public health concerns. The Centers for Disease Control and Prevention (CDC) reported that there were approximately 48 million cases of foodborne pathogens related illnesses each year, resulting in 128,000 hospitalizations and 3,000 deaths in the US (CDC, 2011). In addition, it was estimated that the average national costs of foodborne illness were \$93.2 billion (from \$33.0 to \$176.3 billion) and \$55.5 billion (from \$33.9 to \$83.3 billion) dollars per year based on the enhanced and basic models, respectively (Scharff, 2015).

Food loss is another remarkable economic concern for the food industry. In recent years, hunger, resource conservation, and the environmental and economic costs associated with food waste have raised public awareness of food loss. According to the Food and Agriculture Organization (FAO), there are around 1.3 billion tons of foods lost or wasted every year in the US, which is roughly one third of the food produced globally for human consumption (Gustavsson et al., 2011). The spoilage microorganisms on the foods introduced from processing or other routes are the major cause of food loss (Otoni et al., 2016). Therefore, improving food safety and quality is significantly important in reducing food waste.

Meat production in North America was approximately 18, 16, and 12 million tons of chicken, cattle, and pig, respectively (Gustavsson et al., 2011). However, food losses of meat and meat products in industrialized regions such as Europe and the US are severe, especially

at the retailers and consumers level. Food waste at the consumption stage accounts for approximately half of the total meat losses (Gustavsson et al., 2011). In developing countries in Africa and Asia, meat losses at the production level are even more severe than at the consumption stage. The high losses in agricultural production are explained by high animal mortality rates, caused by diseases such as avian influenza and other diseases in livestock breeding. Therefore, reduction of the microbial loads associated with foodborne pathogens as well as spoilage-related microorganisms in poultry and beef meats is critical to protecting consumers' health and preventing economic loss in the food industry. Additionally, controlling diseases in livestock breeding is important to safe meat production.

Major foodborne pathogens in meat

Staphylococcal food poisoning (SFP) is one of the most common food-borne gastrointestinal diseases in the world. It follows the ingestion of staphylococcal enterotoxins (SEs), which are mainly produced by *Staphylococcus aureus* (Loir et al., 2003). *S. aureus* is commonly found on the skin, hair, and in the noses of up to 25% of healthy people and animals. It is an important pathogen due to its combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. *S. aureus* usually does not cause illness in healthy people, but it has the ability to produce toxins that can cause food poisoning (CDC, 2016). SFP causes around 241,000 illnesses each year in the US (Scallan et al., 2011). From 1998 to 2008, an estimate 458 cases of outbreaks caused by *S. aureus* in the US were reported (Bennett et al., 2013). Infected consumers usually have the acute symptoms such as severe nausea, abdominal cramps, vomiting, and diarrhea (CDC, 2016). Humans who carry *S. aureus* can contaminate foods by poor hygienic handling. Meat and meat products provide a favorable environment for the growth of *S. aureus* and are one of the most vulnerable foods associated with SFP (Bennett et al., 2013; Hennekinne et al., 2012). As *S. aureus* multiplies in food, it produces toxins. Although *S. aureus* is easily killed by heat, the produced toxins

are heat stable and cannot be destroyed by regular cooking. Since foods contaminated with *S. aureus* toxins do not look or smell bad, the chances of infections are increased (CDC, 2016).

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, facultative anaerobic bacterium. *E. coli* O157: H7 is the most frequently isolated serotype of enterohemorrhagic *E. coli* (EHEC) from ill people in the US, Japan, and UK (Lim et al., 2010). In the US, the most frequent route of *E. coli* O157:H7 infections is through consumption of contaminated foods and water (Rangel et al., 2005). This bacterium usually can shed in uncooked beef, unpasteurized milk and juices, contaminated raw fruits and vegetables, or water for several weeks and sometimes even months (Buchanan and Doyle, 1997). Cattle are the major reservoir of *E. coli* O157: H7, and contaminated ground beef is the most common vehicle for *E. coli* O157:H7 outbreaks. Beef products may be contaminated during slaughter, and the process of grinding beef may transfer pathogens from the surface of the meat to the interior. A previous study on the prevalence of *E. coli* O157: H7 in dairy cattle in Washington State showed that this pathogen was found in 8.3% of fecal samples from dairy cattle (Hancock et al., 1994). Infected *E. coli* O157: H7 can cause severe bloody diarrhea, abdominal cramps, vomiting, hemolytic uremic syndrome (HUS), and even death. According to an estimate from the CDC, *E. coli* O157: H7 infections caused 4,928 illnesses, 1,272 hospitalizations, 299 physician-diagnosed HUS cases, and 33 deaths during 2003-2012 in the US (Heiman et al., 2015). Although the total case number of *E. coli* O157:H7 infections is lower than those of other enteric pathogens such as *Salmonella* or *Campylobacter* spp., the diseases caused by *E. coli* O157:H7 showed much higher hospitalization and fatality rates (Mead et al., 1999). Recently, another multistate outbreak of *E. coli* O157:H7 linked to soy nut butter was reported, which led to 12 hospitalizations (CDC, 2017a).

Campylobacter is the third-leading cause of bacterial foodborne illness in the US (Scallan et al., 2011), and poultry exposure is a well-recognized risk factor for infection.

Campylobacter is a gram negative, spiral-shaped, microaerophilic bacterium, which is one of the most common microorganisms causing diarrheal diseases in the US. *Campylobacter jejuni* is the strain associated with the most reported human infections. Although the rate of infection for *Campylobacter* was reduced 30% in 2009 based on the data from the Foodborne Diseases Active Surveillance Network (FoodNet), *Campylobacter* still causes around 14 cases of illness per 100,000 people in the US per year (CDC, 2014). The symptoms of infection with *C. jejuni* are various, ranging from no symptoms to diarrhea (sometimes bloody diarrhea), cramping, abdominal pain, and fever within two to five days after exposure to the organism. It may enter the bloodstream and cause a life-threatening infection for people with compromised immune systems. *C. jejuni* usually survives and multiplies in foods such as contaminated water, raw or unpasteurized milk, raw or undercooked meat, poultry, and fresh produce. Campylobacteriosis associated with poultry is estimated to be responsible for the greatest burden of foodborne disease in the US (Batz et al., 2012). Many chicken flocks infected with *Campylobacter* show no signs of illness. During slaughtering, *Campylobacter* can be transferred from the intestines to the poultry meat in an infected bird. Chicken is the single largest source of infection, with *C. jejuni* found in 47% of raw chicken meat (CDC, 2014), and in 77% chicken livers in the US (Noormohamed and Fakhr, 2012).

Salmonella is the leading pathogen for foodborne infections (Dept of Health et al., 2015; Scallan et al., 2011). It is a gram negative, rod-shaped bacillus that can cause diarrhea in humans. *Salmonella* consists of over 2,300 serotypes of bacteria, but *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common, accounting for half of human foodborne pathogen infections in the US. Most people infected with *Salmonella* develop diarrhea, fever, and abdominal cramps between 12 and 72 hours after infection. According to the CDC, salmonellosis causes around 1.2 million cases of foodborne illnesses and approximately 450 deaths annually in the US (CDC, 2015). Among these, poultry accounts

for 29% of the single food source outbreaks, and 71% of those were due to the consumption of chicken. Poultry causes more outbreaks of *Salmonella* infection than any other food commodity (Cosby et al., 2015). In 2017, there have been already 8 outbreaks with 71 hospitalizations reported for human *Salmonella* infections linked to live poultry in backyard flocks (CDC, 2017b).

Antimicrobial absorbent pads in food packaging

Introduction of antimicrobial food packaging

Many new food packaging technologies have been studied in order to reduce food waste and provide safe food products. Among these, antimicrobial food packaging is one of the most efficient technologies to protect foods in a desirable way. Antimicrobial packaging is a promising form of active food packaging which has been defined as “a type of packaging that changes the condition of the packaging to extend the shelf life or improve safety or sensory properties while maintaining the quality of the food” (Quintavalla and Vicini, 2002).

Antimicrobial packaging has potential to extend shelf life or improve food safety, particularly in meat products. The microbial contamination of food products usually occurs on the surfaces of food during handling and processing. Directly applying antimicrobials on the surface always dilutes its benefits since the active compounds diffuse rapidly into the food mass. Furthermore, chelating effects from nutrients in the food matrix are stronger than those on the contact surfaces, and thus their antimicrobial activities are limited on the surface microorganisms (Quintavalla and Vicini, 2002). Therefore, incorporating antimicrobial agents into packaging materials is more efficient than direct application in food. The antimicrobial compounds usually slowly migrate from the packaging materials to the surfaces of food products. The continual release of antimicrobials maintains a high concentration of active compounds and prolonged contact time with the microorganisms on the surfaces of

foods (de Melo et al., 2012). On the other hand, immobilized antimicrobial packaging such as modified antimicrobial polymer coating contact surfaces have enhanced antimicrobial activity since their active composites cannot be released in the package, and thus would not lose efficacy until they contact with target microorganisms.

There are several forms of antimicrobial in packaging materials, including incorporation of volatile antimicrobial agents into a sachet or absorbent pad, direct incorporation of the active compound into polymers, coating or absorbing it onto the surfaces of polymer, and using inherently antimicrobial polymers (Appendini and Hotchkiss, 2002). Among these, inserting sachets or absorbent pads into packages is one of the most common applications in meat packaging.

Adding sachets into food packaging is mainly dependent on gaseous agents such as ethanol, chlorine dioxide, and various essential oils to inhibit the growth of microorganisms. There are two approaches to producing antimicrobial-releasing sachets: sachets that generate antimicrobial compound in situ and release it; and sachets that carry and release antimicrobials (Otoni et al., 2016). Emitted gases from commercial sachets usually contain oxygen scavengers, carbon dioxide generators, or chlorine dioxide generators. The antimicrobial application of sachets has been reviewed in other papers (Coma, 2008; Otoni et al., 2016).

Fresh food products such as meat or freshly cut melons usually accumulate exudates in packaging containers. The exudates may cause negative sensory perceptions of the products by consumers. Absorbent pads were generally used in the bottom of trays packed with food products, including meat and fruits, to remove the exuded liquids during transportation and storage. Therefore, separating the food products from the juices can maintain appealing appearances of the products.

Meat exuded liquids are nutrient rich broth, and the unsanitary juices immobilized in the absorbent pad could easily promote the growth of microorganisms which may be spread on the outside of the pad. The microorganisms shed in absorbent pads may generate undesirable odors and spoil the quality of food products. In addition, the propagation of foodborne pathogens in the food pad may pose a threat to consumers' health. Reducing the microbial loads in absorbent pads is one of the most important avenues to keeping both the quality and safety of food products. One option to reduce microorganisms is to incorporate antimicrobial agents into food pads.

Antimicrobial absorbent pads

Nanoparticles

Various applications of nanotechnology in food packages have been reviewed (Baltic et al., 2013; de Azeredo, 2013). Nanoparticles below 100 nm have been recognized as having antibacterial activity. The most promising nanomaterials with antibacterial properties are metallic nanoparticles, which have more enhanced antibacterial activities because of their large surface to volume ratio and the crystallographic surface structure (Morones et al., 2005). Silver nanoparticles have been studied intensively for their antibacterial activities.

The antimicrobial activity of nano-sized silver particles (AgNPs) is an area of growing scientific interest. They are known to act as nano-reservoirs of silver ions (He et al., 2003). Absorbent pads incorporated with silver nanoparticles have been evaluated for their antimicrobial activities used in poultry, beef, melon and kiwi fruit packaging (Fernández et al., 2010a, 2010b, 2009; Lloret et al., 2012). Fernández et al. (2009) developed absorbent pads incorporated with AgNPs by immersing the pads in silver nitrate solution, and the silver ions were transformed into nanoparticles in the absorbent pads. The developed absorbent pads with AgNPs soaked in chicken exudates reduced 40% of aerobic mesophiles and lowered the

lactic acid bacteria to below the detectable level. When the developed absorbent pads were used in modified atmosphere packaging containing beef meat, they reduced the levels of the major microbial groups by an average of 1 log CFU/g in the pads during the entire storage period; however, the microbial loads on the surfaces of beef were not significantly affected by the presence of AgNPs coated cellulose materials (Fernández et al., 2010b). Moreover, AgNPs based absorbent pads increased the lag phases of the spoilage microorganisms considerably, and reduced approximately an average of 3 log CFU/g of the microorganisms in the coated absorbent pads. Compared with no antimicrobial effects on the beef, the developed absorbent pads slightly reduced the microbial loads, especially on yeasts in melon cuts (Fernández et al., 2010a). This difference is that melon juices have a lower chelating effect against silver ions in contrast to beef exudates. In addition, silver based nanocomposite absorbing materials reduced up to 99.9% bacteria, yeasts and molds in juices from kiwi fruit and melon, while only 90% of the microorganisms were below the controls in poultry and beef drips (Lloret et al., 2012).

AgNPs from these coating materials were able to inhibit microbial growth in meat or fruit exudates. However, considering the proteins chelating effect, using these cellulose in water-like broth such as fruit drips is more favorable to achieving the desired antimicrobial effects. In the US, there are no specific regulations for active packaging. Antimicrobials in food packaging that may migrate to food are considered food additives and must meet the food additive standards (Appendini and Hotchkiss, 2002). The European Food Safety Authority limits the concentration of silver ions in food to below 0.05 mg/kg (EFSA, 2004), which is usually not high enough to achieve expected antimicrobial effects. Therefore, both matrix effects and regulations should be taken into account for AgNPs application in foods.

In addition, copper nanoparticles have been drawing attention with their effective antimicrobial efficacy and low cost (Llorens et al., 2012a, 2012b). Llorens et al. (2012)

developed absorbent pads incorporated with copper nanoparticles by in-situ reduction of copper sulphate on cellulose fibers. The cellulose/copper composites exhibited a strong antifungal potential against spoilage related yeasts and molds in drips exuded from pineapples, reducing around 4 log cycles of the microbial loads on average.

Bacteriophage

Recently, Gouvêa et al. (2016) developed absorbent pads containing bacteriophage for potential use in refrigerated food products. Bacteriophages act as regulators of microbial growth, and they can infect bacteria under favorable conditions. The absorbent pads used in chilled meat trays contained a mix of six bacteriophages isolated and characterized by this work group. The developed system with three different bacteriophage concentrations were incorporated in absorbent pads individually and evaluated for their antimicrobial activities against *S. Typhimurium* in the pad at various storage temperatures. Results revealed that the food pads with bacteriophages at 10^9 , 10^8 , and 10^7 plaque forming units (PFU)/mL could reduce 4.36, 3.66 and 0.87 log cycles of *Salmonella* at 15 °C, respectively (Gouvêa et al., 2016). Temperature has no effect on phage growth at the range of 4 °C to 37 °C (Sillankorva et al., 2004), but it affects the host microbial growth, and thus may interfere with the adsorption of bacteriophage on the bacterial cell wall. At 10 °C, an average reduction of 0.55 log of microorganisms was achieved when the three concentrations of bacteriophage were used as described above (Gouvêa et al., 2016). In this context, although the phages on the pad remained viable during the treatment time of 48 h, the author emphasized the method of incorporating phage in the pads need to be improved to keep the bacteriophages viable longer in the package for further practical application.

Lone et al. (2016) also developed bacteriophage based packaging materials to control the growth of bacterial pathogens in cantaloupes, ready-to-eat meat, alfalfa seeds and sprouts.

Bacteriophage cocktails were introduced into the materials by two approaches: encapsulation onto paper, and immobilization on cellulose membranes. As a result, the counts of *Listeria monocytogenes* in cantaloupes dropped below the detection level by the adding free phage cocktail, and reduced to around 1 log CFU/g when immobilized *Listeria* phage cocktail was applied. In addition, bacteriophage coating materials caused 1 log cycle reduction in *E. coli* O104:H4 on the germinated sprouts, and under the detection limit in alfalfa seeds (Lone et al., 2016).

Natural products and others

Essential oils have been widely investigated and reviewed for their unique volatile nature and effective antibacterial activity (Burt, 2004; Hammer et al., 1999). Absorbent pads incorporated with oregano essential oil were developed and evaluated for their antimicrobial property when applied on chicken drumsticks packaging stored at 4 °C (Oral et al., 2009). Results showed that the absorbent pads containing oregano essential oil reduced the microbial loads, and extended the shelf life of fresh chicken drumsticks by approximately 2 days. Compared with nonvolatile antimicrobial agents such as nanoparticles and bacteriophages, the main advantage of volatile antimicrobials is that there is no need for direct contact between the food and packaging materials (Pereira de Abreu et al., 2012). Volatile antimicrobials incorporated in the absorbent pads could be easily released and contact all food surfaces. Volatile compounds increased the contact area and reduced chelating effects in the food matrix, and thus exhibited higher antimicrobial activity than those in solutions (Inouye et al., 2003).

In addition to the novel antimicrobials mentioned above, some traditional antimicrobial agents used in the poultry industry were also applied into absorbent pads to reduce the microbial loads on beef. Lim et al. (2004) designed absorbent pads containing

three food-grade antimicrobial agents: cetylpyridinium chloride (CPC), acidified sodium chlorite (ASC), and potassium sorbate (PS). The antibacterial properties of the developed absorbent pads against *L. monocytogenes*, *E. coli* O157:H7, and *S. aureus* on inoculated beef meat were evaluated. Results showed that great reductions of foodborne pathogens on meat surfaces were achieved when CPC or ASC coated absorbent pads were used and when the agents were sprayed on beef under storage conditions. Hansen (1988) patented an absorbent pad comprising a mixture of citric acid, malic acid, and sodium lauryl sulfate. These treated pads were able to significantly reduce the microbial load in *in vitro* test.

The scheme of antimicrobial absorbent pad architecture is illustrated in Figure 1.1. Although antimicrobial absorbent pads present many advantages over other active packaging systems, some limitations need to be considered. Many antimicrobial agents have undesirable odors that cannot be neglected. When antimicrobials are incorporated into absorbent pads, they could negatively affect the sensory properties once packages are open. Another concern is consumers' acceptance of a "non-food" chemical addition such as silver nanoparticles. Furthermore, the migration of incorporated antimicrobials from package to food products is not symmetrically studied in the food model. More safety data on the developed absorbent pads is needed for practical application.

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

Compound MC (Figure 1.2) is a member of N-halamines, and it was first synthesized by Tsao et al. (1991). It showed effective antimicrobial activity not only *in vitro* determination but also in a field test. MC has been studied in many areas, including the food safety and poultry production. Worley (1992) reported that MC could completely disinfect the inoculated *Salmonella* Enteritidis on the surfaces of egg shells within 72 h of contact. Elder and Reid (1993) used MC in hydroponic application by soaking radish seeds. Results

indicated that MC functioned efficiently in surface sterilization of the radishes without negatively affecting their germination and growth. Similarly, in MC treated alfalfa seeds, the microbial loads on the seeds and the germinated sprouts were significantly reduced without affecting the germination (McPherson, 2016). Delaney et al. (2003) applied MC in seawater for disinfecting pathogenic protozoan *Perkinsus marinus*, which causes extensive mortalities in Eastern oyster. The MC compound damaged the permeability of the parasites outer membrane, and thus altered the osmoregulatory functions. The author indicated that MC has the potential to be used in recirculating systems to prevent the spread of parasites in oyster hatcheries.

Other than food related applications, there are several studies in the use of MC for human health and poultry health. Worley et al. (2011) applied MC in contact lens soaking solutions for inactivating pathogenic microorganisms to prevent eye infections without causing significant bleaching of the dyes incorporated into the contact lenses. The same research group also coated MC into polypropylene nonwoven fabrics to protect against airborne microbial contamination. Results revealed that the coated materials exhibited superior antibacterial efficacy after exposure to bacterial aerosol for 3 h, causing approximately 3 to 4 log reductions compared to the uncoated samples (Demir et al., 2015). Ren (2015) treated MC solutions on various surfaces of equipment and facilities in a chicken house to inactivate the contaminated foodborne pathogens. Results showed that a 1% MC treatment was able to completely inactivate *S. Typhimurium* and *C. jejuni* at 10^6 CFU per square inch within 2 h of contact time. MC coated surfaces had a high stability and long-lasting antimicrobial activity up to four weeks in storage. In addition, the oxidative chlorine ions in MC molecules would not dissociate until in contact with microorganisms and the molecules are not volatile (Demir et al., 2015).

Avian influenza virus in chicken

Introduction

Avian influenza (AI) virus belongs to the *Influenzavirus A* genus of the *Orthomyxoviridae* family. The *Orthomyxoviridae* family has five genera, including *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* and *Isavirus*. AI virus is a single-stranded, enveloped RNA virus. At present, only *Influenzavirus A* are known to be able to infect birds (Alexander, 2007). Type A influenza viruses are further divided into subtypes based on the antigenic relationships in the surface glycoproteins, haemagglutinin (HA), and neuraminidase (NA). There are 16 distinct haemagglutinin (H1-H16) and 9 neuraminidase (N1-N9) subtypes that have been isolated and identified from avian species.

Influenza A viruses infecting poultry can be divided into two groups: highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI). HPAI is a systemic disease with a flock mortality rate close to 100%. To date, only certain subtypes of H5 and H7 are able to cause HPAI (Alexander, 2007). All viruses of the 16 subtypes can cause LPAI in susceptible avian. LPAI is a mild respiratory disease with low mortality in poultry unless exacerbated. It indicates that HPAI viruses arise by mutation from LPAI viruses in poultry (Alexander, 2007). The factors of the mutation from LPAI to HPAI are not known, and it sometimes happens rapidly, while in other cases it circulates in poultry for months before mutating. Although it is impossible to predict the occurrence of the mutation, it can be assumed that the more LPAI circulated in the poultry, the higher chance that mutation to HPAI will occur (Alexander, 2007).

Clinical disease

The symptoms of AI virus infections are extremely variable, ranging from no obvious clinical signs to 100% mortality depending on other factors, including host species, age, sex, concurrent infections, acquired immunity and environmental factors (Easterday et al., 1997).

Poultry infected with LPAI viruses have common signs, including reduced egg production, mild to severe respiratory difficulties, digestive and urinary abnormalities, and decreased performances. Avian species infected with HPAI viruses have clinical signs of hemorrhages on the serosal and mucosal surfaces of viscera organs and cardiovascular and nervous systems (Swayne and Halvorson, 2008). However, clinical signs vary depending on the extent of damage. In most cases, chickens are found dead before observation of any clinical signs.

Outbreaks and economic losses

Economic losses from AI diseases vary depending on the strains of viruses, species of poultry infected, amounts of farms involved, control methods used, and eradication strategies (Swayne and Halvorson, 2008). Most outbreaks and economic losses resulting from HPAI or LPAI occur in commercially raised poultry farms. The reports of losses from some cases of AI outbreaks are shown in table 1. Direct losses from HPAI outbreaks include birds losses, depopulation, cleaning, disinfection, and surveillance costs. Indirect costs mainly indicate the loss in poultry exports, income losses of employees, and increased consumer costs from the reduced supply of chicken products. Generally, LPAI outbreaks lead to less loss compared to HPAI outbreaks, since the infected poultry could be under control with lower mortality rates. The costs from LPAI are mortality losses, medication used, cleaning and disinfection, and a delayed schedule for new birds (Swayne and Halvorson, 2008).

The consequences of AI outbreaks are not only economic losses to the poultry industry and a shortage of proteins for developing countries, but also serious threats to human health due to the risk of an emerging pandemic virus from the poultry reservoir. In general, transmissions of AI virus mostly occur within the same species, occasionally between closely

related species. On rare occasions, complete AI virus genes or segments have been found transferred to humans (Easterday et al., 1997).

Intervention strategies

To reduce the impacts on poultry health and economic losses, it is important to control and eradicate AI infections in poultry. Three different modes of influenza transmission have been identified, which are droplet, airborne, and contact transmission (Weber and Stilianakis, 2008). The primary introduction of AI virus into a poultry population is from wild bird activities. Then the spread of AI virus is mainly through human related activities, including the movement of staff, vehicles, equipment, and other fomites that have not been adequately sanitized. If disinfection, footwear, clothing, vehicles, crates, farm equipment and other materials are not handled properly, infection will continually happen in the avian population, posing a threat to the poultry industry as well as to public health. Cleaning and disinfecting are essential to controlling the spread of AI virus (De Benedictis et al., 2007).

Inactivation of AI viruses

To prevent the introduction of AI virus in poultry farms and limit the secondary spread of this virus, biosecurity programs are considered the most important tool. Disinfection through either physical methods or chemical reagents during the biocontainment process helps prevent the virus from spreading. There are many reports on methods of physical inactivation of AI virus, including using temperature, pH and radiation, but results are not consistent. Regarding virus sensitivity to chemical disinfectants, influenza virus belongs to category A virus, which is an enveloped virus (Holl and Youngner, 1959). This structure makes all AI virus very sensitive to the major disinfectant agents.

Physical inactivation

AI virus can survive outside the host for certain periods of time depending on the conditions of the environment. AI virus is susceptible to heat and dryness. Chumpolbanchorn et al. (2006) showed that AI virus at 25 °C in chicken manure lost its infectivity within 24 h, and at 40 °C in 15 min. Another study showed that the AI virus treated at 56 °C for 30 min was completely inactivated, while only 1 min was needed to reduce the virus to undetectable levels at 70 °C (Zou et al., 2013). Similar results were observed by Wanaratana et al. (2010), where the H5N1 virus was completely inactivated at 70 °C for 10 min. Swayne (2006) reported that the highly pathogenic AI H5N1 virus in the raw chicken meat was completely inactivated after exposure at 70 °C for 5 s. Jeong (2010) reported AI H1N1 virus was completely inactivated at 70 °C for 5 min, or at 90 °C in 1 min. Ultraviolet (UV) light irradiation did not show any effect of inactivation against the virus even after 4 h exposure time in chicken fecal manure (Chumpolbanchorn et al., 2006). Zou et al. (2013) showed that UV light irradiation on the AI virus was effective under 30 min of exposure or longer within 75 cm distance. UV radiation treatment effectively inactivated the AI H1N1 virus on filtering facepiece respirators (FFRs) as either in droplets or in aerosol particles form after 15 min of exposure under the light within 25 cm distance. The log reduction in aerosol form was 4.70, and 4.92 for droplet form (Heimbuch et al., 2011). In addition, no viable viruses were detected after 30 min of warm moist heat treatment, which were 4.80 and 4.98 average log reductions in the aerosol and droplet tests, respectively (Heimbuch et al., 2011). UV light treatment is not considered an efficient tool to disinfect equipment due to its poor efficacy of killing AI virus as well as its high cost (De Benedictis et al., 2007). The author also pointed out that the advanced antiviral activity created by warm moist heat technology was a result of the homogeneously distributed environment on the entire surfaces of the FFRs (Heimbuch et al., 2011).

Chemical inactivation

Aldehydes are widely used as fumigation disinfectants for poultry production, including formaldehyde and glutaraldehyde (De Benedictis et al., 2007). A study showed that most of the H5N1 isolates lost more than 50% of their infectivity when exposed to formalin or glutaraldehyde for 10 min (Wanaratana et al., 2010). Compared to formaldehyde, glutaraldehyde is more suitable for applying in field because of its lower toxicity and shorter residual activity (De Benedictis et al., 2007).

Oxidizing agents such as hydrogen peroxide are one of the major disinfectants used in poultry industry. However, most of chemicals from this group usually cannot effectively inactivate AI virus. One study showed hydrogen peroxide at 50% strength was unable to inactivate the H5N1 isolates regardless of the storage temperature or storage time (Wanaratana et al., 2010). The same results occurred with 3% iodine complex treated AI H5N1 virus, which none of the virus lost its infectivity (Wanaratana et al., 2010). Virkon[®]-S is one of the most effective products against the AI virus. Commercial available Virkon[®]-S containing 0.5% dipotassium peroxodisulphate, an oxidizing agent, showed a complete inactivation of H7N9 virus (Zou et al., 2013). However, 10 days old Virkon[®]-S was no longer able to reduce the infectivity of AI virus, even after 1 h exposure (Suarez et al., 2003).

The disinfectants of chlorine and its derivatives are effective against AI viruses. However, many limitations need to be taken into account when using chlorine types of chemicals, such as corrosiveness, pH dependence, and the presence of organic matters etc. Sodium hypochlorite at 1% strength successfully inactivated most H5N1 isolates at 25 or 37 °C even after 14 days of storage (Wanaratana et al., 2010). Similarly, Zou et al. (2013) showed 0.5% sodium hypochlorite was able to completely kill H7N9 virus within 5 min. Rice et al. (2007) revealed that maintaining free chlorine residual at 0.52-1.08 mg/L was sufficient to inactivate the highly pathogenic AI H5N1 by more than 3 log within 1 min. On the surfaces of metal, wood and plastic, 750 ppm of sodium hypochlorite completely inactivated

all the attached AI H7N2 virus (Lombardi et al., 2008). Using household bleach at the recommended concentration completely killed the AI virus with 1 h treatment (Suarez et al., 2003). However, when it was used with the combination of some anti-freezing compounds, the disinfectant activity was impaired (Davison et al., 1999). Therefore, the conditions must be taken into account before application to achieve the optimal efficacy of these products.

Phenol compounds are another class of disinfectants widely investigated for the inactivation of the AI virus. A study showed that 20% chlorinated xyleneol combined with acids was able to effectively reduce the infectivity of most H5N1 viruses at 25 and 37 °C after 14 days of storage, with the exception of the CUK-2 isolate. CUZ-2 isolate had 100% infectivity after exposure to phenol at 37 °C within 10 min (Wanaratana et al., 2010). For these types of disinfectants, their antiviral effects were not affected by the addition of anti-freezing agents such as ethylene glycol or propylene glycol (Davison et al., 1999).

There are other types of disinfectants commonly applied in killing AI virus. Quaternary ammonium compounds (QACs) showed higher antimicrobial efficacy against AI H5N1 virus. When QACs were combined with glutaraldehyde, their antiviral effectiveness was stronger than that of any of the chemicals used individually (Wanaratana et al., 2010). Acetic acid is widely used due to its mild corrosiveness and it is safer for employees compared to other acids. Acetic acid at 1% reduced $10^{6.2}$ EID₅₀/mL of low pathogenic AI H7N2 virus attached on galvanized steel within 10 min contact time (Alphin et al., 2009). Acetic acid applied on wood and plastic surfaces completely inactivated the attached H7N2 virus in 10 min (Lombardi et al., 2008). Seventy five percent ethanol completely inactivated the AI H7N9 virus after 5 min exposure (Zou et al., 2013). Copper ions have been widely recognized as an antiviral agent against AI virus. It was reported that the infectivity of the AI H1N1 virus was significantly decreased after the viruses were dropped on a copper surface

(Noyce et al., 2007). Similarly, another study revealed that 25 μ M copper ions reduced approximately 3 and 4 log of H9N2 virus within 3 h and 6 h, respectively (Horie et al., 2008).

Before applying disinfectants in the field, various factors need to be considered, such as the antimicrobial mechanism of the agent, hardness of water, contact time, concentration, environmental temperature, toxicity to handlers, corrosiveness, cost, presence of organic matters, and type of equipment (De Benedictis et al., 2007). The physical and chemical properties among AI viruses are similar, and different strains have minimal variations on the inactivation, but this area should still be examined (Swayne, 2006).

Antimicrobial air filters

As mentioned above, the spread of AI virus has caused severe poultry diseases as well as human illnesses in past years. Transmission of AI virus may easily occur by direct contact between infected and susceptible birds, or through indirect contact, including aerosol droplets. Previous studies showed that airborne pathogens can reproduce in large quantities, especially in ventilation systems, and the AI virus can be viable for days, with increasing resistance to usual cleaning methods (Cozad and Jones, 2003; Weinstein and Hota, 2004). Therefore, AI virus is a hazard concern in ventilation systems. Early elimination of the AI virus in poultry farms is a critical avenue to reduce the chance of transmission. Many disinfectants used in chicken houses can achieve this goal, but most of disinfectants' antimicrobial activities are negatively affected by the presence of organic matters such as feces. Even if the viruses in the poultry were inhibited by the disinfectants at the beginning, the survived viruses or newly introduced viruses in the farm still can infect poultry after the disinfectants evaporated. Therefore, preventing the entrance of AI virus into poultry farms is an important approach to reducing the chance of transmission. Regular air filters can limit the introduction of some bioaerosols. Pathogens usually can survive for days or even months; in addition, the organic

or inorganic materials deposited on the filters contribute to the microbial growth. The accumulated microorganisms on the filters can enter the farm later and thus infect the birds. The virus-accumulated air filters also pose a health threat to the public if not handled or disposed of properly. Therefore, there is a need to develop antimicrobial surfaces for air filter systems to reduce the risk of infections from airborne pathogens. These materials can be produced by incorporating antimicrobial agents into both ventilation systems and individual respiratory protective devices. Previous studies have investigated various antimicrobial air filters, including silver and silver nanoparticles, silver nitrate, carbon nanoparticles, N-halamines, and natural antimicrobials such as silk sericin, plant extracts and chitosan (Desai et al., 2009; Jung et al., 2011a; Magaraphan et al., 2003; Sharma et al., 2009; Zhu et al., 2012b).

Antibacterial filtration applications

Yoon et al. (2008) coated silver particles onto active carbon filters (ACF) by using the electroless deposition method to determine the antimicrobial efficacy. Disk-diffusion and micro broth dilution methods were used to test the bactericidal effects of the developed filters. Results showed that the silver-deposited ACF filters completely inhibited *E. coli* and *Bacillus subtilis* in 60 min and 10 min, respectively. While on the pristine ACF filters, both types of bacteria multiplied. Silver deposition did not influence the physical properties of ACF filters, such as filtration efficiency and pressure drop, but the adsorptive efficacy was decreased over time. Therefore, before the application of silver-deposited ACF filters, the concentration of silver needs to be optimized with regard to the types and concentrations of the bioaerosols to be inactivated.

Silver nitrate

As an antimicrobial agent, silver nitrate was coated on the woven air filters made of polypropylene to determine its antibacterial effect against colonized microorganisms on the filter media (Miaśkiewicz-Peska and Łebkowska, 2011). The antimicrobial activity of the treated filters was evaluated using the incubation column after aerosolized microorganisms passed through the filters. The addition of silver nitrate at a concentration of 1.4×10^{-6} M led to a 42.1% reduction of *Micrococcus roseus*, 94.7% of *B. subtilis*, and 98.7% of *Pseudomonas luteola* within 2 days. These three test bacteria were reduced to undetectable levels in the filter after 7 days of application. The author mentioned that although the developed filter can cause bacterial reduction in living bacterial cells, the application of antimicrobial agents containing silver ions in medicine has induced silver-resistant bacteria. Therefore, microbial adaptation is a challenge for scientists and manufacturers (Miaśkiewicz-Peska and Łebkowska, 2011).

Chloride salts

Two types of antimicrobial chloride salts were applied to the filter media made of microfiber glass acrylic materials to assess their antimicrobial activities (Verdenelli et al., 2003). Kirby Bauer agar diffusion method was used to estimate the antimicrobial inhibition capacity on microbial growth. It was found that during 180 days of incubation, the chloride salts treated filters reduced the density and variety of recoverable microorganisms, but antimicrobial activity is strongly influenced by the time. In addition, the treated filters exhibited higher filtration efficiency than the controls according to the increase of the “most penetrating particle size (MPPS)” and the vacuum resistance.

Biodegradable compounds

Poly lactide (PLA) and polyhydroxybutyrate (PHB) are biodegradable without impact to the environment, and these polymers are brittle. These two compounds blends show

improved flexibility and hydrolytic biodegradation over their individual components. Eco-sustainable fibrous membranes possessing antimicrobial properties against bioaerosols were obtained from PLA/PHB nanoparticles produced by electrospinning and functionalized with different amounts of ionic liquid (IL) from quaternary ammonium compounds (QACs) (Nicosia et al., 2015). Results showed that higher biocide activity resulted in strong antifungal activity against two mold fungal strains but with an increased pressure drop. Lower concentration on filters exhibited moderate antifungal activity, but the pressure drop was greatly reduced by using the “multi-layering” method.

Chitosan is able to chelate ions in solution and inactivate microbial growth. Desai and co-workers (2009) coated chitosan/PEO blend solutions onto a spunbonded polypropylene substrate by electrospinning and obtained the biodegradable nanofibrous filter media. Within 2 h of exposure, there was less than a 1 log reduction of the inoculated *E. coli* K-12. After 6 h of contact time, the chitosan/PEO blend fibers reduced 2-3 log CFU/mL of *E. coli*. Filtration efficiency of the nanofiber filters was highly dependent on the size of the fibers and the chitosan content. However, one main disadvantage of this filter is lack of mechanical strength to hold vacuum pressure applied during filtration.

Natural products

Sophora flavescens nanoparticles have been intensively studied in the past few years (Choi et al., 2015; Hwang et al., 2015; Jung et al., 2013, 2011b). *Sophora flavescens* is a promising natural antimicrobial agent with high biocidal activity and an abundant supply. Natural products can be processed into nanostructure particles with enhanced antimicrobial properties since nanoscale materials maximize the surface area in contact with microorganisms (Jung et al., 2013). *Sophora flavescens* ethanolic extracts nanoparticles have

been applied to air filters made of fibers or carbon nanotubes to obtain the antimicrobial air filters.

Jung et al. (2011b) coated polyurethane resin fiber filters with *Sophora flavescens* nanoparticles produced by using the aerosol technique of nebulization-thermal drying. Results indicated the relative microbial survival of the test microorganisms (*Staphylococcus epidermidis*, *B. subtilis*, and *E. coli*) reduced with the increase of nanoparticles deposited on the filters within 30 min contact time. The treatment enhanced the bioaerosols' filtration efficiency and antimicrobial activity, but the filter pressure drop increased with the increase of nanoparticles deposited on the filters. The same research group also generated the antimicrobial filtration by coating electrosprayed *Sophora flavescens* nanoparticles onto it. Compared with the conventional nebulization process, the filters used in this study led to a greater inactivation efficiency against *S. epidermidis* (Jung et al., 2013). Similarly, the pressure drop of the filters increased with higher nanoparticles deposition. However, the filtration efficiency of the filters was not affected by the nanoparticle content. In 2015, Choi et al. (2015) developed a new herbal extract by incorporating nanofiber fabricated by the electrospinning technique. The novel antimicrobial filters inactivated 99.98% of *S. epidermidis* bioaerosols in 30 min contact time. This nanofiber air filter showed superior filtration efficiency, but a slightly higher pressure drop compared to the controls.

The air filter substrates discussed above are fibrous materials. In addition, carbon nanotubes (CNTs) are known to be biocidal, and they have been used to prepare antimicrobial silver nanoparticle composites to produce antimicrobial filters (Jung et al., 2011a). Hwang and co-workers (2015) synthesized an antimicrobial air filter with hybrid nanostructures of multi-walled carbon nanotubes coated with herb extracts nanoparticles by using a twin-head electro spray system (THES). This filter exhibited higher antimicrobial activity than those coated only with natural products like *Sophora flavescens* nanoparticles or

multi-walled CNTs. This filter inactivated 95% of the test *S. epidermidis* bioaerosols passed through the filters while only 70% were inactivated by the other two types of filters.

Moreover, the filtration efficiency and pressure drop performance of the NP/MWCNT-coated filters were higher than those of pristine, NP nanoparticles or MWCNTs coated filters.

The filters mentioned above showed antimicrobial activity, but they also have disadvantages, including non-rapid inactivation, reduced air permeability, decreased mechanical strength, high costs, complicated manufacturing procedures, etc. Therefore, new technology or antimicrobial agents need to be further investigated.

N-halamines

N-halamines are a group of compounds containing nitrogen-halogen covalent bonds. The N-halamine coated materials have many advantages, including high antimicrobial activity, long-term stability, simple rechargeability, and low toxicity with little environmental concern. Zhu et al. (2012) have studied nanofibrous nonwoven fabrics coated with a novel N-halamine polymer. The active chlorine content on the developed membranes is durable and rechargeable with household bleach. Results showed that membranes coated with the N-halamine were able to completely inactivate the inoculated *E. coli* and *S. aureus* within 15 min. However, the air permeability of the nanofibrous membranes was reduced with the increase of nanofiber content. Therefore, if it is used in breathable materials for biological or chemical protections, the content of the membranes needs to be optimized. Not only N-halamine polymers showed potent antimicrobial activity, monomer also presented a strong biocidal property. Demir et al. (2015) incorporated an N-halamine monomer onto polypropylene nonwoven fabrics in order to produce antimicrobial air filters and face masks to combat airborne pathogens (Figure 1.3). The coating process is simple and straightforward by using a pad-dry technique. The antimicrobial efficacies of these modified fabrics were evaluated against *E. coli* and *S. aureus* in aerosolized forms. Results showed that both types

of the bioaerosols were completely inactivated after 3 h exposure with the filters. In addition, samples maintained their antimicrobial properties for at least 6 months in darkness, and the effect of coating on air permeability of the filters was minimal.

Antiviral filters

For antiviral functions, mostly copper and copper oxide based materials were investigated. Imai et al. (2012) incorporated copper ions in zeolite-textile materials and evaluated the activity of inactivating AI virus H5 subtypes. As a result, both the highly pathogenic H5N1 and the low pathogenic H5N3 viruses were inactivated on the developed materials after 30 seconds contact time. The author mentioned that the CuCl_2 treatment did not have an antiviral effect on these two subtypes of AI virus suspended in allantoic fluid for a 48 h incubation period (Imai et al., 2012). Borkow et al. (2007) reported that passage of the viruses through the filters containing copper oxide led to a significant reduction of the infectious titers, ranging from 0.47 to 4.6 log CCID₅₀. Influenza A virus H3N2 had a reduction of 1.77 ± 0.87 log CCID₅₀ compared to the controls as it passed through the treated filters. Borkow et al. (2008) impregnated copper oxide into respiratory protective face masks, conferring them with potent biocidal properties without altering the filtration capacities of the masks. After challenging them with aerosolized human influenza H1N1 viruses for 30 min, no virus was detected in copper oxide containing samples, while 4.67 ± 1.35 log TCID₅₀ were recovered from the control masks. Similarly, the titers of the infectious AI virus from the control masks were 5.03 ± 0.54 log TCID₅₀, while less than 0.97 ± 0.01 log TCID₅₀ were recovered from copper oxide based filters.

Rengasamy et al. (2010) evaluated the survivability of MS2 viral aerosols deposited on four different antimicrobial air filters individually. Results showed that iodinated resin based air filters caused a significant MS2 reduction at 37 °C and 80% relative humidity (RH)

for 4 h compared with uncoated control samples, but no remarkable reduction was observed when aerosols passed through silver-copper, EnvizO₃-shield and TiO₂ based filters. In addition, the inactivation of the MS2 virus by antimicrobial filters is dependent on the storage conditions, which is in accordance with a similar antimicrobial filter study (Heimbuch et al., 2015). When stored at 22 °C and 30% RH, none of the antimicrobial filters showed significant reductions in comparison the controls (Rengasamy et al., 2010).

Virus recovery

For air filters, after the blowing procedure, tested mask coupons were aseptically removed from the equipment and transferred to the extraction medium. After stomaching, the medium was serial diluted and the titers were determined (Borkow et al., 2010). For filters used in suspension, filtered culture was collected and serial diluted (Borkow et al., 2007). The infectious titers in Madin-Darby canine kidney (MDCK) cells, and/or 10-day-old embryonated chicken eggs were determined by using a cytopathic-effect assay and calculated by using the Reed-Muench end point dilution method (Reed and Muench, 1938). Aerosolized MS2 passed through the treated and control filters and was individually collected in a sealed reservoir bag used to incubate samples. After sampling, samples were incubated at room temperature for various times and then serially diluted and plated on agars (Heimbuch et al., 2015). In Rengasamy's study, MS2 was recovered by suspending each respirator coupon in ATCC medium and following up with a plaque assay (Fisher et al., 2009; Rengasamy et al., 2010).

Table 1.1. Economic losses from HPAI and LPAI epidemics [modified from (Swayne and Halvorson, 2008)]

Year	Outbreak	Birds dead or culled	Cost item	Original cost	Reference
HPAI					
1983-84	US- H5N2	17 M	USDA eradication Non-identified industry losses Increased customer costs	\$63M \$15M \$349M	(Fichtner, 1987)
1985	Australia- H7N7	238,518	Eradication cost	\$1.4M	(Cross, 1987; Lasley, 1986)
1999-2000	Italy- H7N1	13M	Compensation indirect cost	\$100M	(Swayne and Halvorson, 2003)
1997	Hong Kong- H5N1	1.5M	Eradication	\$13M	(Swayne and Halvorson, 2003)
2003-2005	Asia- H5N1	220M	Losses to the poultry industries	> \$10B	(World Bank, 2006)
LPAI					
1978	Minnesota US- various LAPI	141 farms	Losses to the poultry industries	\$5M	(Halvorson et al., 1998)
1995	Minnesota US- H9N2	178 farms	Losses to the poultry industries	\$6M	(Halvorson et al., 1998)
1995	Utah US-	2M	Losses to the poultry	\$2M	(Halvorson

	H7N3		industries		et al., 1998;
					Pearson et
					al., 1998)
			USDA eradication	\$81M	
2002	Virginia		Losses to the poultry	\$130M	(Akey, 2003)
	US- H7N2	4.7M	industries	\$1M	
			State government		

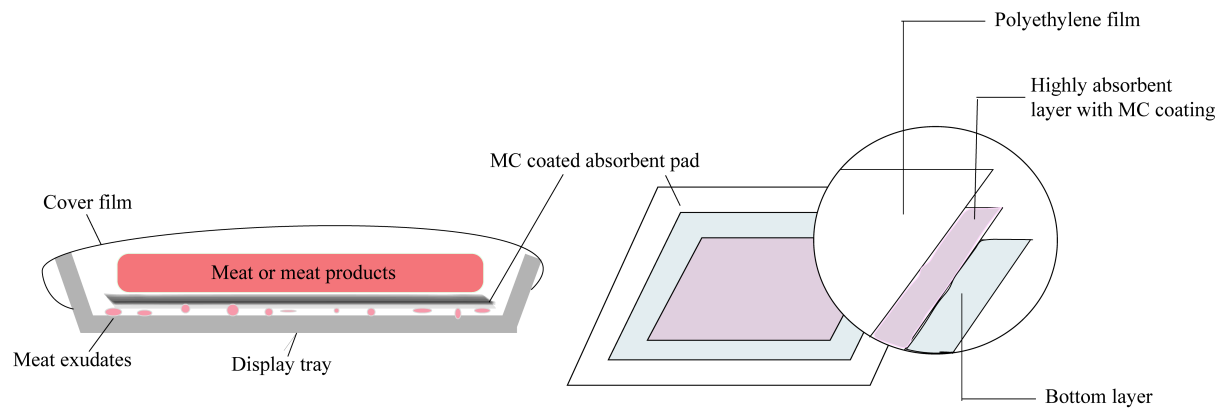


Figure 1.1. Scheme of the absorbent pad architecture

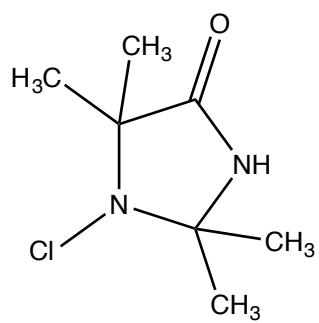


Figure 1.2. Structure of 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC)

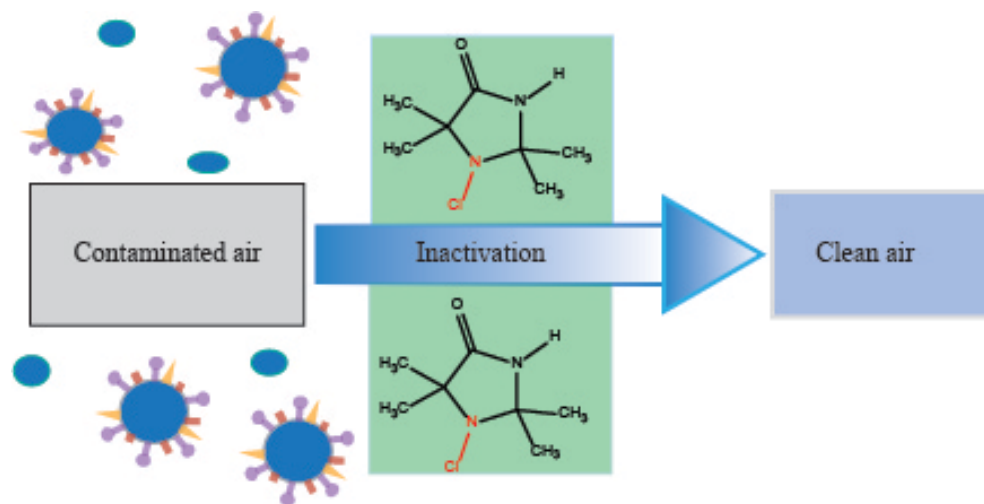


Figure 1.3. Illustration of antimicrobial air filter coated with MC

II. Efficacy of N-halamine compound on reduction of microorganisms in absorbent food pads of raw beef

Abstract

The antimicrobial activity of N-halamine, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC) loaded absorbent pads in meat packaging was investigated. Absorbent pads treated with MC reduced the levels of total aerobic bacteria, *Pseudomonas* spp., and lactic acid bacteria to under the detection limit ($< 2 \log \text{CFU/g}$) for day 0 and day 1. On days 4, 7 and 11, the microbial loads were significantly reduced ($p < 0.05$) in all MC treated absorbent pads. The levels of *Enterobacteriaceae* on the treated samples remained under the detection limit during the entire storage period. Color changes, specially the lightness of packed beef with MC treated absorbent pads, were not significantly increased until day 11, while in the control group there were significant differences with the initial meat from day 1. Microbial loads in meat samples with MC treated pads were also significantly lowered ($p < 0.05$) compared to the control on day 11. MC treated absorbent pads were able to reduce microbial loads in beef samples by 1 log CFU/g on average. MC was able to extend the shelf life of refrigerated raw beef.

Keywords

Antimicrobial, absorbent pad, cellulose, N-halamine, MC, beef

Introduction

Beef is considered one of the most perishable foods due to its high moisture content and abundance of nutrients on its surface for microbial growth. Various microorganisms can utilize the nutrients and colonize on the meat surface, including spoilage and foodborne

pathogens. Raw beef naturally contains liquids and juices, which can accumulate in the packaging container and leak during transportation and storage. The exudates are a sign of an unsanitary product from most consumers' perception. Absorbent pads are widely used in the meat industry packing systems to immobilize the exudates, isolate the meat from unsanitary juices, and create an appealing package. However, immobilized, unsanitary exudates may generate undesirable odors and promote the growth of microorganisms. Therefore, reducing microbial loads in absorbent pads is an important avenue for improving food quality and safety during food packaging and storage (Quintavalla and Vicini, 2002).

Packaging materials can promote food products to consumers in an attractive and hygienic way. To prevent the spread of spoilage and pathogenic microorganisms through meat and meat products, antimicrobial packaging materials have been studied intensively and can be a potential alternative technique to reach this goal (Coma, 2008). Instead of mixing an antimicrobial directly with food, incorporating antimicrobials such as essential oils (Skandamis and Nychas, 2002) and nisin (Cutter et al., 2001; Ercolini et al., 2010b; Natrajan and Sheldon, 2000) into food packaging films or absorbent pads have shown benefits. Traditional applications of antimicrobial agents result in their neutralization on the contact surface or diffusion into the food, reducing their efficacy (Juven et al., 1994). Antimicrobial packaging materials may slowly release the active antimicrobial compounds to the food contact surface, and thus extend its antimicrobial activity during transportation and storage (Quintavalla and Vicini, 2002). In addition, some antimicrobials have negative impacts on physical properties, such as color, and are not allowed to be used in certain food products (Lone et al., 2016). Active antimicrobials in packaging materials that are not directly in contact with food have more applications to improve the safety of the products.

N-halamine is a group of compounds containing one or more nitrogen-halogen covalent bond(s). It has been intensively investigated as potential antimicrobial agents in the

past decade. MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone), is a compound of monochlorinated N-halamine, which was reported as a broad-spectrum antimicrobial agent with long-term biocidal properties (Demir et al., 2015; Lauten et al., 1992; Worley et al., 1992). The structure of MC is shown in Figure 2.1. Demir et al. (2015) showed that MC coated polypropylene nonwoven fabric material was able to inactivate six log of *Staphylococcus aureus* (complete inactivation) and a four log reduction of *Escherichia coli* O157: H7 within 5 min of contact time. MC in a mineral oil suspension that has been used to disinfect *Salmonella* Enteritidis on eggshells within 72 h, and is recommended as a possible disinfectant for the egg-processing industry (Worley et al., 1992). MC coated fabrics in a dark environment are able to retain the initial active chlorine content for up to 6 months (Demir et al., 2015). Moreover, MC is considered a low toxic N-halamine compound (submitted for publications), which has a potential application in food related areas. In addition, MC has been patented in treating fish diseases (Bridges et al., 2007) when used in fish tank and in preventing human infections when contained in fibrous compositions (Worley et al., 2016). Cellulose materials in absorbent pads can be easily incorporated with MC by immersion in 1% MC ethanol solution for industrial applications.

The objectives of this study were to evaluate the effectiveness of MC in reducing the major spoilage-related microorganisms of MC coated cellulose pads in air-permeable packaging materials during 11 days of storage at 4 °C. Additionally, meat quality was also evaluated by measuring the changes of surface color and microbial loads on meat samples during storage. *Staphylococcus aureus* and *Escherichia coli* O157:H7 are the two main pathogens that usually cause foodborne diseases from meat and meat products (Hanson et al., 2011; Millette et al., 2007; Rangel et al., 2005; Scallan et al., 2011; Scanga et al., 2000; Wells et al., 1991). An *in vitro* study of the antimicrobial activities of MC against these two foodborne pathogens was performed.

Materials and methods

Materials

Absorbent pads Dri-loc[®] AC-40 were purchased from Novipax (Reading, PA). The absorbent part is a layer of highly absorbent fluff pulp, separated from food products by a non-permeable top layer polyethylene film and polyethylene film perforated with one-way valves. The absorbency is around 20 grams per absorbent pad (2 g). White foam trays (1004D, length: 9 ¼”, width: 7 ¼”, depth 1 ¼”) were purchased from Genpak[®] LLC (Glens Falls, NY).

MC was supplied by Cangzhou Jincang Chemicals, LTD (China). Tryptic soy broth (TSB), Sorbital MacConkey agar (SMAC), Buffered peptone water (BPW), Baird-Parker agar (BPA) and Plate count agar (PCA) were purchased from BD (Sparks, MD); *Pseudomonas* agar base with ceftrimide-fucidin-cephaloridine (CFC), de Man Rogosa Sharpe agar (MRS), and egg yolk tellurite emulsion were purchased from HiMedia (Nashik, MH). Crystal violet neutral red bile glucose agar (VRBG) was purchased from Hardy Diagnostics (Santa Maria, CA).

Preparation of cellulose-MC hybrid materials

MC was dissolved in 95% ethanol solution to prepare a 1% (10 g/L) stock solution. Then, the stock solution was diluted with 95% ethanol to produce 0.01%, 0.05%, and 0.1% of MC working solutions. Cellulose (2 g/pad) from absorbent pads was soaked in 10 mL of working solutions for 1 min. Then the soaked pads were air dried at room temperature for 48 h. The control was the cellulose treated with 95% ethanol.

Characterization of cellulose-MC hybrid materials

Verification of MC on absorbent pad

MC on cellulose materials was verified by Fourier Transform Infrared Spectroscopy (FT-IR) spectrum (Nicolet 6700 FT-IR Spectrometer, Thermo Scientific, Madison, WI). MC contains prominent C=O bands at 1715 and 1673 cm^{-1} on FTIR spectrum. If MC is successfully incorporated onto the absorbent pad, the MC coated materials will show these unique peaks, which are absent from cellulose alone.

Quantification of remaining active chlorine

Active chlorine loads in the cellulose over the 11 days of storage were determined by the modified iodometric/thiosulfate titration method (Worley et al., 2005). Active chlorine content was calculated according to the following equation:

$$\text{Cl}^+ (\text{atoms/g}) = \frac{6.02 \times 10^{23} \times N \times V}{2 \times W}$$

Where, N and V are the normality (equiv/L) and volume (L) of the sodium thiosulfate (titrant), respectively, and W is the weight of pads in grams.

Antimicrobial activity *in vitro*

A cocktail of *Escherichia coli* O157: H7 ATCC 11229 and *Staphylococcus aureus* ATCC 6538 was prepared. Bacterial suspension was dispersed evenly on 0.05 g cellulose materials, achieving 10^8 CFU/g for each sample. After 2 h, samples were placed into sodium thiosulfate solution to quench the chlorine residuals and wash off the attached bacteria by vigorously vortexing. Then, all the solutions were diluted by BPW (1:10) to make serial dilutions. Diluted samples were spread-plated on Sorbital MacConkey agar and BPA with egg yolk tellurite emulsion to determine surviving *E. coli* O157: H7 and *S. aureus*, respectively.

To determine the antimicrobial activity in the presence of organic matter, 0.05 g samples of cellulose-MC hybrid materials were placed into TSB solutions containing *E. coli* O157: H7 and *Staphylococcus aureus* at a 10^6 CFU/mL level. After incubating at 37 °C for 24 h, the same dilution and plating methods mentioned above were used to enumerate survived bacteria for analysis of antimicrobial activity.

Packaging of beef sample

Top round beef cuts were purchased from the Lambert-Powell Meats Laboratory at Auburn University (Auburn, AL), and were cut the same day into 2 cm thickness pieces (~100 g) under aseptic conditions at 5 °C. A foam tray was packed with a pre-treated absorbent pad, two pieces of meat, and sealed with air permeable overwrap film. Half of the meat samples were packed with MC-treated pads, and another half were packed with 95% ethanol treated absorbent pads as the control group. There were four replicates performed for both the control and MC treated groups. Packed meats were stored under refrigeration (4 ± 0.2 °C) for 11 days. Samples were analyzed on day 0 (3 h after packing), 1, 4, 7 and 11 to determine the microbial populations and color change.

Beef color

To determine the appearance change of beef samples, a chromameter (model CR-410, Minolta, Tokyo, Japan) was used to measure the meat color. The equipment was calibrated by using a standard white plate. Readings of colors were recorded using the standard CIE L* (+light, -dark), a* (+red, -green), and b* (+yellow, -blue) color system. For each beef sample, the area in contact with the polyethylene layers of absorbent pad was measured at least four times.

Microbial loads in absorbent pads

Absorbent pads (2 g) from packed beef samples were homogenized in 1% BPW at 1:10 dilution (w/w) for 1 min at 260 rpm with a stomacher (Stomacher[®] 400 Circulator Seward Ltd West Sussex, UK). The blended samples were 10-fold serial diluted with 1% BPW and spread-plated on agar plates. Total aerobic bacteria and *Pseudomonas* were enumerated on PCA and CFC plates after incubation at 30 °C for 48 h. Lactic acid bacteria were counted on the MRS agar after anaerobic incubation at 30 °C for 48 h.

Enterobacteriaceae were measured from VRBG plates after incubation at 37 °C for 48 h. The data were recorded and transformed to log₁₀ CFU values for analysis.

Microbial loads in beef

Twenty five grams of beef samples were weighed under aseptic conditions, and homogenized in 1% BPW at 1:10 dilution (w/w) for 1 min at 260 rpm with a stomacher. The microbial loads were analyzed using the same method as used with absorbent pads.

Statistical analysis

One-way analysis of variance (ANOVA) (GraphPad Prism, San Diego, CA) was applied in this study. Differences between treatments were analyzed with the Tukey test. Statistical analyses were conducted at the 95% confidence level.

Results and Discussion

Characterization of MC coated cellulose material

The FT-IR spectrum of the MC compound (Figure 2.1) contained prominent bands at 1,715 and 1,673 cm⁻¹, which were not shown in cellulose material of the absorbent pad. Compared with the spectra of cellulose and MC, the specific MC prominent bands found in 1%

MC treated cellulose material confirmed that the MC compound was successfully coated on the cellulose carrier.

Active chlorine loads remained stable over 11 days of storage (Figure 2.2). Oxidized chlorine is the antimicrobial functional group, which is able to kill microorganisms when in contact with their surface. Chlorine contents ranged from the initial 1.2×10^{20} atoms/g to 1.0×10^{20} atoms/g at day 11. There was no significant change ($P < 0.05$) in active chlorine loads over the storage, indicating the stability of the MC hybrid materials, which is similar to a previous study that indicated MC coated polypropylene fabrics retained their initial chlorine loads during a 6-month storage time under dark conditions (Demir et al., 2015).

Antimicrobial test *in vitro*

In BPB solution, cellulose-MC hybrid materials at a 0.01% level resulted in more than two log reductions against both *E. coli* O157: H7 and *S. aureus* (Table 2.1). Cellulose treated with 0.05% MC reduced these two microorganisms to under the detection limit (< 3 log CFU/g) after 2 h of contact.

Due to the abundant nutrients in TSB broth, microbial counts in MC-free cellulose increased by around 3 log after 24 h of incubation (Table 2.1). Cellulose coated with 0.01% MC reduced *E. coli* O157: H7 by about 3-log, and by 5-log with 0.1% MC. There were 0.2 and 3 log reductions of *S. aureus* when contacted with 0.01% and 0.1% cellulose-MC hybrid materials, respectively. Cellulose treated with 1% MC was able to reduce both pathogens to undetectable levels. Compared with the results in BPB solutions, the antimicrobial efficacy of cellulose-MC hybrid materials in TSB was lowered due to the chelating effects of abundant organic matter in the medium. A previous study also showed that organic matter led to the differences in antimicrobial activities of silver nanoparticles when applied in fruit and meat products (Fernández et al., 2010a, 2010b).

Color change of beef during storage

Color change of beef was measured by the differences in lightness (L^*), redness (a^*) and yellowness (b^*) of samples (Table 2.2). L^* values were increased, which indicated beef was becoming darker during the entire storage. The lightness of the controls from day 1 through day 11 had significant differences with that of day 0, while the L^* value of meat in MC treated absorbent pads did not show a remarkable increase until day 11. All beef cuts turned to grayish brown on day 11, when the counts of major spoilage related microorganisms were reaching to 10^8 CFU/g. This phenomenon was observed in another study as well (Fernández et al., 2010b). Redness (a^* value) decreased over time. Both the controls and MC treated groups had significant reductions from day 1. Yellowness or b^* value, did not show any particular trend of change throughout the entire storage. In summary, the values confirmed the negligible effects of MC-loaded absorbent pads on meat color deterioration.

Microbial loads in absorbent pads

During storage, absorbent pads were continually absorbing exudates dripping from meat samples. The control pads accumulated 1.9 g of exudates on day 0 (3 h after packing), 3.9 g on day 1, 5.7 g on day 4, 8.6 g on day 7, and 12.1 g on day 11. The MC treated pads absorbed 2.3 g, 2.8 g, 7.1 g, 7.9 g, and 11.4 g on day 0, 1, 4, 7, and 11, respectively. The accumulation of meat drips was constantly increasing over time, and there were significant differences in absorbent pads with or without the presence of MC. With the accumulation of exudates, microbial populations were constantly increasing in both absorbent pads and beef samples (Figure 2.3 & 2.4).

Total aerobic bacteria counts were the highest among the four major spoilage-related microorganisms, which increased from approximately 5 log CFU/g to 8 log CFU/g over 11

days of storage. There were no bacteria recovered from the MC treated absorbent pads after 3 h packing, which was about 5 log-reduced compared to the control. Similar antimicrobial effects were observed on day 1, with a 5 log reduction as well. On day 4, total aerobic bacteria in MC coated absorbent pads increased to 3 log CFU/g, around 2 logs lower than the control. The total aerobic bacteria on control absorbent pads had similar populations to those in a silver hybrid absorbent pad study (Fernández et al., 2010b). The bacterial populations in MC treated absorbent pads remained over 3 logs lower than those in control groups on days 7 and 11. Overall, significant reductions ($P < 0.05$) of total plate count bacteria were observed in MC treated samples throughout the entire storage period.

Pseudomonas are the predominant microorganisms that contribute to the spoilage of raw beef cuts during aerobic refrigerated storage (Borch et al., 1996; Ercolini et al., 2010a, 2006; Koutsoumanis et al., 2006; Nychas et al., 2008). *Pseudomonas* can degrade glucose and amino acids and produce odor-active molecules during aerobic storage (Dainty and Mackey, 1992; Ercolini et al., 2010a, 2006). It has been observed that a significant decrease of surface glucose level is the first sign of sensory change associated with spoilage (Ellis and Goodacre, 2001). Significant reductions of MC coated absorbent pads were observed on days 1, 7 and 11. On day 0 and day 1, no *Pseudomonas* was detected in the absorbent pads treated with MC, and the bacteria were about 3 logs lower than the controls. *Pseudomonas* populations in the controls reached close to 10^7 CFU/g and 10^8 CFU/g on days 7 and 11, respectively, and the recovered *Pseudomonas* on MC treated pads was around 10^4 CFU/g on these days. There was an average 2.4-log reduction of *Pseudomonas* compared with the controls over 11 d of refrigerated storage.

Lactic acid bacteria (LAB) play an important role in raw beef spoilage during refrigeration (Labadie, 1999). The populations of the LAB in MC treated absorbent pads showed the similar tendencies with total aerobic bacteria, which were not observed until day

4, when the control samples reached over 5 log CFU/g. Thereafter, the populations of lactic acid bacteria in the MC treated samples increased; however, they were significantly lower than the controls over the entire period of storage. MC coated absorbent pads led to an average of 3 log reductions of lactic acid bacteria during the 11 days. *Enterobacteriaceae* are a type of spoilage microorganisms and foodborne pathogens in beef. (Ercolini et al., 2006; Labadie, 1999). The populations of *Enterobacteriaceae* were the lowest among the investigated microorganisms. It reached to 1.9 log CFU/g on day 7 in the control absorbent pads, while there was no *Enterobacteriaceae* detected in MC treated samples. On day 11, the populations increased to 2.9 log CFU/g and 2.2 log CFU/g for the controls and treated absorbent pads, respectively. Prior to day 7, *Enterobacteriaceae* populations were below the detection limit (< 2 log CFU/g); therefore, the results were not presented in graph.

Compared with absorbent pads without meat, the antimicrobial activity of the absorbent pads in the presence of meat is lower. This may be caused by the existence of meat exudate, which contains abundant nutrients (Rotta et al., 2009) supporting the microbial growth and chelation with MC, impairing the antimicrobial activity. This phenomenon has been commonly observed in many antimicrobials applied in foods such as peracetic acid (PAA), chlorine, and other naturally occurring antimicrobial agents. The presence of organic matter such as fatty acids or other components can impair the antimicrobial activity of the agents, as shown by Cutter (1999), in which the antimicrobial activity of triclosan-incorporated plastic (TIP) on meat surfaces was diminished.

Silver pre-treated absorbent pads were able to reduce 1.4, 0.7, and 1.1 log CFU/g of total aerobic bacteria, LAB, and *Pseudomonas*, respectively, during 11 days of storage (Fernández et al., 2010b). MC displayed higher antimicrobial activity during 11 days of storage, with 3.6, 2.4, and 3 log reductions of these three major spoilage microbes, respectively. The cost of the silver nanoparticles is expensive, and the European Food Safety

Authority limits the concentration of silver ions in food to below 0.05 mg/kg (UFSA, 2004) , which is usually not high enough to achieve expected antimicrobial effects. In addition, the technology of preparing MC coated absorbent pads is simple without chemical reactions and heating process. Unlike absorbent pads containing bacteriophages, MC treated materials are not vulnerable to storage conditions such as temperature and moisture content. When the temperature dropped from 15 to 10 °C, the antimicrobial activities of bacteriophage-cellulose reduced by 3 log (Gouvêa et al., 2016).

Microbial loads in beef

Microbial populations in meat samples in the control group had similar trends to the absorbent pads. Total aerobic bacteria was the dominating microorganism among the major spoilage microbes, ranging from 4.6 to 8.3 log CFU/g during 11 days of refrigerated storage. It is in accordance with other studies on beef in the similar aerobic refrigerated conditions (Ercolini et al., 2010b, 2006; Fernández et al., 2010b; Skandamis and Nychas, 2001).

Pseudomonas and LAB reached to around 8.0 log CFU/g in the controls on day 11. When the *Pseudomonas* increased to 7.3 log CFU/g on day 7, the meat appeared slimy and produced noticeable off-odors. After *Pseudomonas* used up the glucose and lactate, it used amino acids, which produced putrid and sulphury odors from sulphur-containing compounds such as dimethyl sulfide (Borch et al., 1996; Nychas et al., 2008). Lactic acid bacteria usually produce milky exudates and sour off-odors. On day 7, noticeable slime was found on most of the meat samples (Franz and von Holy, 1996). *Enterobacteriaceae* was not found on the first day, and then increased to 1.5, 3.6, and 3.3 CFU/g on days 4, 7 and 11, respectively.

Although *Enterobacteriaceae* populations are limited in refrigerated meat, there can be some off odors produced, some described as cheesy (Nychas et al., 2008).

In general, an average reduction of 1.0 log CFU/g was achieved in total aerobic bacteria and *Pseudomonas* recovery, and a reduction of 0.6 log CFU/g of lactic acid bacteria was achieved in meat packed with MC treated absorbent pads. The populations of total aerobic bacteria in the MC treated group were significantly lower than those of the controls after 4 days of refrigerated storage. There was no significant reduction of *Pseudomonas* until day 7, or of lactic acid bacteria until day 11. *Enterobacteriaceae* was not detected during the entire storage time in the treated groups. Previous research showed there were no antimicrobial effects on meat when silver was applied to isolated absorbent pads (Fernández et al., 2010b), making MC a viable agent for this application .

To achieve better antimicrobial effects in meat samples, hurdle technologies are encouraged. A previous study showed that combined applications improved the effects of the original antimicrobials. There is a synergistic effect of volatile compounds of oregano essential oil and modified atmosphere packaging (MAP), which consistently reduced spoilage microorganisms by 1-2 log CFU/g compared to the controls (Skandamis and Nychas, 2002).

In summary, antimicrobial packaging is a promising technique to improve the quality and safety of food products, particularly in meat products. Incorporating effective and long-lasting antimicrobial agents of MC into absorbent pads without direct contact with foods not only extended shelf life, but also improved the safety of foods. To achieve better antimicrobial activity in meat, other techniques such as vacuum packaging and refrigeration should be combined with MC coated absorbent pads.

Table 2.1. Antimicrobial activity of cellulose-MC hybrid materials in absorbent pads against *Escherichia coli* O157: H7 and *Staphylococcus aureus* in Butterfield's phosphate buffer (pH 7.0) and Trypticase[®] soy buffer (pH 7.0) solutions.

MC concentration (g/g)	BPB		TSB	
	<i>E. coli</i> O157: H7*	<i>S. aureus</i> *	<i>E. coli</i> O157: H7	<i>S. aureus</i>
0	6.26± 0.05 ^a	4.50± 0.11	8.73± 0.15	9.41± 0.06
0.01%	3.86± 0.14	3.31± 0.27	5.65± 0.33	9.19± 0.05
0.05%	ND ^b	ND	--	--
0.1%	-- ^c	--	3.94± 0.73	6.63± 0.88
1%	--	--	ND	ND

* Inoculum: *E. coli* O157: H7 2.1×10^6 CFU/0.05g; *S. aureus* 1.4×10^6 CFU/0.05g

a Mean of Log₁₀ CFU/mL ± SD

b Not detected

c Not tested

Table 2.2. CIE L*, a*, and b* color scores of beef meat during refrigerated storage with MC treated absorbent pads.

Absorbent pad treatment	Storage time (days)	Beef color \pm SD		
		L*	a*	b*
Control	0 ^a	40.59 \pm 1.55 A ^b	31.77 \pm 0.62 A	15.96 \pm 0.56 A
	1	43.96 \pm 0.76 B	29.03 \pm 0.62 B	14.32 \pm 0.22 B
	4	44.84 \pm 1.17 B	19.97 \pm 0.94 B	9.78 \pm 0.31 B
	7	45.57 \pm 1.47 B	15.36 \pm 1.51 B	12.25 \pm 0.14 B
	11	47.68 \pm 1.69 B	7.77 \pm 0.18 B	11.73 \pm 0.54 B
MC	0	39.68 \pm 0.78 A	31.52 \pm 0.63 A	13.11 \pm 0.42 B
	1	38.42 \pm 0.89 A	26.96 \pm 2.30 B	14.54 \pm 0.23 B
	4	40.80 \pm 0.90 A	19.47 \pm 1.83 B	8.54 \pm 0.90 B
	7	41.91 \pm 0.45 A	17.88 \pm 1.01 B	9.78 \pm 0.61 B
	11	48.55 \pm 3.65 B	8.02 \pm 0.63 B	10.40 \pm 0.33 B

^a Analyzed after 3 h of contact with absorbent pad.

^b Compared with data in day 0 control group, different superscripts in the same column mean significant differences (P < 0.05)

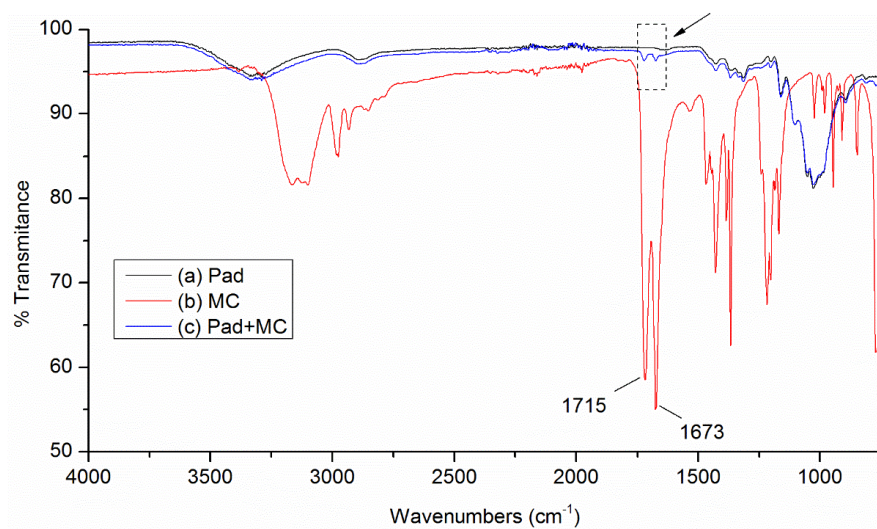


Figure 2.1. Fourier transform infrared spectroscopy (FTIR) spectra of MC coated absorbent pads: (a) absorbent pad, (b) MC, (c) MC coated absorbent pads.

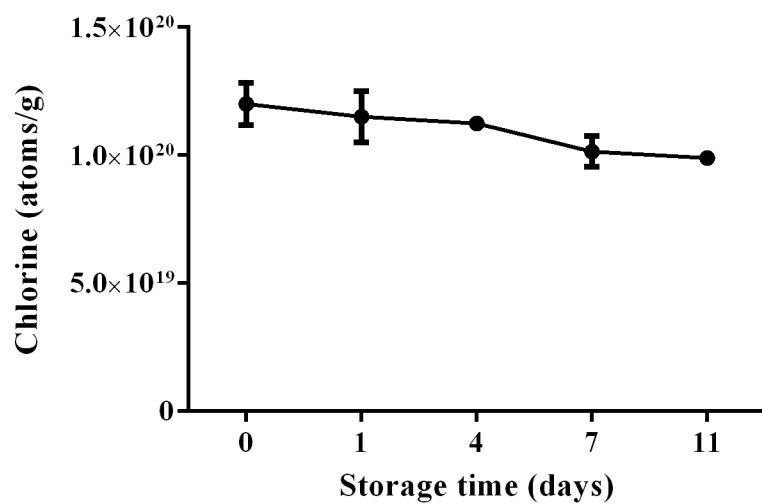


Figure 2.2. Active chlorine contents on MC coated absorbent pads during 11 days of storage.

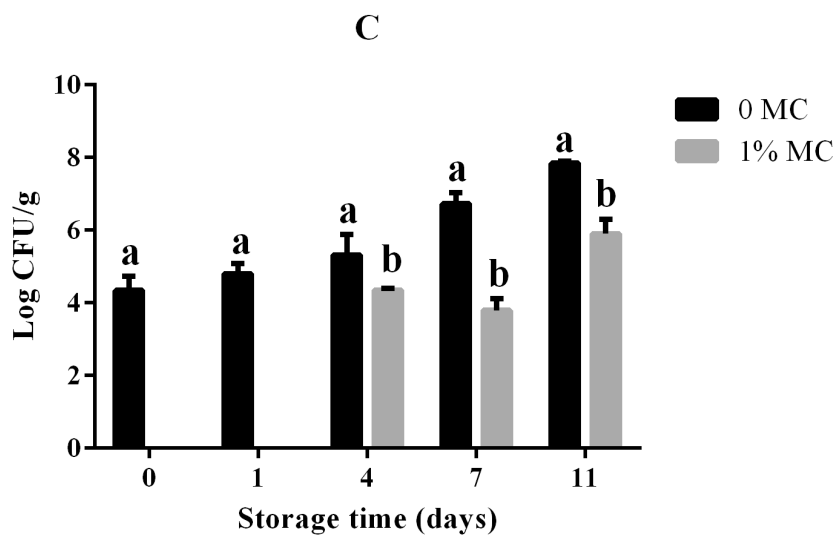
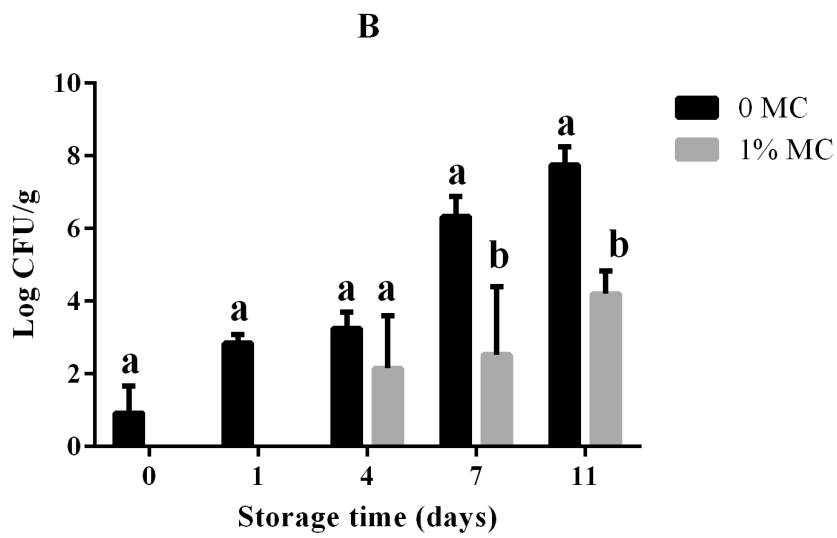
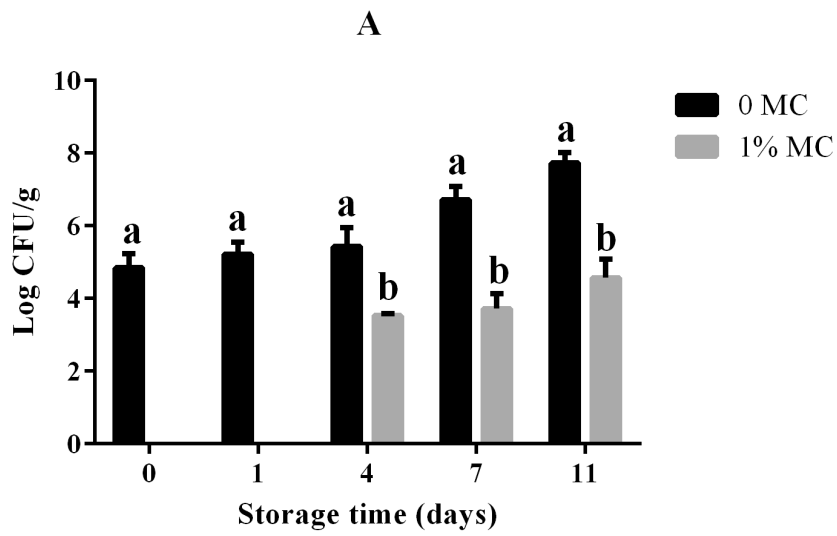


Figure 2.3. Bacterial loads in absorbent pads in contact with beef during 11 days of storage at 4 °C, in the presence or absence (control) of MC. A) Total aerobic bacterial counts, B) *Pseudomonas*, C) Total lactic acid bacterial counts. Each value is the mean of four replicates, and vertical bars represent standard deviations. Different letters in the same sampling day indicate significantly different results at the 95% confidence level.

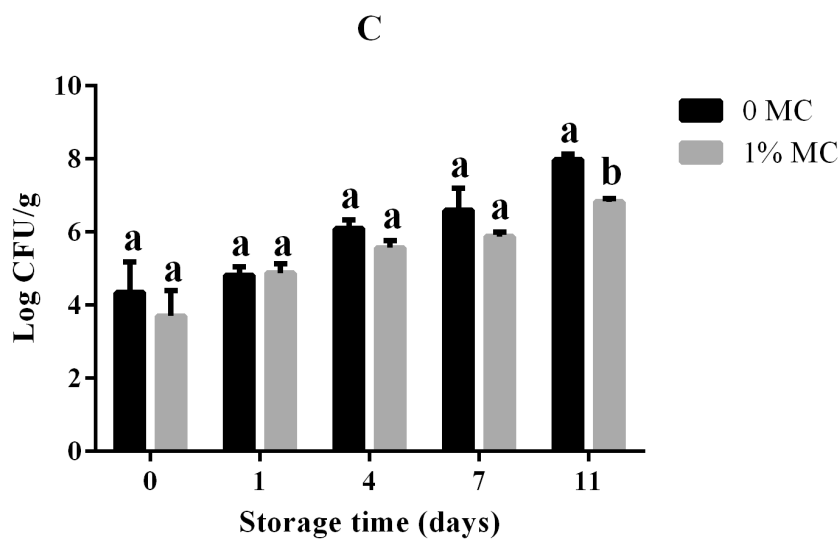
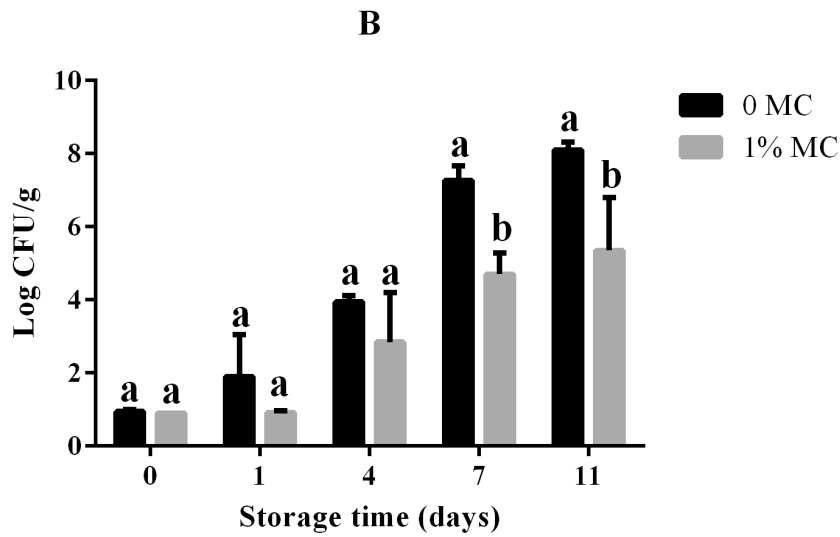
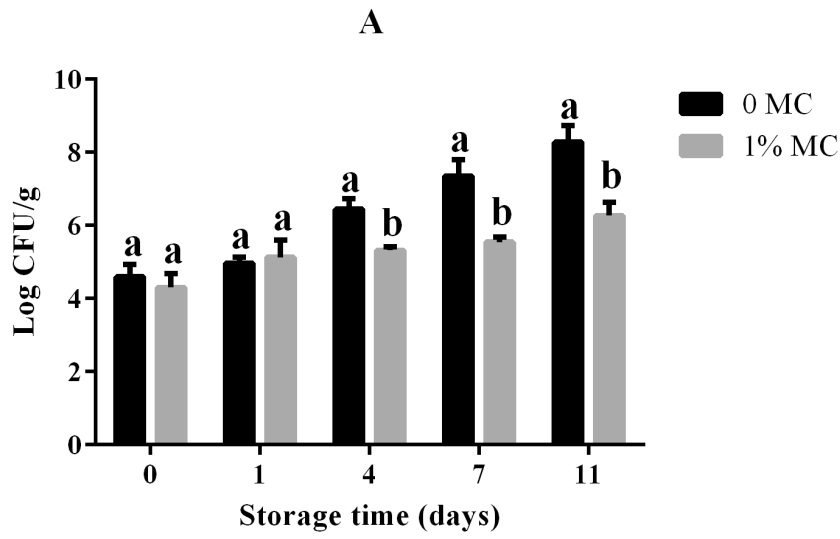


Figure 2.4. Bacterial counts in beef during 11 days of storage at 4 °C, in the presence or absence (control) of MC. A) Total aerobic bacterial counts, B) *Pseudomonas*, C) Total lactic acid bacterial counts. Each value is the mean of four replicates, and vertical bars represent standard deviations. Different letters in the same sampling day indicate significant different results at the 95% confidence level.

III. Absorbent pads containing N-halamine compound for potential antimicrobial use in chicken breast and ground chicken.

Abstract

N-halamines are a group of compounds containing one or more nitrogen-halogen covalent bond(s), and the high-energy halide bond provides a strong oxidative state to inactivate microorganisms effectively. In this study, the shelf life of chicken breast and ground chicken packed with 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC, a member of N-halamine) treated absorbent pads was investigated during refrigerated storage. Fresh, processed chicken meat in packaging trays loaded with or without MC treated absorbent pads were stored at 4 °C for 11 days. The microbial counts in chicken meat as well as in the food pads were analyzed on days 1, 4, 7, and 11. MC treated pads reduced the levels of the main spoilage-related microorganisms (aerobic plate counts, lactic acid bacteria, Enterobacteriaceae, psychrotrophs and *Pseudomonas* spp.) present in the absorbent pads by an average of 3.5 log CFU/g compared to the control. Microbial loads in chicken breast contacted with MC coated absorbent pads were 0.3 log CFU/g lower than those in the control on average, and there was about 0.2 log CFU/g reduction for ground chicken. Neither the color nor pH of the meat was negatively impacted by the presence of MC. An *In vitro* antimicrobial test with 1% MC treated absorbent pads reduced around 4 log CFU/g of *Salmonella* Typhimurium and 7 log CFU/g of *Campylobacter jejuni* after 1 h of contact. The MC treated cellulose materials of absorbent pads lowered these two bacteria to under the detection limit (1 log CFU/mL) in 24 h.

Keywords

Chicken, packaging, antimicrobial, absorbent pad, N-halamines, shelf life

Introduction

The consumption of poultry meat in the U.S. is considerably higher than beef or pork, and the world's consumption of poultry is increasing (Landes et al., 1998; USDA-ERS, 2016). This increase is due to many reasons, including population growth, poultry's relative low cost, and consumer dietary preferences such as low fat and high protein content (The Poultry Site, 2015). However, nutrients in chicken meat can lead to spoilage during transportation and storage. Spoilage of chicken is an economic burden to poultry producers, and it requires the development of new technologies such as active packaging to prolong shelf life and overall quality/safety (Petrou et al., 2012).

Exudates from chicken meat can accumulate in the packaging container and may leak during transportation and storage, producing an unsanitary perception of the products by consumers. Therefore, absorbent pads are widely used in fresh raw meat packaging to remove the exudates, isolate the meat from exuded juices, and thus create appealing products. Nonetheless, the meat juice is a nutrient-rich cultivation broth, and the unsanitary exudate immobilized in absorbent pads could easily promote the growth of microorganisms that may diffuse outside of the pad. The microorganisms shed in food pads may generate undesirable odor, spoil the food quality, and support the propagation of foodborne pathogens. Therefore, reducing microbial loads in absorbent pads is one of the important avenues to improve both the quality and safety of meat. Previous research revealed that incorporating antimicrobials such as silver nanoparticles (Fernández et al., 2010a, 2010b, 2009) and bacteriophages (Gouvêa et al., 2016; Lone et al., 2016) with cellulose materials of absorbent pads was able to control the microbial growth. Although some of those hybrid materials could effectively reduce microbial loads in food pads under certain conditions, they do not meet the food industry demand in the present time in terms of cost and antimicrobial effectiveness.

N-halamine is a group of compounds containing one or more nitrogen-halogen covalent bond(s). Many compounds of this group have been reported as having effective antimicrobial activity (Hui and Debiemme-Chouvy, 2013). Among these compounds, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC, Figure 1.2) has shown excellent long-term biocidal properties (Demir et al., 2015; Lauten et al., 1992; Worley et al., 1992). Worley et al. (1992) showed that MC in a mineral oil suspension was able to disinfect *Salmonella* Enteritidis from the eggshells effectively, and thus it was recommended as a possible replacement as an antimicrobial agent for the edible egg-processing companies. Additionally, MC treated polypropylene nonwoven fabric material of a facial mask inactivated *Staphylococcus aureus* and *Escherichia. coli* O157: H7 in a short contact time and retained its initial active chlorine content for up to 6 months (Demir et al., 2015). Therefore, incorporating MC into absorbent pads might be an alternative approach to reducing the microbial loads during food storage.

The objectives of this study were to evaluate the effectiveness of MC in reducing the major spoilage-related microorganisms of the MC coated cellulose pads in air-permeable packaging materials during 11 days of storage of chicken breast and ground chicken at 4 °C. Meat quality was also evaluated by testing meat surface color, pH, and the microbial loads throughout storage.

Materials and methods

Absorbent pads preparation

The absorbent pads Dri-loc[®] DLSA 100 (5.50" × 7") were kindly donated by Novipax (Reading, PA). Each pad is equipped with a highly absorbent fluff pulp layer to immobilize exudates and a non-permeable top layer polyethylene film to separate the juices from food. The absorption layers can absorb up to 40 mL of exuded fluids. The white foam trays (1004D,

L× W× D= 9¼”× 7¼”× 1¼”) were purchased from Genpak[®] LLC (Glens Falls, NY). MC was supplied by Cangzhou Jincang Chemicals, LTD (China).

At first, MC powder was dissolved in 95% ethanol to produce 0.01%, 0.1% and 1% working solutions. MC coated cellulose materials were prepared by adding 10 mL of the working solutions to 2 g of cellulose in absorbent pads. Treated pads were air dried at room temperature for 48 h. The pads treated with 95% ethanol only served as the controls.

The coating efficiency of the treated absorbent pads was verified by Fourier Transform Infrared Spectroscopy (FT-IR) spectrum (Nicolet 6700 FT-IR Spectrometer, Thermo Scientific, Madison, WI). Meanwhile, since the biocidal activity of N-halamines is a result of the nitrogen-halogen bond(s), the antimicrobial stability of MC-coated pads during storage was determined by measuring the remaining chlorine on the absorbent pads by using the modified iodometric/thiosulfate titration method (Worley et al., 2005).

Bacterial preparation and *in vitro* assay

To determine the efficacy of MC-coated absorbent pads against foodborne pathogens, 100 ppm nalidixic acid (NA)-resistant *Salmonella* Typhimurium and *Campylobacter jejuni* (both isolated and identified from Auburn University Poultry Research Unit, Auburn, AL) were used to challenge the prepared absorbent pads. In this study, *S. Typhimurium* carries nalidixic acid resistance and was grown/differentiated with Trypticase[®] soy broth (TSB, BD Co., MD) and xylose lysine Tergitol 4 agar (XLT4; Acumedia, MI) with 100 ppm NA. *C. jejuni* was grown in Brucella broth (Hardy Co., CA) with FBP (Ferrous sulfate, Sodium biosulfite, and Sodium pyruvate) and Campy-Cefex agar (Acumedia, MI).

For *Salmonella* preparation, 1 loopful of the frozen culture was added into 10 mL TSB, and incubated at 37 °C for 24 h. The recovered *S. Typhimurium* was streaked on XLT4 with 100 ppm NA. After incubating at 37 °C for 24 h, a typical black colony was transferred

into 10 mL of TSB, and incubated at 37 °C for 24 h. The culture was then 10 times diluted with TSB and incubated at 37 °C with shaking at 250 rpm. After 12 h, the culture was centrifuged at 8,000 ×g for 3 min, and the bacteria was resuspended in Butterfield's phosphate buffer (BPB, pH 7.0; BD, MD). Bacteria were further washed 3 times with BPB. The bacterial population was estimated by the absorbance at O.D. _{640 nm} and the cell density was adjusted to 10⁸ CFU/mL with BPB.

A typical *C. jejuni* colony from Campy-Cefex agar (Acumedia, MI) was picked and transferred to 10 mL of Brucella-FBP (Ferrous sulfate, Sodium biosulfite, and Sodium pyruvate) broth (Hardy Co., CA). The culture was incubated at 42 °C for 48 h in microaerophilic condition (5% O₂, 10% CO₂ and 85% N₂). Then, the bacterial suspension was centrifuged at 8,000 ×g for 10 min at 4 °C and resuspended in buffered peptone water (BPW; Hardy Co., CA). A culture of 10⁸ CFU/mL *C. jejuni* was prepared. The final density of the inoculum mixture was 5×10⁷ CFU/mL for each bacterium. A sterile BPB- BPW (1:1) solution was prepared as the control, representing the baseline bacterial counts in absorbent pads.

Ten milliliters of the bacterial cocktail or the control solution was poured on each absorbent pad to mimic the exudates from chicken. The absorbent pads were stored under refrigeration (4±0.2 °C) for 1 h or 24 h. MC free absorbent pads were used to determine the recovery of the inoculated bacteria. Absorbent pads with no inoculated bacteria were used to examine the background of the two microorganisms.

At the end of each desired storage duration, pads were homogenized in 1% BPW at 1:10 dilution with a blender (Stomacher[®] 400 Circulator, Seward Ltd, West Sussex, UK) at 260 rpm for 1 min. Solutions were serial diluted with 1% BPW and each dilution was spread on selective media. *S. Typhimurium* was enumerated from XLT4 agar containing 100 ppm NA plates after being incubated at 37 °C for 24 h, and *C. jejuni* was counted from Campy-

cefex plates incubated at 42 °C for 48 h in the same microaerophilic condition described previously. The data were recorded and reported as log₁₀ CFU per gram of sample for analysis.

Chicken meat packaging

Chicken breast and ground chicken were used in this study. The chicken products were freshly processed, sampled and packed with our pretreated absorbent pads at the Auburn University Poultry Research Unit at 5 °C. The entire procedure complied with Auburn University's regulation and the "Raw meat and poultry product sampling methods" in the USDA-FSIS Microbiological Laboratory Guidebook (FSIS Notice 33-13, 2013).

Two pieces of chicken breast without skin (~ 1.5 lbs.) were packed in a 1004D white foam tray (Genpak[®] LLC, Glens Falls, NY) loaded with pre-treated absorbent pads, and sealed with air permeable overwrap film. Ground chicken was prepared by grinding the mixture of chicken breast and thigh with skin in a ratio of 1:1 using a grinder (Brio 32, MFG Co, Marblehead, OH) at 5 °C. The meat was ground and packed (~ 454 g/tray) in foam trays with absorbent pads under the same conditions as the chicken breast. Four replicates were prepared in each MC concentration for both chicken products. After packing, samples were kept on ice for 30 min before being transported to the laboratory in the Poultry Science Department at Auburn University for further analysis.

Measurement of moisture and fat content of chicken meat

Moisture content of the meat samples was measured following the method in AOAC 950.46 (2016). Briefly, weight loss of meat samples by air drying at 100- 102 °C for 18 h was recorded, and the moisture content was calculated based on the weight loss. Crude fat contents were determined by using the Goldfish apparatus to extract total fats according to the method from AOAC 960.39 (2016).

Microbial loads in chicken and absorbent pads

Once the samples arrived at the laboratory, the packed meat was stored in a refrigerator (Thermo-Kool, Mid-South Industries, Inc., MS) at 4 ± 0.2 °C for 11 d for the shelf-life determination study. On days 1, 4, 7, and 11, microbial populations in both the meat and their absorbent pads were determined.

On each sampling day, 25 g of chicken breast or ground chicken from the packed samples were homogenized with 1% sterile buffered peptone water (BPW) at 1:10 dilution (w/v), at a speed at 260 rpm for 1 min. The homogenates were then 10-fold serial diluted with 1% BPW and were ready for direct plating. Aerobic plate counts (APC) were enumerated on plate count agar (PCA; BD, MD) and *Pseudomonas* spp. were counted from the *Pseudomonas* agar base with ceftrimide-fucidin-cephaloridine (CFC; HiMedia, India) after incubation at 30 °C for 48 h. Lactic acid bacteria (LAB) were determined on the de Man Rogosa Sharpe agar (MRS; HiMedia, India) after anaerobic incubation at 30 °C for 48 h. Enterobacteriaceae were measured from Crystal violet neutral red bile glucose agar (VRBG; Hardy Diagnostics, CA) after incubation at 37 °C for 48 h. Psychrotrophs were evaluated from PCA after incubation at 4 °C for 10 d. Each dilution was plated on selective media in the triplicate, and the plates were incubated under their corresponding optimum conditions. The data were recorded and reported as \log_{10} CFU/g of meat.

Absorbent pads (2 g/pad) from packed chicken breast and ground chicken were transferred to stomaching bags containing 1% BPW at 1:10 (w/v) ratio. Then, samples were homogenized at 260 rpm for 1 min. Microbial loads in the absorbent pads were analyzed by using the same direct-plating methods as mentioned previously.

Color and pH measurement

Color measurement and pH determination of chicken meat were performed on days 1, 4, 7 and 11. The color of each meat piece was evaluated by using a colorimeter (CR-410, Minolta, Tokyo, Japan). Before each test, the equipment was calibrated using a standard white plate. Readings of colors were recorded using the standard CIE L* (+light, -dark), a* (+red, -green), and b* (+yellow, -blue) color system. For each meat piece, five different locations in contact with the polyethylene layer of the absorbent pad were measured, and the mean value was used for analysis.

The pH values of chicken samples were measured according to a previous study (Goulas and Kontominas, 2005). A 10-g sample was homogenized in 100 mL of distilled water by an ultrasonic homogenizer (Sonic VCX-750, Newtown, CT), and the mixture was filtered. The pH of the filtrate was determined by a pH meter (Beckman 390, Indianapolis, IN) at room temperature. Before each test, the pH meter was standardized using pH 4 and pH 7 buffer solutions.

Statistical analysis

GraphPad (version 4.0, Prism, San Diego, CA) was used to analyze data and generate graphs of the results. The differences between treatments were investigated by one-way analysis of variance (ANOVA) and followed by the Tukey test in SAS software package. Statistical analyses were conducted at the 95% confidence level.

Results and Discussion

Characterization of MC coated cellulose material

The FT-IR spectrum of the MC compound contained prominent peaks at 1,217; 1,430; 1,673; and 2,974 cm^{-1} , which were not shown in the original cellulose material of the

absorbent pad (Figure 3.1). Compared with the spectra of cellulose and MC treated absorbent pad, the specific peaks of MC in 1% MC treated absorbent pads confirmed that MC was successfully incorporated with in the cellulose carrier. Titration results showed that chlorine contents in MC treated absorbent pads were 1.4×10^{20} , 1.3×10^{20} , 1.2×10^{20} , 1.2×10^{20} atoms/g on days 1, 4, 7, and 11 respectively. No significant reductions ($P < 0.05$) of active chlorine contents over 11 days of storage indicated the antimicrobial activity of the MC treated pads is stable.

Antimicrobial test *in vitro*

Antimicrobial effects of MC coated absorbent pads against foodborne pathogens *S. Typhimurium* and *C. jejuni* after 1 h and 24 h contact are illustrated in Table 3.1. Initial populations of *S. Typhimurium* and *C. jejuni* were 7.51 and 7.50 log CFU/g, respectively. After 1 h contact, absorbent pads treated with 0.01% MC had slight reductions of *S. Typhimurium* (0.04 log CFU/g) and *C. jejuni* (0.08 log CFU/g) compared to the controls. An MC concentration of 0.1% significantly lowered *C. jejuni* to 4.58 log CFU/g in 1 h contact, which was around a 3 log reduction. Absorbent pads treated with 1% MC reduced *Salmonella* to 3.90 log CFU/g, and lowered *Campylobacter* to under the detection limit (1 log CFU/mL).

When the samples were incubated at 4 °C for 24 h, *S. Typhimurium* and *C. jejuni* in the controls increased to 7.50 log CFU/g and 7.67 log CFU/g, respectively. There were no significant differences ($P < 0.05$) in the bacterial loads between the absorbent pads treated with 0.01% MC and the control. Absorbent pads coated with 0.1% MC led to a 0.5 log reduction of *Salmonella* and a 4.4 log reduction of *Campylobacter*. However, in 1% MC treated absorbent pads, both microorganisms were reduced to under the detection limit after 24 h.

Color and pH changes of meat

On the initial day, results showed that the average moisture contents of chicken breast and ground chicken were $71.3 \pm 0.51\%$ and $66.0 \pm 0.33\%$, respectively. Crude fat contents were 3.5% for chicken breast and 9.0% for ground chicken in wet basis.

The CIE color values of chicken meat in contact with absorbent pads with or without MC were shown in Table 3.2. L^* values increased gradually over storage, which is similar to previous studies of chicken breast with air chilling (Patsias et al., 2008), and ground chicken treated with various antimicrobials (Chen et al., 2014; Radha krishnan et al., 2014). Ground chicken had a significant higher L^* value ($P < 0.05$) compared to chicken breast, which may be due to its lower moisture and higher fat content. Chicken meat that contacted MC incorporated absorbent pads displayed lower L^* values than the controls. However, no significant differences were observed. Ground meat appeared more red (a^*) than the chicken breast, and there were no significant differences between the meats with MC treated absorbent pads and the controls. b^* value, or yellowness, did not show any particular trends over the storage.

Table 3.3 showed the pH changes of chicken breast and ground chicken in the package containing absorbent pads with or without MC throughout the refrigerated storage. The initial pH of chicken breast was around 5.7, and the pH increased over the storage time. A significant increase ($P < 0.05$) in pH (6.3) of breast meat was observed on day 11. Ground chicken samples had a higher initial pH 5.92 and 5.95 for the controls and the treated groups, respectively. At the end of storage time, pH values reached 6.36 and 6.23 for these two groups and were significantly increased compared to those on day 1. Other studies also show similar pH values in raw chicken meat (Radha krishnan et al., 2014) and ground chicken products (Yang and Chen, 1993) by day 11 under the same storage conditions. In general, meat in the package containing the control absorbent pads had a higher pH than that in the

MC treated samples during the storage. The increase of pH may be due to the utilization of amino acids after glucose depletion caused by bacteria (Gill, 1983). However, significant differences between meat in the packages containing absorbent pads with and without the MC compound were not observed.

Microbial loads in absorbent pads

The meat exudates provide a nutrient rich broth, and thus microbial counts in the control absorbent pads increased over time. In the absorbent pads used with chicken breasts, initial populations of APC, *Pseudomonas*, and psychrotrophs in the controls were around 3.8 log CFU/g, while LAB and Enterobacteriaceae were under the detection limit (2 log CFU/g) on the first day. After 11 days of storage at 4 °C, APC and *Pseudomonas* reached 9.4 log CFU/g, while psychrotrophic bacteria and Enterobacteriaceae were 8.4 and 7.1 log CFU/g, respectively. LAB reached to 4.8 log CFU/g on day 11, which was the lowest population among these five microorganisms (Figure 3.2). The microbial loads of these spoilage bacteria in the control absorbent pads were comparable to the results in a beef study under similar experimental conditions (Fernández et al., 2010b).

The antimicrobial effects of MC on spoilage microorganisms in the absorbent pads with chicken breasts are shown in Figure 3.2. Antimicrobial activities of MC treated pads were determined immediately as exuded juices impregnated the pads. The bacterial loads of APC and PSY in MC treated absorbent pads were under the detection limit on days 1 and 4, and rising to around 7 log CFU/g on day 11. There were average reductions of 4.1 and 3.7 log CFU/g throughout the storage of APC and PSY, respectively. *Pseudomonas* and Enterobacteriaceae in MC treated absorbent pads in contact with breast meat were not detected until day 7, and then reached to 7.0 and 5.3 log CFU/g on day 11, respectively, significantly lower than the controls ($P < 0.05$). There were an average of 4.6 and 2.3 log

reductions on these two microorganisms compared to their controls. LAB in MC treated absorbent pads was under the detection limit during the entire storage period, and it was 2.8 log CFU/g of the control.

Similarly, microbial counts on ground chicken with loaded absorbent pads were increased over time (Figure 3.4). Initial populations of all the tested microorganisms were below the detection limit on the first day. After 11 days of storage, the microbial loads of APC, *Pseudomonas*, and psychrotrophs in the pads were approximately 9.0 log CFU/g, and 6.2 log CFU/g for Enterobacteriaceae. In addition, populations of microorganisms in control absorbent pads packed with ground chicken were lower than those pads with chicken breast.

No aerobic bacteria in MC treated pads was detected up to day 4 and then increased to 4.1 and 4.5 log CFU/g on days 7 and 11, respectively. A significant reduction of 3.2 log CFU/g was achieved compared to its control on average. *Pseudomonas* and psychrotrophs in MC treated pads were not detected until day 11. The microbial loads reached a maximum of 4.0 log CFU/g for both microorganisms on day 11, with significant reductions achieved throughout the entire storage time. *Pseudomonas* and psychrotrophs were reduced 3.9 and 3.8 log CFU/g on average, respectively. The populations of Enterobacteriaceae in treated groups were 3.2 log CFU/g, which was significantly lower than in the control. LAB in the MC treated and control absorbent pads were under the detection limit and are not presented in the figure.

Under similar experimental conditions, microbial load reductions in silver hybrid absorbent pads loaded with beef (Fernández et al., 2010b) and cantaloupe melon pieces (Fernández et al., 2010a) were more gradual. Silver loaded absorbent pads in contact with raw beef reduced 1.4 log CFU/g APC, 0.7 log CFU/mL of LAB and 1.1 log CFU/g of *Pseudomonas* on average. A remarkable reduction of 3.0 to 3.5 log CFU/g was achieved for total aerobic bacteria and psychrotrophs in silver treated absorbent pads in contact with

melon pieces, which are slightly lower than those microorganisms found in food pads packed with breast chicken (4.1 and 3.7 log CFU/g) and ground chicken (3.2 and 3.8 log CFU/g). Considering the high cost and strict limitation of silver ions (UFSA, 2004) used in food, MC is a promising antimicrobial agent to be applied in absorbent pads. In addition, bacteriophage-cellulose absorbent pads were able to reduce 3 log CFU of *S. Typhimurium* in 2 days, but the incorporation and storage conditions for the pads were stringent to maintain the viability of the bacteriophage (Gouvêa et al., 2016; Lone et al., 2016). The technology of preparing and storing MC coated absorbent pads is straightforward, without special requirements for either moisture or temperature.

Microbial loads in meats

Initial populations of APC, *Pseudomonas*, and psychrotrophs in chicken breast were all around 3.5 log CFU/g, except LAB and Enterobacteriaceae, which were not detected on the first day (Figure 3.3). After 11 days of storage, bacterial populations of APC, *Pseudomonas*, and psychrotrophs in chicken breast with control absorbent pads increased to 9.0 log CFU/g. The populations of LAB and Enterobacteriaceae were 5.5 and 7.2 log CFU/g, respectively, on day 11. Microbial loads were close to results in other studies with the same product under similar storage conditions (Chouliara et al., 2007; Patsias et al., 2008; Radha krishnan et al., 2014). Microbial counts in chicken breasts with MC treated absorbent pads were slightly lower compared to those counts in the control group. In general, an average reduction of 0.4 log CFU/g for all the major microorganisms in chicken breast throughout the refrigerated storage period was achieved; however, there were no significant differences (Figure 3.4). Compared to the antimicrobial effects of raw beef loaded with silver immobilized absorbent pads (Fernández et al., 2010b), MC treated absorbent pads showed more remarkable antimicrobial activity during refrigerated storage.

Initial microbial loads in ground meat were slightly higher than those in chicken breast (Figure 3.5). Populations of APC in ground chicken with control absorbent pads were around 4.0 log CFU/g on day 1, while *Pseudomonas* and psychrotrophs were 3.8 log CFU/g. LAB and Enterobacteriaceae were 2.8 log CFU/g and 2.6 log CFU/g, respectively. Throughout the refrigerated storage times, aerobic plate counts, *Pseudomonas*, and psychrotrophs reached to approximately 9.0 log CFU/g. LAB and Enterobacteriaceae increased to 5.0 and 7.6 log CFU/g, respectively. Overall, microbial loads in ground chicken were higher than those in chicken breast, which may be the result of the grinding process and more surface area exposed to air. The trend of microbial growth of spoilage bacteria in ground chicken was close to the results from other studies (Chen et al., 2014; Irkin and Esmer, 2010) in the same product under similar conditions. Although statistical differences between the two meat samples were not revealed, an average of 0.2 log CFU/g reduction was shown in microbial loads in ground meat containing MC treated absorbent pads.

In conclusion, coating MC on absorbent pads in meat packaging is a promising technology to improve the quality and safety of chicken meat. MC loaded cellulose showed remarkable antimicrobial activities against *S. Typhimurium* and *C. jejuni* during *in vitro* testing. Cellulose incorporated with MC significantly reduced the spoilage microorganisms in absorbent pads used with chicken breast or ground chicken during storage. The microbial loads in the meat packed with MC coated pads were reduced without significant differences. Neither the color nor pH of chicken meat was negatively affected by MC treated absorbent pads.

Table 3.1. Microbial loads of *Salmonella* Typhimurium and *Campylobacter jejuni* after 1 h and 24 h contact time with MC coated absorbent pads at 4 °C.

MC concentration (g/g)	<i>Salmonella</i> Typhimurium		<i>Campylobacter jejuni</i>	
	1 h	24 h	1 h	24 h
0	7.51± 0.03 A ^a	7.61± 0.09 A	7.50± 0.06 A	7.67± 0.01 A
0.01%	7.47± 0.04 A	7.56± 0.09 A	7.42± 0.04 A	7.45± 0.04 B
0.1%	7.46± 0.07 A	7.05± 0.02 B	4.58± 0.50 B	3.29± 0.59 C
1%	3.90± 0.04 B	0.00± 0.00 C	0.00± 0.00 C	0.00± 0.00 D

Inocula: 7.66 log CFU/mL for *Salmonella* and 7.73 CFU/mL for *C. jejuni*.

^a Values with different letters in the same column mean differ significantly (P< 0.05)

Table 3.2. Color of chicken breast and ground chicken during storage in contact with MC treated absorbent pads. CIE L*, a*, and b* color values for chicken meats contacted with cellulose absorbent pads in the presence or absence of MC at 4 °C storage.

Storage time (day)	Meat type	Meat color (Mean ± SD)		
		L*	a*	b*
Day 1	Breast control	57.32 ± 0.38 A ^a	11.62 ± 0.57 A	11.81 ± 0.41 A
	Breast MC	56.85 ± 0.53 A	11.62 ± 0.80 A	14.06 ± 0.81 B
	Ground control	61.23 ± 0.82 C	14.74 ± 0.45 B	14.00 ± 0.42 B
	Ground MC	61.18 ± 0.68 C	15.95 ± 0.93 B	14.67 ± 0.59 B
Day 4	Breast control	58.24 ± 0.73 B	11.47 ± 0.84 A	12.00 ± 0.72 A
	Breast MC	57.31 ± 0.72 B	11.82 ± 0.55 A	14.45 ± 0.98 B
	Ground control	61.97 ± 1.02 C	14.87 ± 0.42 B	13.91 ± 0.56 B
	Ground MC	61.46 ± 0.34 C	15.98 ± 0.68 B	14.37 ± 0.50 B
Day 7	Breast control	58.64 ± 0.87 AB	12.08 ± 0.73 A	13.42 ± 0.39 AB
	Breast MC	57.60 ± 1.36 AB	12.56 ± 0.99 A	13.44 ± 0.91 AB
	Ground control	62.03 ± 0.77 C	15.41 ± 0.69 B	14.35 ± 0.41 B
	Ground MC	61.14 ± 1.39 C	15.05 ± 0.50 B	13.29 ± 0.59 AB
Day 11	Breast control	59.02 ± 0.72 A	11.42 ± 0.84 A	12.73 ± 0.45 AB
	Breast MC	58.99 ± 1.04 A	12.59 ± 0.72 A	12.38 ± 2.51 AB
	Ground control	62.67 ± 0.80 C	14.80 ± 0.48 BC	14.06 ± 0.25 B
	Ground MC	61.83 ± 0.50 C	14.60 ± 0.33 BC	12.88 ± 0.90 AB

^a Values with different letters in the same column mean differ significantly (P< 0.05)

Table 3.3. The pH values of chicken breasts and ground chickens contacted with MC treated absorbent pads throughout refrigerated storage

	Chicken breast control	Chicken breast with MC	Ground chicken control	Ground chicken with MC
Day 1	5.71± 0.01 A ^a	5.70± 0.02 A	5.92± 0.01 A	5.95± 0.01 A
Day 4	5.71± 0.01 A	5.73± 0.02 A	5.95± 0.01 B	5.92± 0.01 A
Day 7	5.78± 0.02 A	5.73± 0.03 A	5.95± 0.00 A	5.91± 0.01 A
Day 11	6.27± 0.03 B	6.21± 0.01 B	6.36± 0.01 B	6.23± 0.03 B

^a Values with different letters in the same column mean differ significantly (P< 0.05)

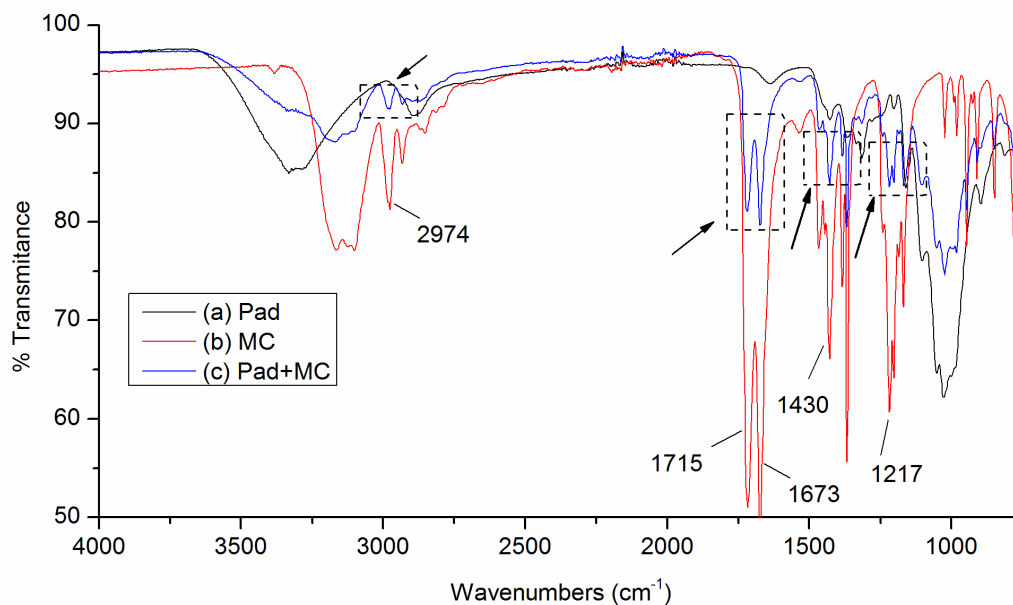


Figure 3.1. Fourier transform infrared spectroscopy (FTIR) chromatograms of MC treated cellulose materials: (a) absorbent pad, (b) MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone), (c) absorbent pad modified with MC.

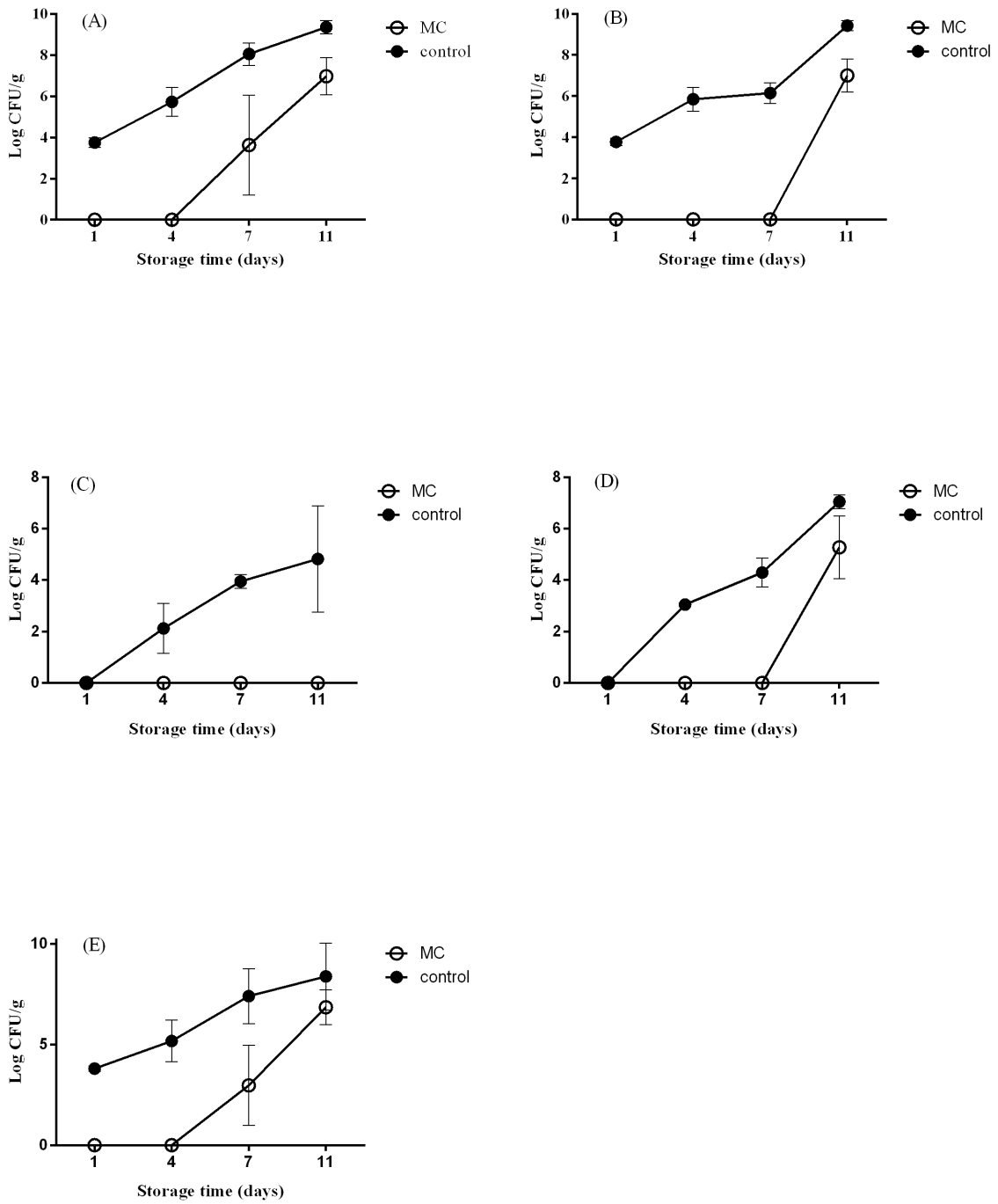


Figure 3.2. Microbial loads in absorbent pads in contact with chicken breast meat throughout 11 days storage at 4 °C in the presence or absence (control) of MC. A) Aerobic plate counts, B) *Pseudomonas* spp., C) Lactic acid bacteria counts, D) Enterobacteriaceae, and E)

Psychrotrophs. Each value is the mean of four replicates, and vertical bars represent standard deviations.

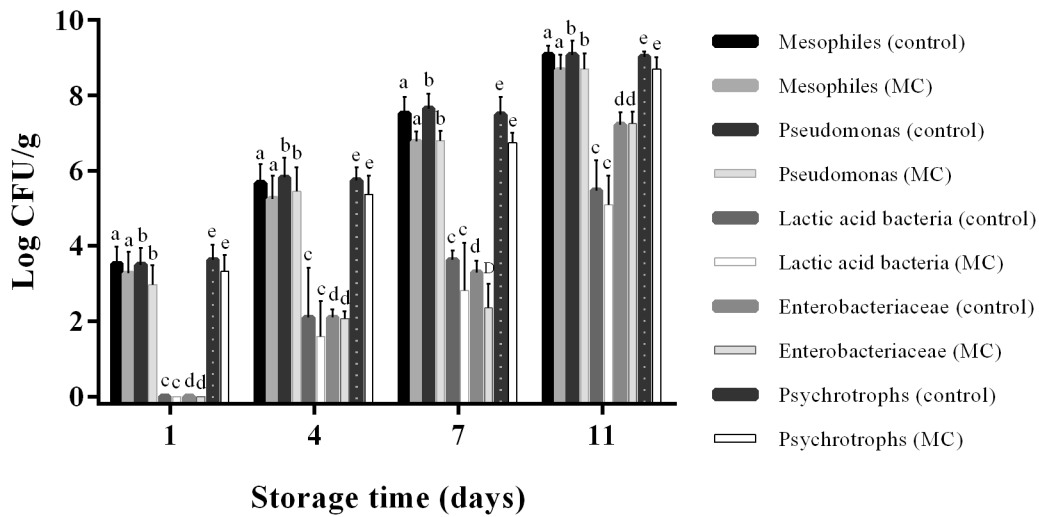


Figure 3.3. Microbial loads of five major spoilage microorganisms in chicken breast meat over 11 days storage at 4 °C in the presence or absence (control) of MC. Each value is the mean of four replicates, and vertical bars represent standard deviations. Different letters in the same sampling day indicate significant differences of the same microorganism between the control and MC treated samples at the 95% confidence level.

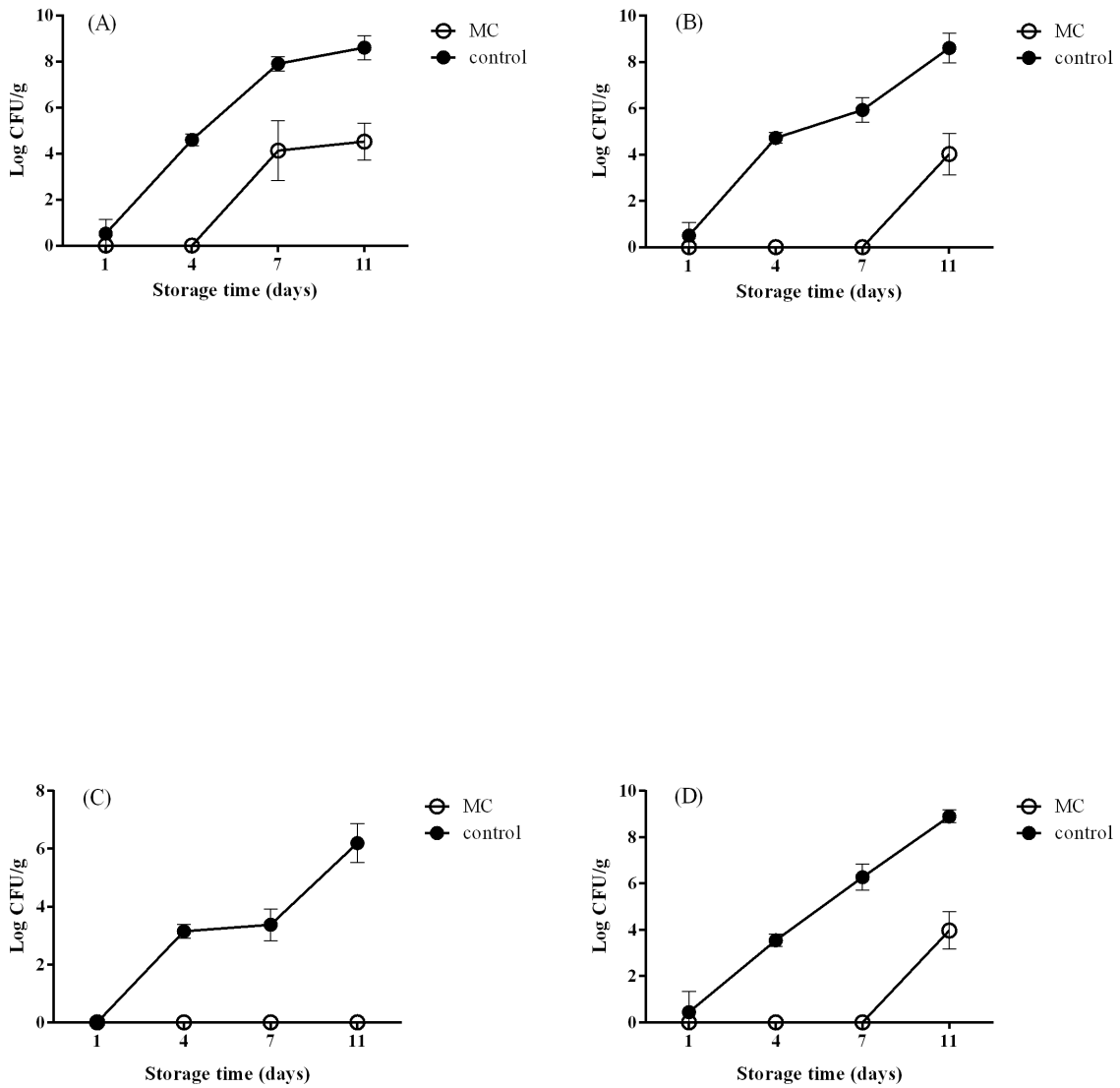


Figure 3.4. Microbial loads in absorbent pads in contact with ground chicken throughout 11 days storage at 4 °C in the presence or absence (control) of MC. A) Aerobic plate counts, B) *Pseudomonas* spp., C) Enterobacteriaceae, and D) Psychrotrophs. Each value is the mean of four replicates, and vertical bars represent standard deviations.

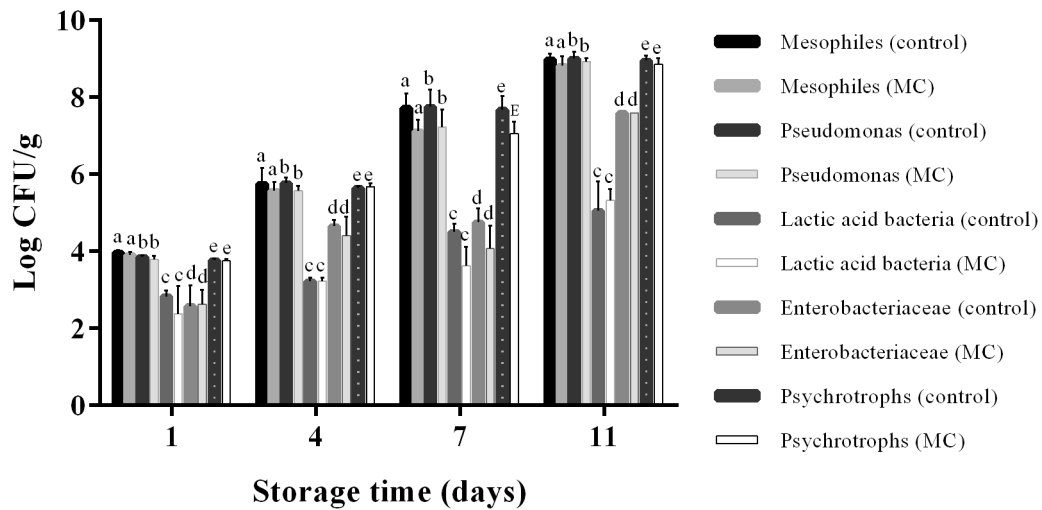


Figure 3.5. Microbial loads of five major spoilage microorganisms in ground chicken over 11 days storage at 4 °C in the presence or absence (control) of MC. Each value is the mean of four replicates, and vertical bars represent standard deviations. Different letters in the same sampling day indicate significant differences of the same microorganism between the control and MC treated samples at the 95% confidence level.

IV. N-halamine incorporated antimicrobial nonwoven fabrics for use against avian influenza H1N1 virus

Abstract

Airborne pathogens are one of the most common approaches leading to poultry diseases. Prevention of microbial pathogens such as avian influenza (AI) virus from entering the chicken hatchery house is critical for reducing the spread and transmission of viruses. Many studies have investigated the incorporation of antimicrobial agents into air filters to prevent viruses from entering into the indoor environment. N-halamines are one of the most effective antimicrobial materials and have been applied on many surfaces against a broad spectrum of microorganisms. In this study, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC, a member of N-halamine) was coated on nonwoven fabrics to render the antimicrobial activity against AI virus. Results showed that MC exhibited a potent antiviral activity either in the suspension or in the air. Higher concentration of MC was able to completely inactivate AI virus and disrupt their RNA, preventing them from being detected by real time reverse transcriptase-polymerase chain reaction (rRT-PCR) assay. The coating fabrics remarkably reduced the AI virus on the MC treated fabrics in a short time. Furthermore, in viral aerosol challenge test, the aerosolized AI virus was completely inactivated when they went through the filters coated with MC compound. In addition, the active chlorine in the MC compound is stable and the coating process of these materials is straightforward and inexpensive. Therefore, these results provide a novel approach to reduce the airborne pathogens in the environment for poultry production.

Keywords

Avian influenza viruses, bioaerosols, N-halamines, air filters, antiviral activity

Introduction

The avian influenza (AI) virus has caused severe poultry diseases around the world. The outbreaks of both high pathogenic and low pathogenic AI virus led to significant economic losses including direct cost such as depopulation, disinfection, indemnities paid for the birds, etc. and indirect cost such as loss in poultry exports, income loss and increased consumer costs (Swayne and Halvorson, 2008). Although it is rarely happened, AI virus has exhibited interspecies transmission from poultry to humans and caused fatal illness in humans such as H5N1 virus outbreaks occurred in Asian countries (Chan, 2002; Claas et al., 1998; Li et al., 2004; Swayne and Halvorson, 2008).

The spread of AI virus in poultry farms may easily occur by direct contact between the infected and susceptible birds, or through indirect contact including aerosol droplets. Therefore, biosecurity actions are important to prevent AI virus infection in the poultry flocks. Many studies reported that major disinfectants such as household bleach could effectively inactivate AI virus if used properly (Prince et al., 2001). However, when the virus was shed in feces or feed with a significant amount of organic matters, most of their antiviral activities were impaired. Although the viruses in the poultry are eliminated at the beginning, the later introduced viruses from outside of farm will continue to spread and transmit. AI virus can be introduced into farms by various routes including infected birds, water, clothing, delivery vehicles, etc. (Swayne and Halvorson, 2008). Therefore, the prevention of AI virus into poultry farms is a critical avenue to eliminate or reduce the AI disease.

The use of individual respiratory protective devices such as N95 filtering facepiece respirator (FFR) has been proven to interrupt or reduce the spread of respiratory viruses (Jefferson et al., 2008). The nonwoven fabrics layer materials were the key components of FFR for the prevention properties. Therefore, this nonwoven layers from the facemask could be used in the ventilation systems of the chicken house or chicken hatchery to prevent the

transmission of bioaerosols pathogens from outside of broiler houses. The filter can only block viruses from entering the broiler houses without disinfection function, and thus may pose a health risk with incorrect handling or disposal. Airborne viruses and pathogens accumulated on the surface of the filters may survive for weeks or even months, and then contaminate the birds and workers if they are not handled and disposed correctly. Therefore, the development of air filter with antimicrobial function is necessary.

Considering the indoor air quality and safety, antimicrobial air filtration technology has been widely studied to control the bioaerosols. Previous research have investigated various antimicrobial agents, including silver (Sharma et al., 2009; Yoon et al., 2008), silver nitrate (Miaśkiewicz-Peska and Łebkowska, 2011), copper, carbon nanotubes (CNTs) (Jung et al., 2011a), N-halamines (Demir et al., 2015; Qian and Sun, 2003; Zhu et al., 2012) and natural antimicrobials such as silk sericin (Magaraphan et al., 2003), plant extracts (Choi et al., 2015; Jung et al., 2013, 2011b), and chitosan (Desai et al., 2009). However, most of these materials require sophisticated process to manufacture, and did not present a significant antimicrobial activity. Most of them were investigated on antimicrobial activity against aerosolized bacteria, while few materials mentioned above were evaluated on antiviral properties, especially on AI virus. Copper based antimicrobials have been mostly reported for their antiviral effects. Borkow et al. (2007) showed that copper oxide based polypropylene filters did not cause a large reduction of human H3N2 virus. The same research group (Borkow et al., 2010) introduced the copper oxide into respiratory face mask containing polypropylene layer, and the human H1N1 and avian H9N2 virus were not recovered after 30 min of aerosolized viruses introduced to the mask. For these copper related coating textiles, the procedures of synthesis were complicated and required some high risk chemicals such as formaldehyde (Borkow and Gabbay, 2004). To solve these problems, there is a need to find

materials that can inactivate AI virus rapidly and effectively, and can be manufactured in a simple and inexpensive technology.

N-halamine is group of compounds containing one or more nitrogen-halogen covalent bonds. N-halamine is increasingly drawing public's attention due to its superior antimicrobial efficiency, and has been systematically reviewed intensively (Dong et al., 2017). Many compounds of this group have been reported possessing potent antimicrobial activity (Hui and Debiemme-Chouvy, 2013). The antimicrobial mechanism of N-halamines involves the direct transfer of oxidative halogen to a cell after contact. The transferred halogen oxidizes the amino acids in the cell membrane and inactivates the microorganism. Molecules of N-halamines are stable and do not dissociate free oxidative halogen until contact with microorganisms (Kenawy et al., 2007; Worley et al., 1988). Among these N-halamine compounds, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC, Figure 1.2) has been proven to have high antimicrobial activity and can be used to treat edible eggs, fish tank, water and equipment surfaces in chicken houses (Lauten et al., 1992; Ren, 2015; Worley et al., 1992). In addition, MC showed potent and long-lasting antibacterial effect when introduced to nonwoven fabrics (Demir et al., 2015).

In this study, we coated nonwoven fabrics with MC to test the antiviral activity against AI H1N1 virus in suspension, and the aerosolized virus that went through the modified air filters. The modes of action against AI H1N1 virus, and chlorine loadings of MC have been determined.

Materials and Methods

MC treated filter

US National Institute for Occupational Safety and Health (NIOSH) certified N95 respirator (3M, 1860) was used as MC carrier materials in this study. NIOSH certified N95

respirators are at least 95% efficient at filtering particles with the size of around 300 nm. The model contained three layers: a) external layer A made of polypropylene, b) internal layer B made of nonwoven fabrics, and c) made of polyester. The internal nonwoven fabrics layer is the key component designed to prevent the passage of pathogens or particles through the mask based on its pore size. Therefore, we incorporated MC compound into layer B. The materials were cut into swatches with a diameter of 2.54 cm. The photos of MC coated air filter swatches are shown in Figure 4.1. The swatches were soaked into MC solutions at 0.1 and 1% (w/v) concentrations with 95% ethanol for 1 min, and then air dried at room temperature. Internal nonwoven fabrics layer from mask treated with 95% ethanol only served as the control. The swatches were sterilized by UV light overnight before coating.

Active chlorine loadings

The coating efficiency of MC on these treated nonwoven fabrics was verified by Fourier Transform Infrared Spectroscopy (FT-IR) (Nicolet 6700 FT-IR Spectrometer, Thermo Scientific, Madison, WI). Since the hydrogen bonded to N-halamines provides the antimicrobial activity, active chlorine loadings in the MC coated swatches were measured by modified iodometric/thiosulfate titration method (Worley et al., 2005).

The virus propagation

The avian influenza (AI) H1N1 virus (A/blue-winged teal/AL/167/2007) isolated from hunter-killed waterfowl from a wildlife refuge in Alabama (Dormitorio et al., 2009) was used in this study. The virus was propagated in allantoic fluid (AF) of 9-day-old specific pathogen free (SPF) embryonated chicken eggs. After 48 h post-inoculation, the AF containing the viruses was harvested and stored at -80 °C until use. Each time before use, the 50% embryo infectious dose per mL (EID₅₀/mL) titer of virus was determined based on the Spearman-Kärber methods (Reed and Muench, 1938). Stock virus was thawed quickly and

diluted to the desired concentration with phosphate buffer saline (PBS), and kept on ice during use.

In vitro antiviral evaluation of MC and sodium hypochlorite

MC and sodium hypochlorite working solutions were prepared. The test method was modified from Zou et al. (2013). MC stock solution was diluted with sterile distilled water to achieve 0.5%, 0.05% and 0.005% concentrations, producing 1000, 100 and 10 ppm of oxidative chlorine, respectively. Each diluted solution of 450 μL was mixed with 50 μL fluid containing $10^{7.5}$ EID₅₀/mL AI virus, and then 4.5 mL of sodium thiosulfate were added to each mixture to terminate the disinfection after 5, 10, 30 and 60 min of treatment. An aliquot of 100 μL of solution was inoculated into six embryonated chicken eggs to determine the recovery of AI virus, and the remaining suspension was used for RNA extraction.

Antiviral activity of MC coated air filters

A modified “sandwich test” method was used to determine the antiviral activity of MC coated nonwoven filters (Worley et al., 2005). An aliquot of 100 μL virus suspension containing $10^{7.5}$ EID₅₀/mL AI virus was added in the center of fabrics coupon, and a second identical coupon was placed on the top. A sterile weight was placed on top of it to ensure a good contact of the two samples. After predetermined contact times, oxidative chlorine on swatches were quenched by 5 mL of 0.02 N sodium thiosulfate solutions to terminate the further disinfection. Viruses were washed off by vigorously vortexing for 10 min. The solutions were filtered through 0.45 μm filter paper, and the control group was diluted from 10^{-1} to 10^{-4} with PBS to determine the titer of recovery viruses. All the diluted control groups and MC treated solutions were individually injected to six embryonated chicken eggs to determine the virus survival.

Challenge with aerosolized viruses

Tested fabrics were challenged with aerosolized AI H1N1 viruses based on the modified ASTM Method F 2101. 01 “Standard Test Methods for Evaluating the Bacterial Filtration Efficiency of Medical Face Mask Materials, Using a Biological Aerosol of *Staphylococcus aureus*”. Before each test, swatches were preconditioned in 25 °C, and a relative humidity of 85% for 4 h. The viral aerosol challenge test apparatus scheme is illustrated in Figure 4.2. Air carried aerosolized viruses by using a nebulizer at airflow rate of 500 mL/min. Airflow was past through the MC coated and uncoated fabric samples which were clamped in the chamber. There was about 31.4 mL of avian influenza virus solutions were aerosolized after 6 h blowing. The air flow was terminated after 6 h, and the system was settle down for 10 min. Then nonwoven coupons were aseptically removed and transferred to 5 mL of 0.02 N sodium thiosulfate solutions to terminate the further disinfection action. After 10 min of vortexing at a full speed, the suspension with viruses was filtered with 0.45 um filter paper, and injected into six 9-day-old embryonated eggs. For the control, the filtered culture was diluted from 10^{-1} to 10^{-3} , and all the dilutions were injected into embryonated eggs as well. The recovery of AI virus in infectious embryos was determined using the method as described in the following section.

Recovery of AI virus in embryonated eggs

The filtered solutions of the treated samples and the controls were injected to 9-day-old embryonated chicken eggs by allantoic route to determine the survival of AI virus. Each solution at 0.1 mL was inoculated into six replicated embryonated eggs, and then the eggs were incubated at 37.5 °C, 65% humidity for up to 5 days. During incubation, eggs were candled every day to check the viability of embryos. The deaths within the first 24 h after inoculation were considered as non-specific and discarded. At the end of incubation, all the eggs were placed into 4 °C refrigerator overnight. Then, AFs from those eggs were harvested

and checked the presence of live virus by using standard hemagglutination assay (HA test) with 0.5% chicken red blood cells (Alexander, 1997).

Negative controls were eggs without any treatments. Toxicity control of the chemicals was the embryos injected with all the media used except viruses to test the sterility and toxicity of the chemicals. Each control group has 6 replicates. Virus recovery controls were the eggs inoculated with diluted avian influenza virus (from 10^0 to 10^{-4} dilution) with PBS as mentioned above.

All media and equipment including the glassware of apparatus were sterilized by autoclaving. All tests were performed under a biological safety cabinet. Cabinet and apparatus were disinfected using 70% ethanol and followed by UV radiation overnight prior to and after the experiment.

Real-Time reverse transcriptase-polymerase chain reaction (rRT-PCR)

Real time reverse transcriptase-polymerase chain reaction (rRT-PCR) was used to evaluate the ability of MC and sodium hypochlorite to inactivate AI virus. The remaining virus culture from *in vitro* test was used for RNA extraction and rRT-PCR. Type A specific influenza test targeted to the influenza matrix gene were employed based on method developed by Spackman (2002) with some modifications. This protocol targets the matrix gene of AI virus. The sensitivity of this test has been shown to be as low as 10^{-1} 50% egg infectious doses (Spackman et al., 2002).

Results

Incorporation of MC with nonwoven fabrics

The coating efficiency of MC into the nonwoven fabrics was confirmed by Fourier transform infrared (FT-IR) characterization. The FT-IR spectrum of MC compound contained

prominent peaks at 1,673, and 1,715 cm^{-1} , which were not shown in the uncoated nonwoven fabrics of air filters (Figure 4.3). Compared with spectra of uncoated fabrics and MC treated samples, the specific peaks of MC in 1% MC treated samples indicated that MC was coated on fabric successfully. Chlorine contents in MC treated filters were 0.2%, and 1.9% (wt/wt) for 0.1% and 1% MC treated samples, respectively.

In vitro antiviral activity test of MC

Results of the antiviral *in vitro* evaluation showed that MC at 0.5% and 0.05% concentrations were able to effectively inactivate AI virus in a short exposure time. The viruses were inactivated from the initial dose of $10^{7.5}$ EID₅₀/mL to undetectable levels after 30 min contact with 0.5% MC and 2 h with 0.05% MC solutions (Table 4.1).

Mode of action

To further evaluate the antiviral mechanism of MC, the commonly used disinfectant of sodium hypochlorite was used as the reference compound. Titration results revealed that MC at 0.5% concentration had the same chlorine content with that in 0.2% sodium hypochlorite, which was at 1000 ppm chlorine level. These two disinfectants at the same active chlorine content showed the same antiviral activity. Within one hour exposure, virus was not recovered in all samples of undiluted and 10 times diluted solutions using the standard egg inoculation procedures. When the 0.5% MC or 0.2% sodium hypochlorite were diluted by 100 times, they were not able to inactivate AI virus.

Since different disinfectants have different modes of action for inactivation of virus, real time reverse transcriptase-polymerase chain reaction (rRT-PCR) test was used to determine if the RNA of AI virus was disrupted by MC or sodium hypochlorite in this study (Table 4.2). Results displayed that the influenza virus RNA was not detected when treated with the undiluted solutions of either MC or sodium hypochlorite. However, when viruses

were in contact with 10 times or 100 times diluted chemicals, the RNA was identified using rRT-PCR test.

Antiviral activity in a sandwich test

Antiviral activity of MC coated air filters was determined by using the sandwich contact test. Virus recovered from challenged uncoated nonwoven fabrics swatches caused 14 out of 24 embryos infected after 1 h contact time, which was determined as $10^{4.5}$ EID₅₀/swatch. These reductions from inoculum were due to adherence of virus to the materials, not inactivating the AI virus. Antiviral results of 0.1% and 1% MC treated fabrics at different contact time intervals were summarized in Table 4.3.

Both concentrations of the coated fabrics exhibited a significant viral reduction against AI virus. Coating fabrics with 1% MC (CI% = 1.90 w/w) inactivated the entire inoculated AI virus within 5 min of contact time. On the 0.1% MC (CI% = 0.20 w/w) treated fabrics, it showed lower inactivation rate, but still had effective antiviral activity against AI virus in 10 min contact (4/6). More than half of embryos survived at 1 h treatment.

Antiviral activity in viral aerosol challenge test

In order to further evaluate the application of these filters in field, the antiviral activities of MC coated and uncoated fabrics against AI virus were determined by a viral aerosol challenge test. The results of fabrics loaded with different MC concentrations against AI virus are shown in Table 4.4 and 4.5, respectively. Untreated fabric samples were served as the controls. It showed that both concentrations of MC coated fabrics had remarkable effectiveness in inactivating aerosolized AI virus. The experiments were performed at different times, but the average virus titer recovered from the control fabrics was stable which was approximate $10^{3.5}$ EID₅₀/sample after 6 h of nebulization. Even with lower MC coating materials (CI% = 0.20, Table 4), AI virus was still completely inactivated in both trials.

For the purpose of capturing virus in bioaerosols passed through the control and coated fabrics, two layers of the untreated nonwoven fabrics were individually put behind each of the test samples. Results from Table 3 and Table 4 revealed that no viable AI virus were recovered from the second filter materials, indicating that the coating fabrics were able to capture all the aerosolized viruses, and effectively in inactivating all the virus attached to the MC coating air filter materials.

Discussion

Coating efficiency

The results of FT-IR spectrum, titration data from the MC coating fabrics, and their antiviral data showed that MC was successful coated on fabric samples. In addition, MC was adsorbed on the nonwoven fabrics rather than chemically bonded to the samples, therefore it would not alter the structure of the coated fabrics and MC cannot be removed mechanically.

Antiviral activity *in vitro* test and antiviral mechanism

The results of antiviral activity *in vitro* test indicated that MC was effective in inactivating AI virus. AI virus is type A virus, which could be easily inactivated by major disinfectants with proper application (Prince et al., 2001). Previous studies revealed that 75% ethanol, 0.5% household bleach (sodium hypochlorite), 0.075% calcium hypochlorite and 0.5% Virkon[®]-S (dipotassium peroxodisulphate) significantly inactivated avian influenza (H7N9) virus within short period of exposure time (Jang et al., 2014; Lombardi et al., 2008; Zou et al., 2013). Sodium hypochlorite as a reference compound in this research showed that it had similar antiviral activity with other studies (Suarez et al., 2003; Wanaratana et al., 2010). Results of mechanisms tests performed in this study (Table 2) were comparable to that in a study conducted by Suarez (2003), which household bleach at a higher concentration was able to disinfect AI virus to undetectable level by rRT-PCR detection, but it was not

happened at lower house bleach levels. It explained that the chlorine of sodium hypochlorite needs to interrupt a specific site on RNA to prevent the detection by rRT-PCR method; however, it can disrupt any site of the virus to cause the inactivation (Suarez et al., 2003). It is the reason that lower concentration of sodium hypochlorite was able to inactivate AI virus, but only high levels of solution could reduce the virus to undetectable level by rRT-PCR. Since MC showed the same results on viral inactivation and RNA disruption with sodium hypochlorite, and both of them have the active chlorine as the antibacterial group. Therefore, MC is assumed that it had the same mode of action of sodium hypochlorite. This mechanism demonstrated the phenomenon that the concentration dependent for inactivating AI virus with these two disinfectants was lower than that to degrade the RNA.

Antiviral activity of MC coated fabrics

Sandwich test revealed that no AI virus was detected from 1% MC treated air filter swatches after 5 min contact time. Lower MC loaded fabrics were also able to effectively inactivate the contacted viruses. Although currently there is no study available on the investigation of either MC compound or MC coated fabrics against viruses, several studies demonstrated the effectiveness of antimicrobial activity against bacterial pathogens of MC and its coated materials. Demir et al. (2015) revealed that MC coated nonwoven fabrics inactivated all the inoculated *Staphylococcus aureus* in 5 min and *E. coli* O157:H7 within 10 min. Results from our another study showed that MC treated cellulose effectively reduced the food spoilage related bacteria in absorbent pads for meat packaging (T. Ren, M. Qiao, T.S. Huang, and J. Weese, submitted for publication).

Viral aerosol challenge test

Currently, there is no study investigated the antiviral effects of N-halamine against AI virus, but several researches were tested the antibacterial efficiency. Nonwoven fabrics

coated with 0.1% MC (Cl%= 0.20, w/w) completely inactivated the entire attached AI virus. This finding is in agreement with a previous study showing that chlorine concentration of MC coated fabrics at 0.47% (wt/wt) reduced the aerosolized *S. aureus* and *E. coli* O157: H7 to undetectable levels (3-5 log reduction). Many researches were investigated on antimicrobial coating fabrics for air filters, but most of the studies were related to antibacterial activity and only quite few were on antiviral efficacy. Compared with current investigation of antiviral coatings on fabrics, MC coating showed a greater inactivation against AI virus. When 5% (wt/wt) copper oxide-based filters was challenged with $10^{7.5}$ CCID₅₀ influenza A virus, there were still $10^{6.7}$ CCID₅₀ of viruses recovered from the treated filters, which it did not cause a large reduction in the viral infectivity titers (Borkow et al., 2007). However, the same group reported that avian H9N2 virus was not recovered from the facemasks containing copper oxide particles after challenged with aerosolized viruses for 30 min (Borkow et al., 2010). Another study working on copper ions incorporated in zeolite-textile materials showed that more than 3.5 log titer reduction against AI H5N3 virus in embryonated eggs (Imai et al., 2012). As mentioned in the previous section, most of coating procedures for these materials are complicated and expensive. The shelf life of these materials was not reported either in current research.

All the embryos injected with MC were alive as the negative controls (chemical free) after 5 days post incubation, which indicated that MC did not have negative effect on embryos. In addition, the dissociation constant of chlorine in MC compound is lower than 10^{-11} , and thus it does not produce or emit chlorine ions easily. The shelf life of MC coatings was evaluated by Demir et teal. (2015), and results showed that the oxidative chlorine on coating fabrics were stable for at least six months in dark storage. Therefore, the treated air filters can be safe for use in hatchery house or chicken house to create a clean environment. Furthermore, since the nonwoven fabrics used in this study were the internal materials in N95

face respirators, which would not be directly contacted with the skin of consumer, MC could be applied in this face mask to prevent the transmission of viruses and other pathogens.

In summary, nonwoven fabrics were successfully coated with 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC) by using a simple pad-dry technique. The coating efficacy and the active chlorine content on coated fabrics were determined. MC was able to inactivate AI virus effectively in suspension, and higher MC concentration was able to disrupt the RNA of AI virus and prevent it from being detected by rRT-PCR test. Air filter fabrics coated with MC were remarkably reduced the virus in suspension or aerosolized virus attached to the fabrics. Since MC coated fabrics possessed potent antibacterial and antiviral properties, face respirator and other air filtration devices can be applied with MC to prevent the spread and transmission of airborne pathogens. It should be pointed out that the production of these coated materials is simple and straightforward, and do not add any significant costs to the price of the uncoated fabrics.

Table 4.1. The antiviral efficacy of MC against avian influenza A (H1N1) virus.

MC concentration	Contact time (min)			
	5	30	60	120
0	NT ^a	NT	NT	6/6
0.005%	6/6 ^b	6/6	6/6	6/6
0.05%	6/6	6/6	1/6	0/6
0.5%	6/6	0/6	0/6	0/6

^a NT= not tested

^b Infected embryo(s)/total inoculated embryos

Table 4.2. The effect of MC on inactivating avian influenza A (H1N1) virus after 1 hour contact and its detection by rRT-PCR.

Concentration/dilution	1/1	1/10	1/100
0.5% MC	(-) ^a /(-) ^b	(-)/(+)	(+)/(+)
0.2% Sodium hypochlorite	(-)/(-)	(-)/(+)	(+)/(+)

^a Negative or positive by virus isolation

^b Negative or positive for rRT-PCR

Table 4.3. The effect of MC-coated nonwoven fabric filters on inactivating avian influenza A (H1N1) virus.

MC concentration	Contact time (min)			
	5	10	30	60
0	NT ^a	NT	NT	14/24 ^b
0.1%	6/6 ^c	4/6	4/6	2/6
1%	0/6	0/6	0/6	0/6

^a NT= not tested

^b The recovery of avian influenza viruses from the control was $10^{4.5}$ EID₅₀/swatch

^c Infected embryo(s)/total inoculated embryos

Table 4.4. Antiviral efficacy of 0.1% MC treated nonwoven fabrics against avian influenza A (H1N1) virus bioaerosols.

Samples	Exposure time (h)	Infected embryonated eggs/total inoculated eggs	
		Trial 1	Trial 2
Control	6	14/24 ^c	13/24
MC treated CI ⁺ %= 0.20	6	0/6 ^d	0/6
Filter ^a	6	0/6	0/6
Filter ^b	6	0/6	0/6

^aThe Millipore sterile filter material behind control fabrics.

^bThe Millipore sterile filter material behind MC-coated fabrics.

^cThe recovery of avian influenza viruses from the control was 10^{3.5} EID₅₀/swatch

^d Infected embryo(s)/total inoculated embryos

Table 4.5. Antiviral efficacy of 1% MC treated nonwoven fabrics against avian influenza A (H1N1) virus bioaerosols.

Samples	Exposure time (h)	Infected embryonated eggs/total inoculated eggs	
		Trial 1	Trial 2
Control	6	14/24 ^c	15/24
MC treated CI ⁺ %= 1.90	6	0/6 ^d	0/6
Filter ^a	6	0/6	0/6
Filter ^b	6	0/6	0/6

^aThe Millipore sterile filter material behind control fabrics.

^bThe Millipore sterile filter material behind MC-coated fabrics.

^cThe recovery of avian influenza viruses from the control was 10^{3.5} EID₅₀/swatch

^d Infected embryo(s)/total inoculated embryos

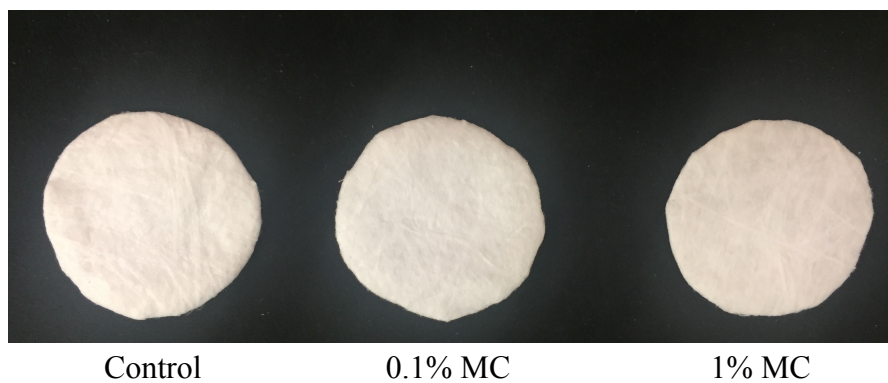


Figure 4.1. Nonwoven fabrics coated with 0, 0.1% and 1% MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone)

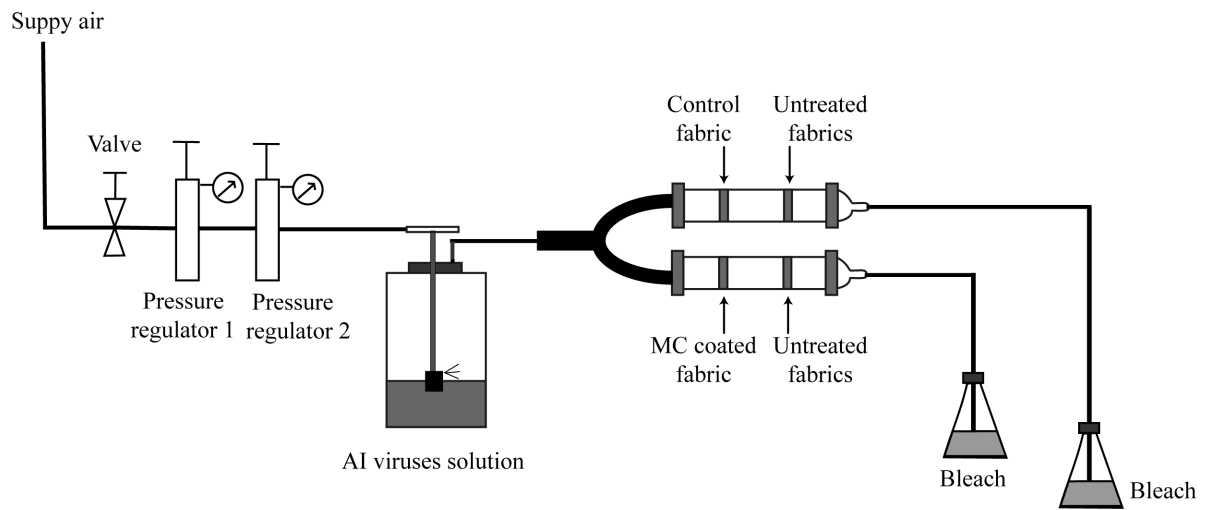


Figure 4.2. Schematic diagram of treated nonwoven filters for antiviral activity tests against aerosolized avian influenza (H1N1) viruses.

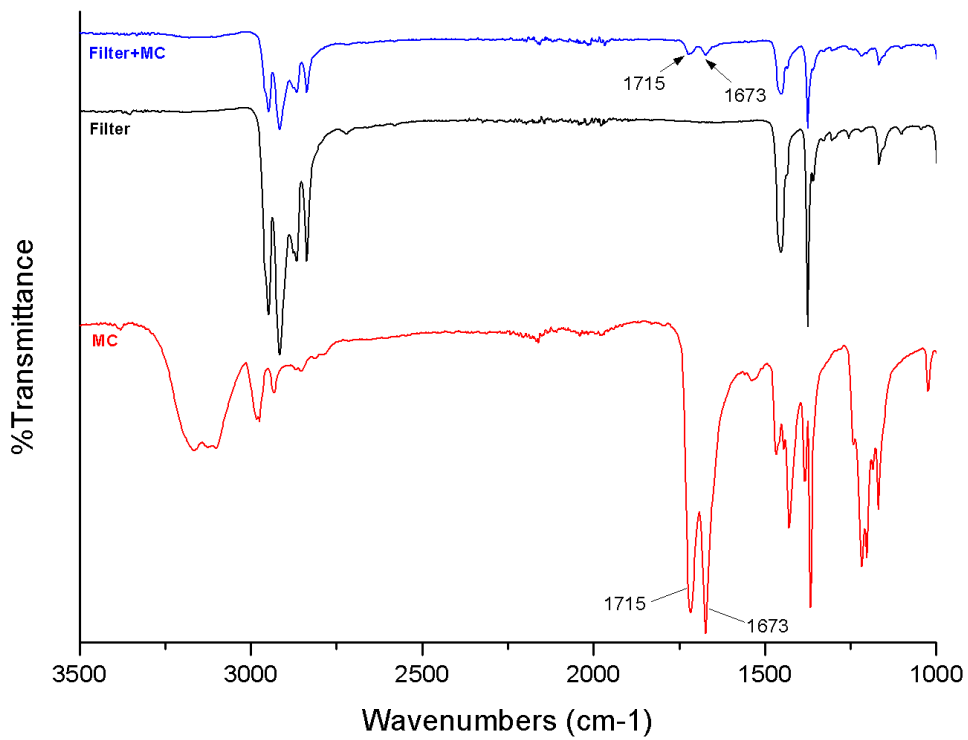


Figure 4.3. Fourier transform infrared spectroscopy (FTIR) chromatograms of MC treated nonwoven fabrics: (black) nonwoven fabrics, (red) MC, (blue) nonwoven fabrics treated with MC.

V. Conclusion

N-halamine compound, 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC), was successfully coated on the absorbent food pads. The coating of MC was using a simple pad-dry technique, and the procedure is inexpensive. The effects of coating on biocidal activity and physical properties of meat samples were investigated. Both coated absorbent pads in chicken and beef meat revealed significant antimicrobial efficacy against foodborne pathogens and spoilage related microorganisms. In addition, the MC coatings did not have negative impact on the quality (pH and color) of the meat.

When the air filtration materials were coated with MC compound using the same pad-dry technique, it showed remarkable antiviral activity against avian influenza viruses either in suspension or aerosolized virus deposited on the fabrics. Higher MC was able to disrupt the RNAs of AI virus and reduced them to undetectable limit by rRT-PCR method. The MC coatings did not significantly affect the air permeability of the air filters. Since MC coated fabrics possessed potent antibacterial and antiviral properties, face respirator and other air filtration devices can also be applied with MC to prevent the spread and transmission of airborne pathogens.

Considered the superior antimicrobial activity of MC coated materials, the straightforward and inexpensive coating procedure, long stability, and no release of any chlorine gas, they have the potential to be applied as antimicrobial materials in the food and poultry industry.

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