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ANTIMICROBIAL EFFICACY OF EDIBLE SOY PROTEIN ISOLATE FILMS AND COATINGS INCORPORATED WITH HOP ETHANOL EXTRACT AND THE INFLUENCE ON SHELF-LIFE AND SENSORY ATTRIBUTES OF BOLOGNA

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ANTIMICROBIAL EFFICACY OF EDIBLE SOY PROTEIN ISOLATE FILMS AND COATINGS INCORPORATED WITH HOP ETHANOL EXTRACT AND THE INFLUENCE ON SHELF-LIFE AND SENSORY ATTRIBUTES OF BOLOGNA

DISESSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Department of Animal and Food Sciences at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

ANTIMICROBIAL EFFICACY OF EDIBLE SOY PROTEIN ISOLATE FILMS AND COATINGS INCORPORATED WITH HOP ETHANOL EXTRACT AND THE INFLUENCE ON SHELF-LIFE AND SENSORY ATTRIBUTES OF BOLOGNA

There is demand for improved security of refrigerated ready-to-eat meats. Antimicrobial edible films and coatings could function as an added barrier against post-processing contamination. Hops and hop extracts are known for their antimicrobial efficacy which is attributed to key antimicrobial components including humulones, lupulones, xanthohumol and various terpenoids. Yet, hop ethanol extract has not been studied as an antimicrobial to incorporate into edible protein films and/or coatings. The overall objective of this research was to evaluate hop ethanol extract as an antimicrobial agent incorporated into edible soy protein isolate (SPI) films and coatings, and the influence on the shelf-life and sensory attributes of bologna.

Hop ethanol extract was examined for minimum inhibitory concentration before the extract was incorporated into a 6% SPI solution at 0, 10, and 20% levels to determine antimicrobial efficacy as a cast film and simulated coating via zone of inhibition against *Listeria monocytogenes* strains ATCC 4644, UKADL and ATCC 49594. The results showed that hop ethanol extract alone was inhibitory of all three strains. Moreover, the hop ethanol extract, when incorporated at 10 and 20% (v/v) into edible soy protein isolate (SPI) films and simulated coatings, exhibited antimicrobial action against all three *L. monocytogenes* strains. Key antimicrobial components, as mentioned above, were identified in the hop ethanol extract via mass spectrometry.

The SPI with 10% incorporated hop ethanol extract (SPI+10%hop) antimicrobial coating was applied to bologna, prepared in lab without *L. monocytogenes* inhibitors, where it exhibited a significant (*P* ≤ 0.05) bacteriostatic effect against strain ATCC 4644. The SPI+10% hop coating was then applied to a commercial bologna to examine effects on shelf-life and sensory attributes. Significant differences (*P* ≤ 0.05) were found in instrumental red and yellow colors, however not in sensory color. There was no significant difference (*P* > 0.05) found in measured lipid oxidation between the bologna with no coating, SPI coating or SPI+10%hop coating. The incorporation of hop did exhibit a slightly bitter taste. Overall, these findings indicate that the SPI+10%hop antimicrobial coating functioned as an inhibitor of *L. monocytogenes* while producing minimal effects on shelf-life and sensory attributes of bologna.

KEYWORDS: Antimicrobial, edible film coating, ready-to-eat, soy protein, hops

Jamie Renee Greene Skudlarek
December 12, 2012
ANTIMICROBIAL EFFICACY OF EDIBLE SOY PROTEIN ISOLATE FILMS AND
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December 12, 2012
(Date)
To my mom, husband, family and friends,

Thank you.
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CHAPTER 1  
INTRODUCTION

Food safety is becoming a major concern for the consumer and food industry. Ready-to-Eat (RTE) products are considered to be of high risk of biological threat agent contamination because there is no further processing or heat treatment necessary before consumption. *Listeria monocytogenes* is often a culprit in RTE food outbreaks because of its abundance in the environment, ability to withstand cold temperatures, and its tolerance of low oxygen atmospheres often used in the packaging of RTE foods. According to Kristo and others (2008), post-processing contamination is accountable for about two-thirds of all microbiologically related Class I recalls in the USA. Edible films and/or coatings incorporated with GRAS (Generally Recognized As Safe) compounds found to be effective against biological threats such as *L. monocytogenes* could add another hurdle to the safety measurement of RTE meat, fish and cheese. These edible films and coatings should also appeal to consumers who desire to have more natural foods and less environmental impact.

Edible films and coatings have been used for decades to increase food quality and shelf-life. Starches, oils, waxes, gums, proteins, and gelatin are used for a variety of coatings/casings for items such as fresh fruits, vegetables, nuts, fried foods, confections, cereals, meat products, and drug tablets (Krochta 2002). These packaging techniques prevent or limit the migration of moisture and gases which could result in unwanted color and/or flavor of food products. Quintavalla and Vicini (2002) stated that the purpose of
edible films/coatings would be for shelf-life extension and enhanced food safety through controlling microbial growth.

Incorporating antimicrobial agents into a film or coating used to package a food product would provide an extra barrier against harmful contaminating microorganisms, whether the contamination was intentional or accidental. Antimicrobial edible films and coatings, which are considered active packaging, are receiving increased research interest (Quintavalla and Vicini 2002). Hutton (2003) stated that packaging may be termed active when it performs some desired role in food preservation other than providing an inert barrier to external conditions. Han (2005) suggested that antimicrobials may be incorporated into a packaging material initially then have opportunity to migrate into the food via diffusion. However, there are still some issues for edible antimicrobial packaging. According to Lesiow and Xiong (2008) active packaging has faced some challenges in that there is a lack of knowledge about their effectiveness and consumer acceptance, along with their economic and environmental impact.

Several factors should be considered when producing an antimicrobial incorporated edible film/coating. Campos and others (2011) state that because of possible loss in antimicrobial activity and film characteristics, the following parameters should be considered when selecting an antimicrobial: the effectiveness against the target microorganism and the possible interactions among the antimicrobial, the film-forming biopolymer, and other food components present. For example, Min and Krochta (2005) performed turbidity testing on lactoferrin, lactoferrin hydrolysate and lactoperoxidase against *Penicillium commune* by dissolving the antimicrobials in different concentrations into inoculated peptone, incubating at 23°C for 5 d and measuring optical density at 600
nm by spectrophotometer to measure microbial growth. Although they found these antimicrobials to be effective in turbidity tests, when they performed disk diameter testing on whey protein films incorporating lactoferrin, lactoferrin hydrolysate and lactoperoxidase against *Penicillium commune*, they found the films to be ineffective with the exception of lactoperoxidase at a level of 59 mg/g.

Quintavalla and Vicini (2002) stated that antimicrobial packaging, although extremely challenging, could have a significant impact on shelf-life extension and food safety of meat and meat products. Ku and Song (2007) incorporated nisin in corn zein films at 4,000, 8,000 and 12,000 IU/mL of film forming solution. They found that the antimicrobial activity of these films against *L. monocytogenes* increased with increasing concentrations of nisin resulting in a 1.4 log cycle reduction. Kristo and others (2008) examined known *L. monocytogenes* inhibitors such as sodium lactate, potassium sorbate and nisin incorporated into sorbitol-plasticized sodium caseinate films. They found that coatings containing nisin were most effective in reducing growth of *L. monocytogenes*, followed by those containing potassium sorbate. The least effective was sodium lactate, which was only slightly effective even at inclusion of 40% (w/w dry basis).

A large body of research has been devoted to the inclusion of natural bacteriocins, enzymes, organic acids and essential oils into edible films, including: apple skin polyphenols in apple films (Du and others 2011); p-aminobenzoic and sorbic acid in whey protein films (Cagri and others 2002); oregano-clay composite in whey protein films (Sothornvit and others 2009). In addition, plant-derived antimicrobial compounds obtained from various herbs, spices, fruits and vegetables are considered natural compounds, depending on the extraction method. It has long been recognized that some
essential oils have antimicrobial properties and that the recent interest in ‘green’
customerism has led to a renewed interest in these substances (Burt 2004).

Essential oils have been highly investigated as antimicrobials incorporated into
edible films/coatings. Rojas-Grau and others (2007) found cinnamon, cinnamaldehyde,
carvacrol, citral and lemongrass oil incorporated into alginate-apple puree edible films at
0.1 and 0.5% to be effective against *E. coli* 0157:H7. Seydim and Sarikus (2006) tested
1.0 - 4.0% (w/v) ratios of oregano, rosemary and garlic essential oils for zones of
inhibition against various bacteria including *E. coli*, *Staphylococcus* and *Salmonella*.

However, the use of essential oils could be very limited if the aromatic component is
considered and other extraction methods should be utilized to obtain other antimicrobial
plant components, such as weak acids and polyphenols. For example, the hop plant is
known to contain antimicrobial components such as alpha acids (humulones) and beta
acids (lupulones), and xanthohumol which is a polyphenol. By extracting with an ethanol
solution, these hydrophobic antimicrobial components would be obtained along with
small amounts of various terpenoids (essential oils) which are known to have
antimicrobial activity. Hop ethanol extract has not been investigated as an antimicrobial
in edible films/coatings.

There is some research on the application of antimicrobial edible films and/or
coatings to various food products. However, there is a lack of research on application of
antimicrobial edible films and coatings to RTE meats and poultry with only a few studies
reported. These include p-aminobenzoic acid and/or sorbic acid in whey protein films on
bologna and summer sausage (Cagri and others 2002), nisin and/or calcium propionate in
zein films on RTE chicken (Janes and others 2002), p-aminobenzoic acid in whey protein
casing on hot dogs (Cagri and others 2003), nisin with grape seed extract and green tea extract in SPI film coated turkey frankfurters (Theivendran and others 2006), and nisin with grape seed extract, malic acid and EDTA on turkey frankfurters (Gadang and others 2008). These studies have only evaluated antimicrobial activity, and have not considered the effect the coatings may have on shelf-life and sensory attributes. According to Gennadios (1997), in most of the published application studies, the assessment of protein film/coating functionality did not include sensory evaluation.

The purpose of the work in this Ph.D. dissertation research was to identify a novel GRAS plant extract that produced little color and aroma change of the edible film, while still acting as an efficient antimicrobial agent when incorporated into the edible film/coating. To accomplish the goal the following objectives were proposed:

1. To produce a GRAS plant extract with antimicrobial efficacy against *L. monocytogenes*.

2. To incorporate said plant extract into an edible protein film so that the antimicrobial EFC is effective against *L. monocytogenes*.

3. To examine antimicrobial efficacy of said antimicrobial EFC against *L. monocytogenes* when coated onto a RTE food.

4. To valuate storage and sensory qualities of said antimicrobial EFC when coated onto a RTE food.
CHAPTER 2

LITERATURE REVIEW

2.1 Ready-To-Eat Meats

The Food Safety and Inspection Service define ready-to-eat (RTE) meat and poultry products, either shelf-stable or non-shelf-stable, as products that have been processed so that they may be safely consumed without further preparation, i.e., without cooking or application of some other lethality treatment to destroy pathogens. Refrigeration until consumption is required for non-shelf-stable RTE products to prevent the growth of both pathogenic and spoilage organisms (FSIS 2001). Bologna is a cooked, smoked type of sausage that is considered RTE (Table 2.1). These foods, largely refrigerated RTE meats, are susceptible to post-processing contamination, and can be dangerous to the consumer because there is no further cook or bacterial kill step before RTE meats are eaten. *Listeria monocytogenes* is a common culprit in contamination of RTEs. According FSIS (2007), 6,907 lbs of RTE turkey products were recalled for contamination of *L. monocytogenes*.

2.1.1 *Listeria monocytogenes*

*L. monocytogenes* is a versatile, ubiquitous Gram-positive bacterium that can grow in low oxygen atmosphere and refrigeration temperature. These factors distinguish *L. monocytogenes* as a dangerous causative agent of foodborne illness in RTE foods. This bacterium causes an illness known as Listeriosis, which can cause fever, muscle aches,
vomiting and diarrhea, and can be potentially fatal in newborns, the elderly and the immune compromised. This illness is especially harmful for expecting mothers because of the intercellular movement of the bacterium. Although the expecting mother may show only mild or minimal symptoms, the fetus could be suffering from the illness.

2.1.2 Control of *L. monocytogenes* in RTEs

The United States Department of Agriculture Food Safety and Inspection Service (FSIS 2003) gives manufacturers of RTE meats the option of three alternatives to inhibit growth of *L. monocytogenes*:

- **Alternative 1:** Employ both a post-lethality treatment and a growth inhibitor for *Listeria monocytogenes* on RTE products. Establishments opting for this alternative will be subject to FSIS verification activity that focuses on the post-lethality treatment’s effectiveness. Sanitation is important but is built into the degree of lethality necessary for safety.

- **Alternative 2:** Employ either a post-lethality treatment or a growth inhibitor for the pathogen on RTE products. Establishments opting for this alternative will be subject to more frequent FSIS verification activity than those in Alternative 1.

- **Alternative 3:** Employ sanitation measures only. Establishments opting for this alternative will be targeted with the most frequent level of FSIS verification activity. Within this alternative, FSIS will place increased scrutiny on operations that produce hotdogs and deli meats. In a 2001 risk ranking, FSIS and FDA indentified these products as being high-risk products for listeriosis.

Post-packaging pasteurization is a technique used on RTE meats to control possible *L. monocytogenes* contamination after the mandated post-lethality step.
Additionally, techniques such as radiation, high pressure processing (HPP), microwave processing and active antimicrobial packaging are other techniques being used to control post-lethality contamination. These techniques are generally applied after packaging is complete.

Kristo and others (2008) stated that in order to control unwanted contamination of microorganisms in foods during storage and distribution, antimicrobial substances may be added to the product formulation, coated onto the surface of food or incorporated into the food packaging materials. Antimicrobials specifically effective in preventing growth of *L. monocytogenes* i.e., sodium diacetate, potassium lactate, and sodium lactate, are allowed in RTE products.

Active antimicrobial packaging, both edible and non-edible, is promising in the defense against *L. monocytogenes* contamination. Non-edible films are impregnated with antimicrobial agents (organic acids and their salts, nitrites, chlorides, phosphates, epoxides, alcohols, parabens, bacteriocins, etc) usually in a multi-layer system (Ozdemir and Floros 2004). Antimicrobial edible films and/or coatings are gaining substantial interest with the promise of food safety, reduced packaging, biodegradable packaging and decreased environmental impact.

### 2.2 Edible Films and Coatings

Edible films and coatings are not a new technology. Edible films and coatings have been used for centuries in the food industry, for example, waxes on fruits and nuts, and the natural intestines and cellulose used for meat casing. However, a renewed interest
has been established in the development of edible films and coatings into a type of active packaging, antimicrobial packaging.

### 2.2.1 Definition of Edible Films and Coatings

Edible films and coatings can be defined differently in that edible films are pre-formed by casting and drying to produce an edible film which can then be placed on a food product, whereas edible coatings are sprayed, dipped, brushed, etc onto a food surface as a solution. There are several ways to cast edible films such as skimming, solvent casting, plate casting, extrusion and thermal compaction. Yet, the general production and purpose of edible films and coatings is the same.

### 2.2.2 Production of Edible Films and Coatings

Edible films and coatings can be produced from a number of edible, biodegradable materials primarily polysaccharides, proteins, lipids and biopolymers of the before mentioned. These components are put into solution usually with water and/or alcohol. The use of other solvents, heat and/or changes in pH may be necessary for proper formation of edible films and coatings. Plasticizers such as glycerol and sorbitol are sometimes needed to add flexibility to edible films, particularly with edible protein films.

The formation of a desired edible film or coating may be dependent on various physical, chemical and/or enzymatic treatments employed to modify the functional properties of the protein being used. These may include thermal treatments. Several studies have demonstrated the advantages of thermal treatments to promote unfolding of
proteins and formation of intramolecular and intermolecular cross-links. In general, heat treatment tends to increase tensile strength while reducing water solubility and water vapor permeability. Promotion of cross-linking may also be done with the use of enzymes such as transglutaminase, mainly microbial transglutaminase.

According to Torres (1994), protein films and coatings are prone to cracking during handling and storage; therefore plasticizers are incorporated to add strength and flexibility. Gennadios (1997) summarized that plasticizers act by physiochemically associating with the polymer by entering between polymeric chains and reducing cohesion within the film network, hence extending and softening the film structure. The downside is that plasticizers usually decrease the ability of the film or coating to act as a barrier to moisture and gases. Therefore, an optimum point must be found when using plasticizers.

Major film forming proteinaceous materials include corn zein, wheat gluten, soy protein isolate, cottonseed flour, whey protein concentrate and isolate, casein, milk protein, egg albumen, collagen and fish myofibrillar protein. Whey, soy and collagen proteins are of interest in the production of edible films and coatings because of the sustainability of their application. Collagen or gelatin films and coatings are used commercially to encapsulate low-moisture or oil-phase food ingredient and pharmaceuticals (Krochta 1997).

2.2.2.1 Whey protein isolate (WPI). Whey protein compromises 20% of milk protein and is the portion that remains soluble after casein is precipitated out at pH 4.6 (Krochta 1997). Because of their globular shape, whey proteins require heat denaturation to form films. Whey protein isolate is extremely hydrophilic. Whey protein films are
found to be transparent, flexible and excellent oxygen and aroma barriers, although low moisture barriers (Mchugh and others 1993).

2.2.2.2 Maize zein. Maize or corn zein is fractionated from corn gluten which is separated from the corn germ, fiber and starch in the corn wet-milling process (Krochta 1997). Zein protein is a prolamin, meaning it has high content of uncharged amino acids such as leucine, alanine, proline and glutamine. This makes the protein more hydrophobic and soluble in 70% ethanol. Although zein protein can form films, these are highly brittle and require plasticizers. In general, zein films have relatively good water vapor barrier qualities but have a yellow color.

2.2.2.3 Soy protein isolate (SPI). Soy Protein is globular in nature and is further classified into 2S, 7S, 11S and 15S fractions (Krochta 1997); the main components being conglycinin (7S) and glycinin (11S). While both of these fractions are tightly folded, alkaline conditions and heating cause dissociation and subsequent unfolding due to deamination, since soy protein is high in asparagine and glutamine residues.

Soy protein isolate (SPI) is produced by dilute alkali extraction from defatted soy protein meal then pH precipitation by adjustment to 4.5 (Krochta 1997). SPI is soluble in water and commonly used to produce edible films. SPI with the help of some plasticizer produces transparent and flexible films. However, SPI films tend to have low water barrier qualities due to their solubility.

2.2.3 General Purpose of Edible Films and Coatings

According to Krochta (1997), the purpose of edible films and coatings is to inhibit the migration of moisture, gases, aromas, and lipids; to carry food ingredients (e.g.,
antioxidants, antimicrobials, and flavors); and/or to improve the mechanical integrity or handling characteristics of foods. Preventing oxidative moisture and gases from reaching the food product would lead to less oxidation of the food product, reducing rancidity and other unwanted or adverse effects. Desired properties of edible films and coatings which have been investigated include mechanical, water vapor barrier, oxygen and carbon dioxide barrier, aroma barrier and oil barrier properties. Mechanical properties measured to produce desired edible films include tensile strength and elongation, and are typically measured of edible films that are previously cast and dried.

Krochta (1997) stated that edible films and coatings could have an impact on overall packaging requirements of foods; potentially reducing or eliminating the complexity of the packaging thereby reducing packaging waste. This is one of the benefits of the biodegradable and/or edible packaging, along with the fact that edible films and coatings can be produced using natural ingredients. According to Gennadios (1997), there are two major drivers for developing edible films and coatings from proteins; including, opportunities for adding value to underutilized agricultural materials and concerns over the potentially adverse environmental impact of synthetic materials. Recently, interest lies in producing active packaging edible films and coatings by incorporating antioxidants and antimicrobials to improve storage and shelf-life, and to reduce bacterial contamination. Because food safety is of high importance to the current consumer, antimicrobial edible films and coatings are a hot topic.

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2.3 Antimicrobial Edible Films and Coatings

Due to increased consumer awareness of foodborne disease, investigation of antimicrobial edible films and coatings, a type of active packaging, is of increasing interest. According to Campos and others (2011), edible films and coatings do not pretend to replace traditional packaging materials, but instead provide an additional hurdle or stress factor to be applied for food preservation; however, the end result may help reduce the cost or amount of traditional packaging. The application of an antimicrobial edible film or coating holds the promise of exerting a more localized functional effect without increasing the overall concentration of the additive such as when it is blended into the food or applied to the entire non-edible packaging. Also, slow diffusion, gradual liberation, or leaching of the antimicrobial (active component) would also be a possibility with antimicrobial films or coatings as it is with non-edible packaging.

A substantial amount of research has already been done on incorporating antimicrobials into edible films and coatings (Cagri and others 2001; Hoffman and others 2001; Ko and others 2001; Min and Krochta 2005; Min and others 2005; Sanjurojo and others 2006; Seydim and Sarikus 2006; Gucbilmez and others 2007; Ku and Song 2007; Rojas-Grau 2007; Du and others 2008; Kristo and others 2008; Sivarooban and others 2008; Du and others 2011). The majority of these findings have focused on incorporating a known antimicrobial compound into an edible film or coating solution and performing examinations such as comparison of optical densities aka turbidity tests or measuring zones of inhibition aka disk diffusion method to determine antimicrobial activity. Cowan
(1999) stated that the two most commonly used screens for determining antimicrobial susceptibility are the broth dilution assay and the disc or agar well diffusion assay. Some popular antimicrobials being incorporated into edible films and coatings include natural products such as enzymes, bacteriocins, organic acids and their salts, and various plant extracts.

2.3.1 Enzymes

Lysozyme is a popular enzyme examined in EFCs. According to Gucbilmez and others (2007) lysozyme is one of the most frequently used antimicrobial enzymes incorporated into packaging material in general. Lysozymes are effective against Gram-positive bacteria. Gucbilmez and others (2007) found zein films with incorporated lysozyme and chickpea albumin extract were effective against *E. coli* and *B. subtilis*. Padgett and others (1998) found that the use of lysozyme with chelating agents in biodegradable zein and soy protein films increased the effectiveness against Gram-negative bacteria.

The lactoperoxidase system, which has been identified as a natural antimicrobial found in human secretions including saliva, was incorporated into edible whey protein films by Min and Krotchta (2005). They found that an inclusion of 10 mg/ml of lactoperoxidase system into the whey protein films was effective at inhibiting the growth of *Penicillium commune*. In 2005a, Min and others examined the effects of the lactoperoxidase system in whey protein films against *Salmonella enteric* and *E. coli* O157:H7. And in 2005b, Min and others tested the whey protein coating with incorporated lactoperoxidase system against *L. monocytogenes* and applied the
antimicrobial coating to cold-smoked fish. They found their antimicrobial edible film to be inhibitory of *L. monocytogenes* on agar media as well as when it was applied as a coating on the smoke fish.

### 2.3.2 Bacteriocins

Nisin is a peptide produced by *Lactococcus lactis*. It is effective against a wide range of Gram-positive bacteria, but is not particularly effective against Gram-negative bacteria unless a chelating agent is used to weaken the protective cell wall. Its mode of action is to create pores in the cell membranes of bacteria, destroying the integrity of the bacterial cell membrane and leading to death of the bacterial cell (Raybaudi-Massilia and others 2009). Pore formation leads to depletion of proton motive force and loss of cellular ions, amino acids and ATP (Davidson and Zivanovic 2003).

According to Quintavalla and Vicini (2002), nisin, a natural bacteriocin produced and derived from *Lactococcus* sp., is a well known antimicrobial agent and is already incorporated into certain foods and food packaging. Many, including Hoffman and others (2001) with nisin in zein films and Ko and others (2001) with nisin in whey protein, soy protein, egg albumen and wheat gluten films, have studied the inclusion of nisin into edible films and coatings. In 2001, Ko and others investigated the physical and chemical properties of soy protein isolate and wheat gluten films and their efficacy against *L. monocytogenes* to show that films with greater hydrophobicity i.e. wheat gluten resulted in a greater inhibition of the bacteria. Sivarooban and others (2008) found that nisin (10,000 IU/g) when used in combination with grape seed extract and EDTA can reduce *L.
monocytogenes, E. coli O157:H7 and Salmonella typhimurium by 2.9, 1.8 and 0.6 log CFU/mL, respectively.

Although no longer novel, nisin is still a promising antimicrobial that continues to be examined in edible films and coatings, especially now that research is moving into the next phase of applying antimicrobial edible films and coatings to foods to examine subsequent effects on antimicrobial activity, shelf-life and sensory attributes of the food being manipulated. Janes and others (2002) found that zein coatings with incorporated nisin can prevent the growth of L. monocytogenes on RTE chicken. Theivendran and others (2006) studied the effect of nisin incorporated whey protein coatings when applied to turkey frankfurters that had been previously inoculated with L. monocytogenes, E. coli O157:H7 or Salmonella typhimurium. They found a 2.3 log/g reduction of L. monocytogenes after 28 d storage at 4°C when a whey protein coating containing 6,000 IU/g nisin. Ming and others (1997) fixed nisin to a cellulose casing and found complete inhibition of L. monocytogenes when applied to ham, turkey breast and beef.

2.3.3 Organic acids

Many organic acids and their salts, when used within the specified daily intake, are considered generally recognized as safe (GRAS), and are key antimicrobials used within the food industry. These include sorbic, lactic, citric, p-aminobenzoic (PABA) and acetic acids. According to Brul and Coote (1999), the antimicrobial activity of lactic acid occurs through the diffusion of the lactic acid molecules into the microbial cells until equilibrium is reached based on the pH gradient, causing membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and accumulation
of toxic anions and ultimate death of the cell. Jay and others (2005) suggested similar movement of the undissociated forms of the organic acids into the bacterial cells, decreasing cytoplasm pH and interfering with proton motive force of the cell membrane.

Many of these acids are currently used as ingredients or additives to reduce microbial growth and extend shelf-life, making them ideal candidates for inclusion into edible films and coatings. It is common for a 2% lactic acid wash to be used in a large meat-processing facility to reduce the bacterial load on the outside of the carcass (Dormedy 2000). Accordingly, Cagri and others (2001) found zones of inhibition against *L. monocytogenes* when incorporating 1.5% p-aminobenzoic or sorbic acid into whey protein films. Also, Hoffman and others (2001) found bactericidal and bacteriostatic activity of corn zein films incorporated with lauric acid. Kristo and others (2008) found that sodium caseinate films incorporated with various percentages of sodium lactate and potassium sorbate were effective against *L. monocytogenes*, with potassium sorbate being the more effective.

### 2.3.4 Plant Extracts

Plant extracts are becoming increasingly popular ingredients in active edible films and coatings research because of the consumer demand for more natural food ingredients. Also, it is ancient knowledge that many plant extracts contain antimicrobial capabilities. Several books and reviews have covered the health benefits of the many different plant extracts and oils found all over the world. Plant extracts contain many constituents that may lend to their antimicrobial efficacies such as phenolic compounds, terpenoids, essential oils, weak acids, etc. According to Hammer and others (1999), the antimicrobial
activity of plant oils and extracts has formed the basis of many of their commercial applications such as in raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. In general, a substantial amount of research has examined various plant extracts for their antimicrobial efficacy against pathogenic bacteria. From these studies, scientists can deduce which antimicrobial plant extracts are suitable for incorporation into the edible films and coatings being evaluated.

Plant extract is a very broad term. The composition of the extract depends on the treatment of the plant being extracted, solvent used and the process of extraction. Heat, pH, atmosphere, polarity and solvent type are just some of the parameters that will have an effect on the composition of the plant extract. These parameters should be considered both before and after the extraction process. Cowan (1999) divided useful antimicrobial phytochemicals derived from plants into several categories; phenolics and polyphenols, (simple phenols and phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, coumarins), terpenoids and essential oils, alkaloids, lectins and polypeptides, and mixtures and other compounds.

Essential oils are popular plant extracts being examined as antimicrobials in edible films and coatings. There are several suggestions as to how essential oils would act upon a bacterial cell. Some of these suggestions are reviewed and summarized into six general modes of action (a – f) by Burt (2004) (Fig 2.1). Burt (2004) goes on to state that it is most likely that the antibacterial activity of the essential oils is not attributable to one specific mechanism but more likely the combination of several. Accordingly, Seydim and Sarikus (2006) studied whey protein edible films incorporated with oregano, rosemary and garlic essential oils. By measuring zones of inhibition, they found that the oregano
essential oil was the most effective against various bacteria at 2% inclusion vs. the garlic and rosemary. Rojas-Grau and others (2007) found that alginate-apple puree edible films incorporated with cinnamon, cinnamaldehyde, carvacrol, citral and lemongrass oil at 0.1 and 0.5% were effective against E. coli 0157:H7. Emiroglu and others (2010) applied edible soy films incorporated with thyme and oregano essential oils on fresh ground beef patties to find significant reductions in various bacteria. Du and others (2008) examined tomato puree films incorporated with percentages of carvacrol against E. coli O157:H7 to find zones of inhibition and determined that 0.75% inclusion result in optimum effectiveness.

One should also consider other components, including polyphenols, peptides and weak acids, of the plant as antimicrobial compounds. Du and others (2011) examined apple puree edible films containing apple skin polyphenols against Listeria monocytogenes, E. coli O157:H7 and Salmonella enteric. Different extraction methods should be utilized to obtain other antimicrobial plant components, such as weak acids and polyphenols. For example, the hop plant is known for its antimicrobial components including alpha acids (humulones) and beta acids (lupulones), and xanthohumol which is a polyphenol. By extracting with an ethanol solution, these hydrophobic antimicrobial components would be obtained along with amounts of various terpenoids (essential oils) which are known to have antimicrobial activity. Hop ethanol extract has not been investigated as an antimicrobial in edible films/coatings.

2.3.4.1 Hops. The hop plant (Humulus lupulus) is highly desired for its female flower or cone, especially in the production of beer. The female flower is where the bittering compounds, alpha acids (humulones) and beta acids (lupulones), are found.
These are weak acids that isomerize easily. Other constituents, including xanthohumol and various hydrocarbons, are found in hops and thought to contribute to the antimicrobial efficacy. According to Jirovetz (2006), essential oil of hops cones showed antimicrobial activities against Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis*, as well as the Gram-negative bacteria *Escherichia coli* and *Salmonella* sp. They also found that some hydrocarbon components such as myrcene, alpha humulene, beta caryophyllene and beta pinene showed antimicrobial activity against these bacteria.

Hops are highly recognized for their antimicrobial abilities. According Vanhoucke (1964), hop cones, due to anecdotal evidence of having antimicrobial and antifungal activities, have been used in traditional medicine since the middle ages. Teuber and Schmealreck (1973) state that many investigators have noted hop resins acting mainly on Gram-positive bacteria while Gram-negative bacteria remained resistant or only affected by high concentrations. According to Larson and others (1996) growth of *L. monocytogenes* was inhibited in culture media and in certain foods by four hops extracts containing varying concentrations of alpha and beta acids. Some research has investigated the mode of action of the more prominent hop components, the alpha and beta acids.

2.3.4.2 Hops antimicrobial mode of action. Keukeleire (2000) stated that hop alpha and beta acids have bacteriostatic activity against Gram-positive bacteria. He goes on to contribute this activity to the interference of the prenyl groups, a characteristic side chain of the acids, with the function of the cell plasma membrane; with the beta acids exhibiting stronger bacteriostatic effect because it contains three prenyl groups. He
concludes that this is an important process in wort boiling, leading to sterile beer. This is supported by earlier research from Teuber and Schmealreck (1973), who found that the bactericidal activity of lupulone, humulone, isohumulone and humulinic acid against *Bacillus subtilis 168*, Gram-positive bacteria, is resultant of primary membrane leakage. Simpson (1992) demonstrated that trans-iso-humulone appeared to affect the transmembrane pH of *Lactobacillus brevis*, claiming that this may be the general mechanism of antimicrobial action.

### 2.4 Application of Antimicrobial Edible Films and Coatings

Many studies have identified antimicrobial efficacy of edible films and coatings incorporated with various antimicrobial agents mainly by measuring differences in optical density and zones of inhibition. Some have even placed bacteria directly onto a cored film sample for a scheduled time then massaged, plated and incubated to obtain a plate count. This method is not practical considering that the bacteria have no media in which to grow except for the waterless film on which they are placed. If a technique like this were used, it would be necessary to apply the film to a food or food simulating media to eliminate the possibility of the bacteria not growing due to lack of nutrients.

However, there is a lack of research on application of antimicrobial edible films and coatings to RTE meats and poultry. A handful of studies have applied antimicrobial edible films and coatings to various RTE meats but have only evaluated antimicrobial activity. These include p-aminobenzoic acid and/or sorbic acid in whey protein films on bologna and summer sausage (Cagri and others 2002), nisin and/or calcium propionate in
zein films on RTE chicken (Janes and others 2002), p-aminobenzoic acid in whey protein casing on hot dogs (Cagri and others 2003), nisin with grape seed extract and green tea extract in SPI film coated turkey frankfurters (Theivendran and others 2006), and nisin with grape seed extract, malic acid and EDTA on turkey frankfurters (Gadang and others 2008). Shelf-life and sensory attributes were not evaluated.

Several factors should be considered when producing an antimicrobial incorporated edible film/coating. Campos and others (2011) state that there could be loss of in antimicrobial activity and film characteristics when incorporating antimicrobials into edible films and coatings. They state that the following parameters should be considered when selecting an antimicrobial: efficacy against the target microorganism and possible interactions among the antimicrobial, film-forming biopolymer and other food components present.

Besides considering possible loss of antimicrobial efficacy, one should also consider the effect the antimicrobial edible film or coating would have on the food product being manipulated; whether placing a dried film on the product or coating the product with an edible film and coating solution by spraying, dipping, etc. Hence, it is essential in the study of antimicrobial edible films and coatings to apply them to a food product and measure effects on shelf-life parameters such as oxidation, color change and moisture loss, as well as sensory attributes such as color, aroma, taste and mouthfeel, etc. Spoilage organisms should also be accounted for to ensure there is no added contamination by the antimicrobial edible films or coatings solution.
2.4.1 Oxidation

Oxidation of food components such as lipids and proteins can drastically alter appearance, flavor and mouthfeel of the food. Lipid oxidation is a common cause of rancidity and off-flavors in foods. According to Kanner (1994), oxidation occurs universally in processed muscle foods due to the abundance of unsaturated phospholipids and pro-oxidative metal ions, and is responsible for the development of rancidity and other off-flavors. Peroxide value and 2-thiobarbituric acid reactive substances (TBARS) are frequently used techniques to measure levels of lipid oxidation. Additives such as vitamin E are often used to reduce oxidative effects and inhibit rancidity and color change.

2.4.2 Color

The color of a food greatly influences the purchase by the consumer. Color can be an indication of quality, freshness and safety. Sensory perception of food is influenced by color; hence, in the sensory portion of this study, visual attributes are examined after taste and mouthfeel. Color of food can be instrumentally measured and expressed as lightness (L*), redness (a*) and yellowness (b*) values. The generally goal of an edible film or coating would be to prolong the natural color of food by limiting moisture and gas exchange. Thus, the initial color of an antimicrobial edible film or coating should be accounted for so that there is minimal color change when it is applied to a food. Gounga and others (2008) found whey protein isolate-pullulan edible coatings to be effective in prolonging surface color development of freeze-dried Chinese chestnuts when compared with uncoated ones.
2.4.3 Moisture

Moisture and texture are qualities that greatly influence the overall palatability of a food. Moisture loss can be detrimental in RTE meat products. Changes in texture can be caused by moisture loss. One of the general purposes of edible films and coatings is to reduce or eliminate the migration of moisture and gases; thus, these qualities should be examined of a food that is coated with an antimicrobial edible film or coating. These attributes can both be measured instrumentally via moisture/fat analyzers and mechanical tenderness analyzers, respectively. Bonilla and others (2012) suggest that edible films and coatings provide better preservation of quality by limiting moisture loss.

2.4.4 Sensory

Although several edible films and coatings have been utilized for centuries and a substantial amount of research has investigated edible films and coatings for new and improved ingredients, production and purpose; in general, there is limited sensory evaluation. According to Gennadios (1997), in most of the published application studies, the assessment of protein film/coating functionality did not include sensory evaluation. The majority of the sensory analysis performed is on coated nuts, fruits and vegetables (Mei and others 2002; Yaman 2002). There has been some research to report sensory attributes of stand-alone edible films and these include whey protein isolate with candelilla wax (Kim and Ustunol 2001) and milk protein based films Chen (1995). Sensory attributes determine the overall acceptability of a food product; hence, if an edible film or coating is going to be applied to a food, then sensory analysis should be performed.
**Table 2.1:** Table of ready-to-eat meat and poultry products (adapted from FSIS 2001).

### EXAMPLES OF RTE PRODUCTS

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dried Products</strong></td>
<td>Basturma, Pastirma, Basturmi, Beef Sticks, Carne Seca, Dried Beef, Dry Duck Breast, Meat/Poultry Jerky,</td>
</tr>
<tr>
<td><strong>Salt-Cured Products</strong></td>
<td>Cappicola, Coppa, Country Ham, Dry Cured Duck, Parma Ham, Prosciutto, Prosciutti.</td>
</tr>
<tr>
<td><strong>Fermented Products</strong></td>
<td>Alessandri (Dry Sausage), Apenino (Dry Sausage), Arles or D’Arles (Dry Sausage), Blockwurst (Semi-Dry Sausage), Cacciatore/Cacciatora (Dry Sausage), Cervelat, Cervelat, Soft, Chorizo, Lebanon Bologna, Pepperoni, Salami (Soft), Salami (Genoa, Italian, German), Summer Sausage, Thuringer, Thuringer(Soft),</td>
</tr>
</tbody>
</table>
| **Cooked or Otherwise Processed Whole or Comminuted Products** | *Meat*  
**Poultry (Products Containing any Amount of Poultry).**  
Chicken Burritos, Chicken BBQ, Chicken Bologna, Chicken Breast, Chicken Franks, Cooked Poultry, Cooked Poultry Rolls, Corn Chowder with Chicken, Entrees/Dinners, Poultry Loaf, Poultry Patties, Poultry Rolls, Poultry Salads, Poultry Soups, Frozen, Turkey BBQ, Turkey Franks. |
| **Thermally-Processed, Commercially Sterile Products** | Canned Spaghetti with Meat Balls, Canned Corned Beef Hash, Canned Ham, Canned Chicken Salad, Canned Soups with Meat or Poultry. |
Figure 2.1: Six general modes of action (a – f) of essential oils adapted from Burt (2004).
CHAPTER 3

PRELIMINARY RESEARCH

This research began with the investigation of incorporating certain spice and herb extracts, found to have antimicrobial properties by the food microbiology lab at the University of Kentucky, into edible protein films. Initial tests involved incorporation of quercetin, dissolved in 95% ethanol, into edible whey protein isolate (WPI) film solution which was composed of a mixture of whey protein isolate, water and glycerol, and produced according to Wang and others (2013). The incorporation of quercetin was not possible due to the severe aggregation of the proteins upon addition of the quercetin/ethanol solution. Also, different plant anthocyanin extracts such as garlic, cardamom and basil were evaluated. These had been previously lyophilized to aide in the incorporation of the extract into the protein solution. Once the extracts were incorporated into the film solution, the mixture was then allowed to dry into an edible film over a 2 d period. Core samples were cut from the films and placed on inoculated lawns of Listeria monocytogenes. The plates were then incubated at 37°C for 24 hrs after which they were observed for zones of inhibition. All plates examined showed no signs of inhibition.

To ensure proper lab techniques were being used, a known antimicrobial was evaluated. Nisin, a natural bacteriocin previously examined in edible protein films, was incorporated into WPI films at concentrations of 3,000, 30,000, 60,000 and 90,000 IU per 15 mL of edible protein solution, similar to Ko and others (2001). The solutions were cast and dried for two days, then cored and examined by disk diffusion assay against a
cocktail of *L. monocytogenes*. Measurable zones of inhibition were observed (Fig. 3.1), indicating that my techniques were correct.

Research in the food microbiology lab had suggested that minimal amounts (<25 ppm) of these compounds were needed to produce a minimal inhibitory concentration (MIC) against certain bacteria including *L. monocytogenes*. However, this was not the case once these concentrations were added to the protein solution. Concentrations up to 100x the recommended MIC amount were used and still had zero effect when the cored film samples were tested for zone of inhibition on microbial lawns. Hydrophobicity of the films versus the extracts was thought to play a role in the diffusion of the compounds from the film; hence the use of other proteins including soy protein isolate (SPI) being more hydrophobic than WPI, and maize zein being extremely hydrophobic. The same amounts of extracts were incorporated into the different protein films and the same results were found.

It was thought that maybe a coating, because when applied was still moist, could allow more diffusion and therefore more efficacy of the antimicrobial compound. So, a ‘simulated coating’ was tested. This was done by placing a drop of 15 µL of the protein solution with antimicrobial onto the inoculated lawns. There was still no effect at such low amounts of incorporation. Finally, going back to the literature review, and noticing the amount of essential oils being effective at high incorporation rates, it was decided to produce SPI film solutions with high concentrations (4 and 10%) of incorporated eugenol and cinnamon oil (Fig. 3.2). These solutions where applied as simulated coatings by dropping 0.5 µL onto modified oxford (MOX) agar plates. The agar plates were previously inoculated with a *L. monocytogenes* cocktail. After 24 h incubation at 37°C,
there were large visible zones of inhibition. The film solutions were then cast and cored film samples were tested on an inoculated agar. The dried/cored films produced zones after 24 hrs, but not as large.

Even with these encouraging results, it was determined that producing antimicrobial edible films and coatings using essential oils would not be practical because, even at minimal incorporation amounts, the essential oil/protein solution gave off a potent aroma. So, a closer look was taken at the list of compounds tested by the food microbiology lab. There was one compound, xanthohumol, in particular that seemed to be effective against *L. monocytogenes* (Cetin-Karaca 2011). However, the use of this compound was hindered by price when purchasing as an isolated form, so the source of the compound was identified. It originates from the hop plant (*Humulus lupulus*). Some quick research on hops suggested that the plant contained more than the one compound, xanthohumol, that was suggested to be antimicrobial. It also contains alpha and beta acids which are well documented for antimicrobial behavior.

Zein and soy protein solutions were prepared to test acetone/chloroform extracted hop oils (Chinook variety) prepared in lab. Solvents were removed via rotovaporation. A boiled hop oil extract vs. a non-boiled hop oil extract was tested. Each oil extract was incorporated at 5% in both types of protein solution making a total of four treatments: 1) boiled hop in soy, 2) non-boiled hop in soy, 3) boiled hop in Zein, and 4) non-boiled hop in Zein. Each treatment was tested as a simulated coating on a *L. monocytogenes* cocktail lawn with 24 hr incubation at 37°C. Some zones of inhibition were observed but not for all treatments (Table 3.1).
These results supported further research with hop extracts. Chinook hops and a strain of barley were extracted with acetone/chloroform followed by rotoevaporation to remove solvents. The water (acetone) and oil (chloroform) portions of the Chinook hops and the oil portion of the barley extract were added to both zein and soy protein solutions at 5% incorporation. All were tested as simulated coatings against *L. monocytogenes* cocktail lawns. Both zein and soy protein solutions and the extracts themselves were tested as controls (Table 3.2). Zones were apparent for 72 hrs incubation at 37°C.

The latest results were promising; however, the extraction solvents used, namely acetone and chloroform, would not conform to Generally Recognized As Safe (GRAS) guidelines. It was decided to make hop extractions with water and ethanol to keep with GRAS guidelines (FDA 2011). After running these extracts through the Biotek spectrophotometer, it was found that the minimum inhibitory concentration (MIC) for the hop ethanol extract was 500 ppm and 25% for the water extract. Both extracts were incorporated into SPI solution at 5, 10 and 20% and tested for zone of inhibition. The water extract did not produce zones for any of the tested concentrations. However, at the 10 and 20% inclusion of hop ethanol extract, zones were produced consistently with 3 and 5 mm, respectively; thus, leading to this dissertation research.

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Figure 3.1: Representative photo of various levels of incorporated nisin into edible whey protein isolate films.
Figure 3.2: Representative photo of zones of inhibition produced by edible soy protein isolate films incorporated with 4 and 10% cinnamon essential oil and 10% eugenol.
### Table 3.1: Zone of inhibition observation and measurements for various treatments of edible coating, essential oil and edible coating with incorporated essential oil.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Zone (yes or no)</th>
<th>Treatment</th>
<th>Zone (yes or no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zein solution</td>
<td>No</td>
<td>Chinook hop oil</td>
<td>Yes ~1.9 cm</td>
</tr>
<tr>
<td>Soy solution</td>
<td>No</td>
<td>Zein Chinook hop oil</td>
<td>Yes ~1.4 cm</td>
</tr>
<tr>
<td>Barley oil</td>
<td>Yes ~0.8 cm</td>
<td>Soy Chinook hop oil</td>
<td>Yes ~1.7 cm</td>
</tr>
<tr>
<td>Zein Barley oil</td>
<td>No</td>
<td>Chinook hop water</td>
<td>Yes ~0.6 cm</td>
</tr>
<tr>
<td>Soy Barley oil</td>
<td>No</td>
<td>Zein Chinook hop water</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soy Chinook hop water</td>
<td>No</td>
</tr>
</tbody>
</table>

### Table 3.2: Zone of inhibition observation and measurements for treatments of soy or zein coating with incorporated boiled or non-boiled hop oil extracts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average zone diameter and/or description of plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy/ boiled hop</td>
<td>Complete bacterial growths over coatings</td>
</tr>
<tr>
<td>Soy/ non-boiled hop</td>
<td>0.6 cm zone of inhibition</td>
</tr>
<tr>
<td>Zein/ boiled hop</td>
<td>Some inhibition directly under film but no zone</td>
</tr>
<tr>
<td>Zein/ non-boiled hop</td>
<td>0.35 cm zone of inhibition</td>
</tr>
</tbody>
</table>

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CHAPTER 4

ANTIMICROBIAL EFFICACY OF EDIBLE SOY PROTEIN ISOLATE FILMS AND COATINGS INCORPORATED WITH HOP ETHANOL EXTRACT AGAINST LISTERIA MONOCYTOGENES

4.1 Summary

There is demand for improved security of ready-to-eat (RTE) meat products. Active packaging such as edible films/coatings with incorporated antimicrobials could function as an added barrier against handling contamination and microbial growth. There is also substantial trend in the food industry to use ‘Natural’ ingredients. Many natural plant extracts have been found to have antimicrobial properties. Hops are known for their ability to inhibit bacterial growth which is mostly attributed to humulones, lupulones, xanthohumol and various terpenoids. This research examined hop ethanol extract as an antimicrobial incorporated into soy protein isolate (SPI) films and coatings by examining zones of inhibition, and applying the antimicrobial coating to bologna samples to observe inoculation growth during storage at 7 ± 1°C and 23 ± 1°C. The results showed that hop ethanol extract, incorporated into edible soy protein isolate coating solution, was inhibitory against Listeria monocytogenes strains ATCC 4644, UKADL and ATCC 49594. When the antimicrobial coating was applied to bologna samples, it exhibited a statistically significant bacteriostatic effect against strain ATCC 4644.
4.2 Introduction

In more recent years, food safety has become a major concern for the consumer as well as the food industry. Ready-to-eat (RTE) foods are considered to be of high risk to biological threat agent contamination because there is no further processing or heat treatment necessary before consumption. *Listeria monocytogenes* is often a culprit in RTE foods because of its abundance in the environment, ability to withstand cold temperatures, and its tolerance of low oxygen atmospheres often used in the packaging of RTE foods. According to Kristo and others (2008), post-processing contamination is accountable for about two-thirds of all microbiologically related Class I recalls in the USA. Edible films and/or coatings incorporated with GRAS (Generally Recognized As Safe) compounds found to be effective against biological threats such as *L. monocytogenes* could add an additional hurdle to the safety measurement of RTE meats, while also appealing to consumers who are concerned about attaining more natural foods and less environmental impact.

Edible films and coatings have been used for decades to increase food quality and shelf-life by acting as a barrier to prevent or limit the migration of moisture and gases. Starches, oils, waxes, gums, proteins, and gelatin are used for a variety of coatings/casings for items such as fresh fruits, vegetables, nuts, fried foods, confections, cereals, meat products, and drug tablets (Krochta 2002). Incorporating antimicrobial agents into an edible film or coating used to package a food product would provide an additional hurdle against harmful contaminating microorganisms.
There have been several studies using nisin, a bacteriocin used to inhibit Gram-positive bacteria, in various edible films and coatings: Ku and Song (2007) studied nisin incorporated gelatin and corn zein films, Kristo and others (2008) investigated nisin in sodium caseinate films, Sanjurjo and others (2006) studied nisin suspended in tapioca starch films. A large amount of research has investigated the inclusion of organic acids and essential oils into edible films including: p-aminobenzoic and sorbic acid in whey protein films (Cagri and others 2002), oregano-clay composite in whey protein films (Sothornvit and others 2009), and cinnamon, cinnamaldehyde, carvacrol, citral and lemongrass oil incorporated into alginate-apple puree edible films at 0.1 and 0.5% to be effective against E. coli 0157:H7 (Rojas-Grau and others 2007). However, the use of essential oils would be very limited if the aromatic component is considered and would only be suitable for food products that have similar and/or compatible aromas.

Also, most research is limited to initial steps of incorporating the antimicrobial into the edible film or coating, and testing for zones of inhibition and/or changes in optical density. Some studies have further applied antimicrobial edible films and coatings to various food products. These include p-aminobenzoic acid and/or sorbic acid in whey protein films on bologna and summer sausage (Cagri and others 2002), nisin and/or calcium propionate in zein films on RTE chicken (Janes and others 2002), p-aminobenzoic acid in whey protein casing on hot dogs (Cagri and others 2003), nisin with grape seed extract and green tea extract in soy protein isolate (SPI) film coated turkey frankfurters (Theivendran and others 2006), and nisin with grape seed extract, malic acid and EDTA on turkey frankfurters (Gadang and others 2008).
The purpose of this research was to identify a GRAS plant extract that produced little color or aroma change, while still acting as an efficient antimicrobial agent when incorporated into the edible film or coating. By producing a hop ethanol extract with 75% ethanol, this goal was achieved. Hops are known for their antimicrobial activity which is attributed to their humulone, lupulone, xanthohumol and various terpenoid components. The hop ethanol extract was identified to be an active antimicrobial which was incorporated into an edible SPI film with minuscule elicited influence on color or odor while retaining antimicrobial efficacy. Bologna samples, produced in lab without *L. monocytogenes* inhibitors, were coated with the SPI+hop and stored at 7 ± 1°C and 23 ± 1°C to find that the antimicrobial coating exhibited strong bacteriostatic activity when applied to a RTE meat.

4.3 Materials and Methods

4.3.1 Bacteria Preparation:

Three *L. monocytogenes* strains, ATCC 4644, ATCC 49594 and University of Kentucky Animal Diagnostic Lab (UKADL), were used for susceptibility testing. The three strains were inoculated and grown in brain-heart infusion (BHI) (Difco Laboratories Inc, Detroit MI) broth from BHI slants. The strains were grown over a two day period of 37°C incubation to achieve a healthy growth of the organism. During experimentation, bacterial lawns and massaged samples were spread on modified oxford (MOX) plates. MOX is a selective medium for the detection of *L. monocytogenes*. The bacteria
hydrolyze aesculin in the MOX agar to produce dark zones around growing colonies. Gram-negative bacteria are completely inhibited by this agar due to added antimicrobial supplements.

4.3.2 Preparation of Hop Ethanol Extract:

A hop ethanol extract was produced using dried hops pellets of the Chinook variety (Hopunion LLC, Yakima WA) and 75% ethanol in a 1:10 w/v ratio, modified from Kong and others (2007). The hop ethanol extract had an average pH of 5.1 ± 0.2. The solution was blended in a Waring commercial blender (Waring® Products, Stamford CT) at medium speed for approximately 30 sec. The solution was allowed to rest for approximately 5 min, until there is noticeable swelling of the pellets, before it was blended at medium speed for an additional 30 sec. After another 5 min of rest, the solution was pulse blended before pouring into a vacuum filter system to remove solids. The solution was then filtered through a 0.22 µm filter to remove possible microbial contamination and finish preparation. The hop extract was stored in sterile containers at 7 ± 1°C until further use.

4.3.3 Edible Protein Solution Preparation:

Soy protein isolate (SPI) was obtained from a commercial source. Edible SPI solutions were prepared using protocol by Wang and others (2012) and Jiang and others (2012), with slight modification. Approximately 65 mL of nanopure (deionized) water was added to a 125 mL flask then zeroed on a scale to add 1.64 g of glycerol drop wise. SPI was weighed to 3.9 g and added to the solution. The solution was stirred for
approximately 30 min to ensure homogenization. After adjusting pH to 8.0 ± 0.2 using a combination probe pH meter (Fisher Scientific Co., Fair Lawn, N.J.), the solution was heated in an 85°C bath for 30 min. After heating, the solution was placed in a cooling water bath while continually stirring to bring the solution down to room temperature.

4.3.4 Bologna Preparation:

Bologna was prepared in the University of Kentucky Meat Processing Facility using a bologna seasoning (Legg’s Old Plantation Seasoning, Calera AL) of which 1 oz contained 6.25% sodium nitrite. There were no *L. monocytogenes* inhibitors such as sodium lactate or sodium diacetate added. The bologna was cased and cooked to internal temperature of 80°C, then sliced into 0.64 cm slices. The bologna slices were portioned into 4 slice aliquots, vacuum sealed in storage bags and placed in a -20°C freezer until further use. The bologna had a final percent moisture of 62.8%, percent fat of 19.8% and pH of 5.9.

4.3.5 Hop Ethanol Extract Evaluation:

The hop ethanol extract was evaluated for minimal inhibitory concentration (MIC) against the three *L. monocytogenes* strains (ATCC 4644, ATCC 49594 and UKADL) via optical density at 660 nm in a calibrated spectrophotometer (BioTek Synergy 4, Winooski VT) using a 96-well plate. The well plate was incubated at 30 °C in the spectrophotometer for 48 h with optical density read every 2 hrs. MIC was determined at the end of the 48 h incubation due to competition of the control, although it is customary to determine MIC at 20-24 h (Barry 1976).
Mueller Hinton broth (MHB) (Difco Laboratories Inc, Detroit MI) was inoculated with freshly grown 24 h cultures by adding approximately 3 drops via sterilized Pasteur pipette. A micropipette was used to add 200 µL of the inoculated MHB to each plate well being evaluated. Two of these wells for each bacterium were used as controls and did not have anything else added. The hop ethanol extract was diluted in 75% ethanol to 5000 ppm so that when 50 µL of this dilution was added to a plate well filled with 200 µL of inoculated broth, the resulting concentration would be 1000 ppm. This plate well was then used to produce a serial dilution of 500, 250, and 125 ppm. The same was done with 75% ethanol for a negative control.

4.3.6 Dried Film and Simulated Coating Evaluation:

Prepared protein solutions with incorporated extracts were evaluated by zone of inhibition to identify susceptibility. The prepared SPI solution was separated into 10 mL aliquots before incorporating the hop ethanol extract and 75% ethanol by percentage (v/v). The hop ethanol extract was incorporated into the SPI solution at 10 and 20%. These were compared to 10 and 20% incorporation of 75% ethanol for a total of four treatments. The hop ethanol extract and 75% ethanol were added slowly, drop-wise to the SPI solution to decrease sudden aggregation. The mixtures were homogenized by stirring at 700 rpm for no less than 15 min. Although the MIC data suggested otherwise, preliminary zone of inhibition results indicated that the hop ethanol extract needed to be incorporated into the SPI solution at ≥ 10% (v/v) to produce zones of inhibition.

All treatments were evaluated as ‘simulated coatings’ and dried/cored films against three strains of *L. monocytogenes* by using the zone of inhibition or disk diffusion
method. The simulated coatings were produced by dropping approximately 15 µL of the prepared protein solution onto an agar plate that had been previously lawn inoculated with the desired bacteria by dipping a swab into the bacteria broth and completely swabbing the agar. Approximately 8 mLs of the remainder of the protein solution was poured into Teflon lined Petri dishes. These dishes were placed under a laminar hood for two days with the Petri lid partially open to allow the solutions to air-dry into films approximately 80 ± 10 µm thick. The dried films were cored to produce 9.5 mm samples that were placed on inoculated plates.

All prepared plates were then incubated for 24 h at 37°C. Zones of inhibition were measured using a slight modification from the NCCLS norm by measuring zones from the edge of the simulated coating or dried/cored film to the edge of the inhibitory zone. This method was used because the diameter of the simulated coatings, usually atypical in circumference, and dried/cored films were larger than 6 mm and this approach could convey a more accurate account of the antimicrobial efficacy. Three plates were made prepared for each strain with all four treatments being tested per plate. This was replicated for an end result being the average of six measurements per treatment per strain.

4.3.7 Applied Coating Evaluation on Bologna Samples:

Four coating treatments were evaluated: 1) no coating 2) soy coating 3) soy coating with 10% incorporation of hop ethanol extract (v/v) and 4) soy coating with 20% incorporation of hop ethanol extract (v/v). These treatments were evaluated for antimicrobial efficacy during storage at room (23 ± 1°C) and refrigeration (7 ± 1°C)
temperature. Samples were either stored at $23 \pm 1^\circ C$ for 24 h, or $7 \pm 1^\circ C$ 1, 3 and 7 days. Day zero samples were measured for all four treatments (Fig 4.1).

On day zero of the experiment, the bologna was thawed by placing in a walk-in cooler at $7 \pm 1^\circ C$ for approximately 5 h. A 2.54 cm meat corer was used to obtain bologna samples for coating weighing approximately $3.5 \pm 0.1$ g. The cored bologna samples were placed on sterilized stainless steel coated test tube racks. A separate rack was used for each coating treatment (10 core samples per treatment). The bologna samples were laid onto the racks horizontally and as level as possible so that the coating solution would not immediately drip off. A bulk protein solution was prepared while the bologna was being thawed. The solution was separated into 30 mL portions in 50 mL beakers. Using a small stirbar, the solution was stirred continuously while the hop ethanol extract was added at 10 and 20% (v/v). The prepared antimicrobial solutions were allowed to stir for at least 15 min to achieve complete homogenization while the bologna samples were being prepped.

Once both the bologna and coating solutions were prepared, spray nozzles, previously sterilized by boiling water bath were used to spray coat the bologna samples. This was done by holding the end of the nozzle approximately 4 cm from each piece and spraying twice to achieve a visible layer of coating, approximately $0.5 \pm 0.1$ g of coating per two sprays. Once the coating was applied, the samples were allowed to dry for 15 min. The samples were flipped using sterilized forceps and the coating process was repeated for the other side of bologna. The finished coated bologna core samples weighed approximately $3.7 \pm 0.1$ g for an approximate gain of $0.16 \pm 0.02$ g of dried coating.
Completely coated, the cored bologna samples were placed on white poly trays (2 samples per tray) and inoculated with 15 µL of diluted *L. monocytogenes* ATCC 4644 broth to achieve an inoculation of approximately $10^4$. The inoculums were spread using sterile hockey sticks. The trays were then wrapped twice with plastic wrap and placed into storage. The room temperature trays were left at $23 ± 1°C$ for 24 h storage, and the refrigerated trays were placed in a walk-in cooler ($7 ± 1°C$) until intended sampling days (1, 3 and 7). Day 0 inoculated samples were immediately placed in dilution using buffered peptone, from which serial dilutions were made and plated on MOX agar.

On subsequent sampling days, the bologna samples were aseptically placed into 18 oz stomacher bags using flamed forceps, and sterile peptone solution was added to make a 10x dilution. The bologna core samples were gently massaged using thumb and forefinger from the outside of the stomacher bag to obtain bacterial growth. Serial dilutions were made as necessary and spread on MOX agar plates to obtain plate counts. The plates were incubated for 48 hrs at $37°C$ before counting. Two bologna samples were prepared for each treatment per holding temperature per day. This was done in triplicate for a total of six measurements per treatment per storage application.

### 4.3.8 Statistical Analysis

Statistical analysis was performed on the coating evaluation by factorial design using 4 treatments x 5 storage applications (temperature by time) using SAS (SAS Institute Inc., Cary, NC, USA) General Linear Model to analyze by Analysis of Variance. Significant differences ($P ≤ 0.05$) were found using least mean squares.
4.4 Results and Discussion

4.4.1 Hop Ethanol Extract Evaluation:

Minimum inhibitory concentration (MIC) was determined at 48 h when it was apparent that the 1000 ppm hop ethanol extract produced complete inhibition, whereas the 75% ethanol control began to have growth for all three *L. monocytogenes* strains (Fig 4.2). The 500 ppm level of hop ethanol extract was also more effective than the 1000 ppm 75% ethanol. There was inhibition at the 250 and 125 ppm levels of hop ethanol extract; however these were not as effective as the 1000 ppm of 75% ethanol. According to Barry (1976) the MIC is determined as the lowest concentration that inhibits visible growth of an organism. Hence the MIC was determined to be 500 ppm for the hop ethanol extract.

4.4.2 Dried Film and Simulated Coating Evaluation:

A control of SPI edible solution with no incorporated antimicrobials resulted in no zones of inhibition as simulated coating or dried, cored film. When the negative control of 75% ethanol incorporated protein solution was evaluated as a simulated coating and film, the results showed no zones of inhibition with 10% inclusion. The 20% ethanol (v/v) protein solution resulted in no zone of inhibition as a simulated coating and was unable to form a dried film for evaluation. The 10 and 20% hop ethanol extract inclusion produced zones of inhibition against each strain of *L. monocytogenes* as both simulated coatings and dried, cored films (Table 4.1). The comparisons between zones of inhibition of 75% ethanol and hop ethanol extract are shown in representative photographs (Fig
4.3. Similar results were observed in dried, cored film samples. The 10 and 20% SPI+hop ethanol extract solutions had an average pH of 7.3 ± 0.2 and 7.0 ± 0.2, respectively. This suggests that pH may not play a role in the antimicrobial mode of action.

These results are similar to the findings of others. Cagri and others (2001) found measurable inhibition zones against *L. monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella typhimurium* DT104 testing whey protein based edible films containing p-Aminobenzoic and sorbic acids. Min and Krochta (2005) found inhibition zones against *Penicillium commune* when testing whey protein films incorporated with lactoperoxidase. Ku and Song (2007) found that antimicrobial activity against *L. monocytogenes* increased with the increase of nisin concentration incorporated into corn zein and gelatin films when measuring zones of inhibition.

**4.4.3 Applied Coating Evaluation on Bologna Samples:**

Temperature plays a very important role in antimicrobial efficacy when considering the preference of the bacteria and the antimicrobial agent being studied. Room temperature studies of antimicrobial edible film efficacy are not directly applicable to non-shelf stable foods needing to be held at refrigeration temperatures. Quintavalla and Vicini (2002) state that generally, increased storage temperature will accelerate migration rates of active agents in film or coating layers, while refrigeration temperature can slow the migration rate. For this reason both room and refrigeration temperatures were evaluated.
4.4.3.1 Inhibition of *L. monocytogenes* at room temperature. The room temperature (23 ± 1°C) 24 hr storage resulted in a statistically significant (*P* ≤ 0.05) 3 log difference in CFU/mL between the controls (no coating and SPI coating), and both SPI coating with 10 and 20% hop ethanol extract treatments (Fig 4.4). The population difference is the result of the bacteria being able to grow at room temperature in the control treatments, where in the hop extract treatments, the bacteria were absolutely inhibited from growing and beginning to expire as a result from the treatment. There was no significant difference (*P* > 0.05) between the 10 and 20% hop extract treatment over 24 hr storage at room temperature suggesting that the added percentage of hop ethanol extract did not increase the antimicrobial efficacy of the coating as the zone of inhibition results suggested. There was no significant difference (*P* > 0.05) within the initial inoculation of the treatments suggesting that the hop ethanol extract did not have an instantaneous bactericidal effect.

4.4.3.2 Inhibition of *L. monocytogenes* at refrigeration temperature. The refrigeration temperature (7 ± 1°C) storage resulted in smaller differences, however still statistically significant (Fig 4.5). The reasoning for the smaller differences between treatments is that the bacteria, although able to survive, acclimate and eventually grow, do not prefer refrigeration temperature; therefore, the control treatments did not grow as quickly as in the room temperature study which produced the larger population difference. The CFU/mL of the control and soy coating treatments did not change over storage period where there was a significant decrease (*P* ≤ 0.05) over the 7 d storage in both the soy with 10 and 20% hop ethanol extract treatments. There was no significant difference in CFU/mL between control and soy treatments; however, there was
significant difference ($P \leq 0.05$) between these treatments and both treatments with incorporated hop extract with an approximate 10 fold difference. Similarly, Cagri and others (2002) found inhibition of *L. monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella typhimurium* DT104 at refrigeration temperature by placing edible whey protein films incorporated with p-aminobenzoic and sorbic acid on inoculated bologna and summer sausage slices.

### 4.5 Conclusion

Overall, these data suggested the antimicrobial edible coating, produced by incorporating hop ethanol extract into edible SPI solution, was highly bacteriostatic. In this study, initial inoculation was high enough ($10^4$) to produce quantitative results; however, in everyday circumstances, most food contamination would be at a much lesser level (only a handful of cells). Although technically it is unknown the effects the tested antimicrobial edible coating would have on a lesser contamination level, this data would suggest that the antimicrobial edible coating would act as an additional hurdle and possibly eradicate normal, lesser levels of contaminating cells; thereby, preventing foodborne illness. Further research should involve better understanding of the mode of action of the hop ethanol extract, then combining the hop ethanol extract with substances that use similar and/or different modes of action to achieve a synergistic or additive antimicrobial affect. For example, nisin works by producing pores in the cell wall of bacteria and a combination of nisin with hop extract may result in a greater population reduction.
Extract hops pellets using 75% ethanol
Determine MIC
Incorporate into soy protein isolate solution
Test for Zone of Inhibition as ‘simulated coating’ and dried/cored film

Coated Bologna Experiment

Thaw and core bologna samples
Place cored bologna samples on sterile test tube racks (10 samples per treatment, each treatment had a separate rack)
Spray coatings onto cored bologna (allowed to dry 15 min per side)
Place control (no coating) bologna on tray (2 per tray)
Inoculate samples by dropping 15 µL of diluted L. monocytogenes broth onto the surface of the bologna sample then spread gently using a sterile hockey stick
Massage and plate Day 0 control samples
Place coated samples onto trays, inoculate and wrap for storage.
Massage and plate Day 0 coated samples
Place other trays in appropriate storage

1) No coating  2) SPI  3) SPI+10%Hop  4) SPI+20%Hop

Day 0  Day 1, 23°C  Day 1, 7°C  Day 3, 7°C  Day 7, 7°C

**Figure 4.1:** Experimental design and flowchart.
Figure 4.2: Comparison of optical density of normal bacteria growth (control), hop ethanol extract at 125, 250, 500 and 1000 ppm, and 75% EtOH at 1000 ppm against L. monocytogenes ATCC 4644, UKADL and ATCC 49594.
Figure 4.3: Representative photos of zones of inhibition compared between soy protein isolate solution with incorporated 10 and 20% hop ethanol extract (10H and 20H) and 10 and 20% ethanol (10E and 20E) as simulated coatings against *L. monocytogenes* strains ATCC 4644, UKADL, and ATCC 49594, respectively.
Table 4.1: The mean of six observations for zone of inhibition (mm) produced by SPI simulated coatings and dried, cored films incorporated with either 10 or 20% hop ethanol extract. Standard deviation is represented by ±.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>ATCC 4644</th>
<th>UKADL</th>
<th>ATCC 49594</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulated coating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPI+10% Hop</td>
<td>3.75 ± 0.42</td>
<td>3.17 ± 0.26</td>
<td>2.88 ± 0.25</td>
</tr>
<tr>
<td>SPI+20% Hop</td>
<td>5.00 ± 0.32</td>
<td>4.50 ± 0.45</td>
<td>3.90 ± 0.22</td>
</tr>
<tr>
<td>Dried, cored film</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPI+10% Hop</td>
<td>3.83 ± 0.26</td>
<td>3.33 ± 0.41</td>
<td>2.00 ± 0.32</td>
</tr>
<tr>
<td>SPI+20% Hop</td>
<td>5.75 ± 0.42</td>
<td>5.00 ± 0.71</td>
<td>3.92 ± 0.66</td>
</tr>
</tbody>
</table>
Figure 4.4: Comparison of CFU/mL of no coating, soy protein isolate (SPI) coating, and SPI with incorporated 10 and 20 % hop ethanol extract coatings against *L. monocytogenes* ATCC 4644 over a storage of 24 hrs at 23 ± 1°C.

*abc within treatment between storage, different letters differ significantly (*P* ≤ 0.05)
*ABC within storage between treatments, different letters differ significantly (*P* ≤ 0.05)
*Standard error was 0.1139 for all observations according to least squares mean
Figure 4.5: Comparison of CFU/mL for no coating, soy protein isolate (SPI) coating, and SPI with incorporated 10 and 20% hop ethanol extract coatings over a period of seven days under refrigeration temperature (7 ± 1°C).

*abc within treatment between storage, different letters differ significantly (P ≤ 0.05)
*ABC within storage between treatments, different letters differ significantly (P ≤ 0.05)
*Standard error was 0.1139 for all observations according to least squares mean
CHAPTER 5

IDENTIFICATION OF KEY ANTIMICROBIAL COMPOUNDS IN HOP ETHANOL EXTRACT USING MASS SPECTROMETRY

5.1 Summary

Hops, hop pellets and hop extracts are commonly used in the production of beer and are known to contain various components with antimicrobial activity, including xanthohumol, humulone, lupulone and their various isomers, along with various terpenoids. Previous chapters have identified hop ethanol extract as having a significant bacteriostatic effect when incorporated into soy protein isolate edible film and coating. In the present chapter study, mass spectrometry techniques, including electrospray ionization (+) with tandem mass spectrometry (ESI-MS/MS) and headspace analysis (solid phase microextraction) using gas chromatography with mass spectrometer detection (GC-MS), were used to identify key antimicrobial components in the hop ethanol extract. Key antimicrobial components were identified via ESI-MS/MS as xanthohumol and various forms of humulone and lupulone. Terpenoids including myrcene, alpha pinene and beta pinene were identified using GC-MS.
5.2 Introduction

The female flowering, fruiting body or cone of the hop plant (*Humulus lupulus*) has been used for centuries to impart various flavors and aromas to beer. The cones, also known as hops, are added during the wort boiling process. This process extracts components from the hops that contribute to the colloidal, foaming and bacteriological stability of the beer, as well as the bitter taste (Keukeleire 2000). According to Vanhoenacker and others (2004), during the boiling process the hop alpha acids or humulones are isomerized into isohumulones, which impart to the bitter taste. The beta acids or lupulones are also believed to add to the bitter taste.

Hop extracts are known for their antimicrobial activity (Bhattacharya and others 2003). Both the alpha and beta acids are known to exhibit antimicrobial properties (Bhattacharya and others 2003; Srinivasan and others 2004), along with prenylflavonoid and xanthohumol (Herath and others 2003; Srinivasan and others 2004). Ceslova and others (2009) reported that hops contained many polyphenolic compounds in addition to bitter-eliciting acids, and the most important hop flavonoids are xanthohumol and related prenylflavonoids as isoxanthohumol, desmethylxanthohumol, 6-prenylneringenin, 8-prenylneringenin, and 6-geranylneringenin. The various hop flavonoids have a positive effect on the human health due to antioxidant, anticancer, antimicrobial and anti-inflammatory properties (Ceslova and others 2009).

Hop can be purchased in the form of whole cones, pellets, or in an extract form typically prepared with ethanol or supercritical CO₂ (Magalhaes and others 2007). Ethanol is a strong solvent with low selectivity, meaning it can be used for the extraction
of numerous compounds, both hydrophilic and hydrophobic. According to Magalhaes and others (2007), ethanol was one of the first solvents to be used in the beginning of the century for the extraction of aromatic and bitter components from hop because ethanol would extract virtually all the lupulin components, pigments, cuticular waxes, and water-soluble compounds.

In the preceding chapters, it was found that a hop extract, produced using hop pellets and 75% ethanol, had a significant bacteriostatic effect. To ascertain that key components, i.e. xanthohumol, lupulone, humulone and their isomers which are believed to contribute to the antimicrobial efficacy, were present in the hop extract, the extract was subjected to mass spectrometry analyses.

5.3 Materials and Methods

5.3.1 Preparation of Hop Ethanol Extract

Hop pellets of the Chinook variety were purchased from Hopunion LLC (Yakima WA). This variety, according to the label, contained 11.8% alpha acids and 3.0% beta acids. An ethanol extract was prepared by weighing 5 g of the hop pellets and blending with 50 mL of 75% ethanol, according to Kong and others (2007) with modifications. The solution was blended in a Hobart mixer at medium speed for approximately 30 sec. The solution was allowed to rest for approximately 5 min until there was noticeable swelling of the pellets, before it was blended at medium speed for an additional 30 sec. After another 5 min of rest, the solution was pulse blended before pouring into a vacuum
filter system to remove solids. The solution was then filtered through a 0.22 µm filter to remove possible microbial contamination. The hop ethanol extract was stored in sterile containers at 7 ± 1°C before use.

5.3.2 Electrospray Ionization (ESI)-MS/MS

Mass spectra (MS) were acquired by the University of Kentucky Mass Spectrometry Facility using the following conditions. Electrospray ionization (ESI) mass spectra were obtained on a ThermoFinnigan LTQ ion trap mass spectrometer (Thermo Scientific, San Jose CA), with sample introduction by direct infusion at 3 µL/min. Full scan mass spectra were recorded in the mass range of 150-700 m/z using positive (+) or negative (-) ion polarity. Instrument parameters included spray voltage: 3.5kV, capillary temperature: 200°C, capillary voltage: 75V, and tube lens voltage: 100V. MS/MS spectra were acquired through collision-induced dissociation (CID) using a normalized collision energy of ~20 – 22% to produce a reduction in the precursor ion relative abundance to <10%. Identification of prominent peaks from the full positive-ion (+) mass spectra was done by determination of molecular weights and examining daughter ions from MS/MS spectra. These were compared with results from other research to further support identification.

5.3.3 Gas Chromatography (GC)-MS

Headspace samples were drawn with an inert needle valve syringe equipped with a 65µm PDMS/DVB fused silica/SS solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) and were analyzed on a Hewlett Packard Model 5890 Series II gas...
chromatograph (Wilmington, DE), equipped with a Hewlett Packard 5971 Series Mass Selective Detector. Approximately 100 µL of hop extract was placed into a 40 mL vial with hole cap PTFE/silicone septa (Supelco, Bellefonte, PA). The sample was allowed to sit at room temperature (23 ± 1°C) for approximately 1 h. Before sampling the headspace, the syringe fiber was conditioned in a neighboring GC at 210°C for 10 min. The syringe was inserted into the septum of the 40 mL vial and the fiber was exposed for 10 min to obtain a sample of the headspace.

5.4 Results and Discussion

5.4.1 Electrospray ionization (ESI)-MS/MS

HPLC with UV detection is routinely used to analyze bitter acids (Zhang and others 2004); however, the instability and structural similarity of the hop bitter acids cause difficulty in routine analysis. MS/MS, with its improved sensitivity and greater selectivity, lends itself a reliable detection technique superior to UV detection, especially for the analysis of minor components in complex matrices (Magalhaes and others 2007). The full scan ESI mass spectra for both positive (+) and negative ion (-) modes are shown in Fig 5.1 and Fig 5.2, respectively. The prominent peaks from the full ESI positive-ion (+) MS include m/z 355, 363, 401, 413, 415, and 417. Additional structural information was obtained from MS/MS analysis of prominent peaks revealed on the full positive-ion (+) mass spectrum, and the spectra are shown in Fig 5.3-5.10, respectively. The negative-ion (-) spectrum was not used in the identification because the positive-ion spectrum resulted in more definition between peaks.
The MS/MS of peak 355 (Fig 5.3) revealed a strong precursor or daughter ion of 299 m/z along with two smaller or less abundant peaks at 179 and 235 m/z. Magalhaes and others (2007) found the same daughter ions using similar equipment, ESI(+) - MS/MS and identified the parent or pseudomolecular ion as xanthohumol and isoxanthohumol; therefore, the peak 355 in the present study was tentatively assigned to xanthohumol and isoxanthohumol. This identification was in agreement with Stevens and others (1999) who reported xanthohumol and isoxanthohumol having the parent ion of 355 m/z and daughter ion of 179 m/z.

The full MS (+) spectra prominent peak at 363 m/z was identified as humulone/adhumulone when comparing the MS/MS spectra (Fig 5.4) with spectra established by Zhang and others (2004) who analyzed the isolated compound. Both their spectra and the MS/MS spectra from the present research revealed daughter ions of 307, 295, 239 and 223 m/z. Although Zhang and others (2004) utilized different equipment (APCI-MS/MS), their spectra were similar to the ones generated by ESI-MS/MS in the present study. Although the spectra were not identical, they are comparable because the same ions are identified in spite of the difference in abundance.

Colupulone is the compound identified at peak 401 m/z of the full MS (+). This was supported by comparison of the MS/MS spectra (Fig 5.5) to the report of Zhang and others (2004) that showed similar daughter ion peaks at 345, 332, 277, 275 and 219. The present identification was further supported by Vanhoenacker and others (2004) who found a strong peak at 399 m/z using APCI negative ionization mode.

The full MS (+) peaks at 413, 415 and 417 m/z are believed to be very similar compounds when their MS/MS spectra are analyzed (Fig 5.6 – 5.8, respectively). The full
MS (+) peak at 415 m/z was identified as lupulone/adlupulone when the MS/MS spectrum (Fig 5.7) was compared to the spectrum of Zhang and others (2004) using (+) APCI-MS/MS. Both spectra reveal daughter ions at 359, 347, 291 and 275 m/z.

Identification was further substantiated by Vanhoenacker and others (2004) who determined adlupulone to have a molecular weight of 414. The MS/MS spectrum of the 417 m/z peak revealed very similar daughter ions (360, 348, 292 and 276 m/z) to the MS/MS spectrum of 415 m/z which could be due to an addition of two hydrogen or the loss of a double bond. Arraez-Roman and others (2006) found a peak at 413 in (-) mode ESI-M/MS in a hop pellets extract and identified it as lupulone by comparison of MS/MS spectrum of an internal standard to MS/MS spectrum of the hop pellet extract revealing daughter ions at 369, 344, 301, 233 and 208 m/z in each spectrum. These results were comparable with the MS/MS spectrum (Fig. 7) of the full MS (+) peak at 413 m/z in this research.

By identifying these compounds, conclusions can be drawn about the mode of action or actions the hop ethanol extract may utilize to inhibit the growth of the Gram-positive bacterium, Listeria monocytogenes. The weak acids (humulones and lupulones) are thought to disrupt the bacterial cell membrane via hydrophobic interactions, mainly via distinct prenyl side groups (Keukeleire 2000). Organic acids such as lactic, citric and sorbic acids have been linked to the disruption of the ion gradient in bacterial cells by entry of the undissociated form into the bacterial cells, further disrupting proton motive force and inhibiting production of ATP (Jay and others 2005), suggesting an alternate mode of action for the hop acids.
5.4.2 Gas chromatography (GC)-MS

The terpenoids myrcene, alpha pinene, beta pinene and caryophyllene were observed in the headspace analysis of the hop ethanol extract. These are labeled in the GC-MS spectrum (Fig 5.9). SPME is used to examine the headspace or volatile components of compounds. By using this technique to sample the headspace over the trapped hop ethanol extract, it can be deduced that certain terpenoids and various other hydrocarbons that essentially make up what is known as the essential oil, are volatilizing from the extract. Raybaudi-Massilia and others (2009) state that essential oils, also called volatile oils, aromatic oily liquids obtained from plant materials, and are composed of a complex mix of compounds including terpenes, alcohols, cetones, phenols, acids, aldehydes and esters. The antimicrobial properties of numerous essential oils have been investigated; however, their mechanisms of action have not been studied in great detail, and when considering the various compounds present in essential oils, it is most likely that their mechanism of action is over several specific targets in a cell and not just one (Raybaudi-Massilia and others 2009). Cowan (1999) stated that the mechanism of action of terpenes, although not fully understood, is speculated to involve membrane disruption by hydrophobic components.

5.5 Conclusion

Various suspected key antimicrobial components of the hop ethanol extract were positively identified, i.e., various forms of humulone, lupulone, and xanthohumol, along with some prominent terpenoids, including myrcene, alpha pinene, beta pinene and
caryophyllene. And, by identifying these compounds, conclusions can be drawn about the mode of action or actions the hop ethanol extract may utilize to inhibit the growth of the Gram-positive bacterium, *Listeria monocytogenes* shown in the previous chapter. Further research would include examination of shelf-life and sensory attributes of commercial bologna with applied soy protein isolate with incorporated hop ethanol extract coating.
Figure 5.1: The full electrospray ionization mass spectrum in positive-ion mode of hop ethanol extract.
Figure 5.2: The full electrospray ionization mass spectrum in negative-ion mode of the hop ethanol extract.
Figure 5.3: MS/MS spectrum of peak at 355 $m/z$. 
Figure 5.4: MS/MS spectrum of peak at 363 m/z.
Figure 5.5: MS/MS spectrum of peak at 401 m/z.
Figure 5.6: MS/MS spectrum of peak at 413 m/z.
Figure 5.7: MS/MS spectrum of peak at 415 m/z.
Figure 5.8: MS/MS spectrum of peak at 417 m/z.
Figure 5.9: Chromatograph from gas chromatography-mass spectrometry analysis of hop ethanol extract.
CHAPTER 6

SHELF LIFE AND SENSORY ATTRIBUTES OF BOLOGNA COATED WITH EDIBLE SOY PROTEIN ISOLATE INCORPORATED WITH HOP ETHANOL EXTRACT

6.1 Summary

Active packaging such as edible films and coatings with incorporated antimicrobials could function as an added barrier against handling contamination and microbial growth. Many herbs and plants, including hops, are known for their ability to inhibit bacterial growth. Previous research found that hop ethanol extract, incorporated into edible soy protein isolate (SPI) coating solution, exhibited antimicrobial action against \textit{L. monocytogenes} as a simulated coating and film; and, when the antimicrobial coating was applied to bologna samples, it exhibited a statistically significant ($P \leq 0.05$) bacteriostatic effect. This experiment analyzed the SPI+hop antimicrobial edible coating for differences in shelf-life and sensory when applied to a store-bought, commercialized bologna. Results indicate a significant difference ($P \leq 0.05$) in instrumental a* and b* color values, yet no significant ($P > 0.05$) difference in sensory detection of atypical color. Although only slightly noticeable, the SPI+hop coating contributed a significant ($P \leq 0.05$) bitter taste. Variation in lipid oxidation was not significant ($P > 0.05$). The SPI+hop coating increased moisture significantly ($P \leq 0.05$). Overall, the findings indicate that the SPI+10%hop antimicrobial coating produced minimal effects on shelf-life and sensory attributes of bologna.
6.2 Introduction

Edible films and coatings have been around for centuries. These include natural casings, cellulose casings, sugar coatings used in cereals and even gelatin coatings used on medicines. Because the latest consumer trend is for more natural, less synthetic foods and food ingredients, there is increased interest in edible films. Consumer trends in food safety also focus interest in active antimicrobial edible food packaging. Microbial contamination not only increases the risk of foodborne illness, but reduces shelf-life of foods (Quintavalla and Vicini 2002). Hence the consumer would have an added line of defense while decreasing environmental impact and use of synthetic ingredients.

The general purpose of edible films and coatings is to preserve quality and shelf-life by acting as a barrier to prevent or limit the migration of moisture and gases. Hence, shelf-life analysis should examine oxidation, moisture loss and color change. Wu and others (2000) evaluated edible wheat gluten, soy protein, carrageenan and chitosan films and coatings on precooked beef patties and found that all coatings were as effective at reducing moisture loss as the control of polyvinyl chloride film.

Although there is the promise of food safety, an edible film or coating that produces detrimental change in food characteristics such as taste, aroma and color, regardless if it was found to be an effective inhibitor of L. monocytogenes, would not be accepted by the consumer. Thus, the evaluation of sensory properties of antimicrobial films and coatings is of high importance to indicate acceptance by consumers. Chen (1995) found milk protein-based films to be generally flavorless and tasteless.
In previous work, hop ethanol extract produced by extracting hops pellets with 75% ethanol (1:10 w/v) then filtering was found to be an effective antimicrobial. Hop ethanol extract at 10% inclusion in soy protein isolate coating solution inhibited *Listeria monocytogenes* when applied to bologna core samples. The initial objective was to identify a plant derived extract that would remain an effective antimicrobial when incorporated into an edible film or coating, while imparting minimal effects on visual, aroma and taste characteristics.

In an effort to further understand the effects the antimicrobial SPI+hop ethanol extract edible coating may have on the visual, aroma and taste characteristics of a RTE meat, a commercial, store-bought bologna was coated with the antimicrobial edible coating and evaluated for shelf-life and sensory characteristics. Commercial bologna slices were either left uncoated, coated with SPI edible solution or coated with SPI incorporated with 10% hop ethanol extract. The treated slices were subjected to a storage time of 10 days at 7 ± 1°C using vacuum packaging under fluorescent lighting and resealable storage bags under dark lighting. Shelf-life indicators such as oxidation, color, moisture and spoilage organisms were analyzed, as well as a full sensory analysis using a trained panel.

6.3 Materials and Methods

6.3.1 Antimicrobial Hop Extract:

A hop ethanol extract was produced using dried hops pellets of the Chinook variety (Hopunion LLC, Yakima WA) and 75% ethanol in a 1:10 w/v ratio, modified
from Kong and others (2007). The hop extract had an average pH of 5.1 ± 0.2. The solution was blended in a Waring commercial blender (Waring® Products, Stamford CT) at medium speed for approximately 30 sec. The solution was allowed to rest for approximately 5 min, until there is noticeable swelling of the pellets, before it was blended at medium speed for an additional 30 sec. After another 5 min of rest, the solution was pulse blended before pouring into a vacuum filter system to remove solids. The solution was then filtered through a 0.22 µm filter to remove possible microbial contamination and finish preparation. The hop extract was stored in sterile containers at 7 ± 1°C until further use.

6.3.2 Edible Protein Solution:

Soy protein isolate (SPI) was obtained from a commercial source. A large batch of edible SPI solution was prepared using protocol by Wang and others (2012) and Jiang and others (2012), with slight modification. Approximately 400 mL of nanopure water was added to a 1000 mL flask then zeroed on a scale to add 1.64 g of glycerol drop wise. SPI was weighed to 3.9 g and added to the solution. The solution was stirred for approximately 30 min to ensure homogenization. After adjusting pH to 8.0 ± 0.2 pH using ColorpHast® pH 2.0 – 9.0 strips (EMD Chemicals Inc, Gibbstown NJ), the solution was heated in an 85°C bath for 30 min. After heating, the solution was placed in a cooling water bath while continually stirring to bring the solution down to room temperature.
6.3.3 Applying Antimicrobial Edible Coating:

The SPI solution was divided into two portions. One portion was incorporated with 10% (v/v) hop ethanol extract while the other remained untreated. Both solutions were stirred for approximately 15 min to ensure complete homogenization. For the sensory analysis, all ingredients used were food grade and all equipment used was thoroughly washed.

The same three treatments, 1) no coating, 2) SPI coating and 3) SPI+hop ethanol extract coating, were used for both shelf-life and sensory analysis. All equipment used, glassware, wire racks, stir bars, etc were previously autoclaved to ensure samples were aseptic as possible. A popular commercial brand bologna was purchased at a local supermarket. Bologna was aseptically removed from the package, dipped or not dipped in the appropriate coating solution depending on the treatment, and laid on sterilized racks to dry for approximately 15 min. Vinyl gloves were used to transfer the coated bologna into vacuum bags. The bags were vacuumed at 95% and placed in appropriate refrigerated storage (7 ± 1°C). Shelf-life and sensory bologna trials were initiated on separate days to keep sample numbers to a minimum to aide in the sterility of the project.

6.3.4 Shelf-life Analysis

To simulate consumer purchase of a commercialized bologna, shelf-life was examined on the same samples over two storage conditions: 1) 7 day storage of vacuum packaging under fluorescent refrigeration (7 ± 1°C) was used to simulate opening of the package of bologna bought by the consumer then placing it in a resealable storage bag before storing it in the refrigerator.
Shelf-life was examined using three measurement parameters; 1) color, 2) oxidation and moisture, and 3) spoilage organisms. Triplicate bologna slices were prepared per treatment (no coating, SPI and SPI+hop) per measurement group, plus 9 slices for baseline values, for a total 36 slices. The 9 slices for baseline (Day 0) values were measured for oxidation, moisture and spoilage organisms. The remaining 27 slices were divided equally between the three measurement groups. Baseline values for color were taken on the outside of the vacuumed slices alienated for color. With the exception of color, which was measured twice on Day 7/0 both outside and inside the vacuum package, all measurements were taken on Day 0 (baseline, vacuum), Day 7/0 (vacuum/resealable storage bag), Day 3 (resealable storage bag), Day 7 (resealable storage bag) and Day 10 (resealable storage bag). The slices reserved for measurement group 1 were left whole to measure color. The slices reserved for measurement groups 2 and 3 were sampled using a thumb and forefinger on the outside of the package to pinch/cut a quarter of the meat and aseptically slide the quarter sample from package for analysis (Fig 6.1). Shelf-life analysis was run in duplicate.

6.3.4.1 Color. A Minolta Chroma Meter CR-300 colorimeter, 1-cm aperture, Illuminant C (Minolta, Osaka Japan) was used to measure color in values of lightness (L*), redness (a*) and yellowness (b*) of the stored bologna. On Day 0, after samples were vacuum packaged, baseline color measurements were taken on the outside of the vacuum package, making sure to calibrate the machine using a piece of the vacuum bag over the white calibration plate. On Day 7 of vacuum packaged, fluorescent storage, the samples were measured for color outside of the vacuum package. Then the package was opened and Day 0 of resealable storage bag, dark storage color measurements were taken
directly on the bologna surface. Day 3, 7 and 10 resealable storage bag samples were measured for color values on the bologna surface without packaging. All samples were measured in triplicate.

6.3.4.2 Lipid oxidation. The quarter slice bologna samples were analyzed for 2-thiobarbituric acid reactive substances (TBARS) at each storage time to provide a measure of lipid oxidation. This was done according to Sinnhuber and Yu (1977). Each quarter slice bologna sample (~ 11 g) was finely ground using a food processor, then approximately 5 g of the sample was placed in a 25 mL screw cap culture tube. Approximately 1.5 mL of 1% thiobarbituric acid (TBA) solution was added to each of the 25 mL screw cap culture tubes containing 5 g of the sample, along with 0.075 N NaOH and 8.5 mL 2.5% trichloroacetic acid (TCA) solution with 0.036 N HCl. The samples were vortexed and heated at 100 °C for 30 min. The samples were then placed in an ice bath and allowed to cool to room temperature before approximately 5 mL of the supernatant were transferred into 15 × 125 mm glass centrifuge tubes. Then, 5 mL of chloroform was added to each sample before vortexing and centrifugation at 1792 × g for 10 min. The supernatant was transferred to a 15 × 125 mm glass centrifuge tube and approximately 2 mL of petroleum ether was added to remove residual cloudiness. Samples were again vortexed and centrifuged at 1792 × g for 10 min. The lower phase was read at 532 nm.

6.3.4.3 Moisture. From the remainder of the finely ground quarter sample, approximately 3 g was used for moisture analysis. To measure percent moisture, two 9 cm glass fiber sample pads (Data Support Co Inc, Encino CA) were zeroed in the DSC HFT 2000 fat and moisture analyzer for beef and pork (Data Support Co Inc, Encino
The 3 g of sample was spread onto the smooth surface of one of the filter pads using a spatula. The other filter pad was then pressed on top, smooth surface towards sample before being placed in the moisture analyzer and percent moisture was measured.

6.3.4.4 Spoilage organisms. The quarter slice sample used for analysis of spoilage organisms was slid from the vacuum package aseptically into a stomacher bag. A 10x dilution was made using buffered, sterilized peptone and massaged. Serial dilutions were produced to obtain accurate plate counts. A micropipettor was used to dispense approximately 1 mL of each dilution onto a labeled petrifilm. The plastic concave device was used to spread the dilution in a circle over the surface of the petrifilm. All prepared petrifilms were then incubated for 48 hrs at 23 ± 1°C.

6.3.5 Sensory Analysis:

6.3.5.1 Sensory panel. This study was approved by The Institutional Review Board (IRB). A taste panel consisted of 9 semi-trained individuals of varying race and age. These panelists were selected based on their general liking of bologna. All panelists signed consent forms that revealed ingredients and associated risks. The panelists were trained to detect atypical attributes compared to a commercial, store bought bologna. Each panelist was sat at a separate cubical and provided with water, unsalted crackers to cleanse the pallet, and a small cup with a few coffee beans to cleanse the olfactory.

6.3.5.2 Sensory cards. Two separate score cards were used to evaluate visual attributes and palatability attributes (Figs 6.6 and 6.7). A 6-point hedonic scale was used on the sensory cards. An even number was selected so that the panel members were forced to make a decision of either ‘slightly like’ or ‘slightly dislike’ instead of having...
the option of ‘neither like nor dislike’. Palatability was evaluated for the following attributes: atypical aroma, bitter taste, chewiness, juiciness, texture and overall acceptance. Visual attributes included moisture, atypical color and overall acceptance. There was a comment line on both cards. The palatability attributes were assessed under red light prior to the panelists observing samples for visual attributes under fluorescent light to avoid potential perception interference of visual response to palatability response.

6.3.5.3 Bologna samples. A total of 28 slices of bologna per treatment (no coating, SPI and SPI+hop) were needed for sensory analysis; 9 slices per storage day (0, 2 and 4), plus one slice per treatment for microbiological analysis. Bologna were treated and packaged three days prior to initial sensory evaluation so that pathogen and spoilage organism analysis could assure that the samples were safe for consumption. One slice of each treatment was randomly picked to run microbial analysis. This was done by stomaching two separate quarter sections of the slice. Serial dilutions were made and plated on petrifilm. To obtain enumeration of pathogenic bacteria, two petrifilms per dilution were stored for 48 hrs in 37°C; and, to obtain enumeration of spoilage bacteria, two petrifilms per dilution were stored for 48 hrs in 23 ± 1°C. The remaining 27 bologna slices were vacuumed packaged at 3 slices per 12x16 in vacuum bag. The vacuum packaged bologna slices designated for sensory were stored at 7 ± 1°C in dark conditions.

On Day 0, each vacuum bag of bologna was opened on one end to remove one slice per bag. The opened vacuum bags, still having two bologna slices, were placed into resealable storage bags which were then sealed and placed immediately back into refrigerated storage. For flavor analysis, the slices were placed in a stack of 6 and the entire stack was cut from the top into 9 pie shaped slices. This resulted in enough pie
slices for each panelist to examine each treatment 3 times with 2 slices per treatment. A bowl of 18 randomly generated three-digit numbers was prepared. These numbers were randomly drawn from the bowl on each day of sampling and assigned to a treatment. The panelists were reminded that the numbers were mixed and redrawn each day. For visual analysis, three bologna slices per treatment were left whole and placed on individual plates. Each plate was assigned one of the three-digit numbers (Fig 6.5).

The panelists were sat in a sensory room in individual booths lit with red light. Each panelist received a total of three plates with three different samples per plate; each sample was assigned a random three-digit number. After panelists were finished with the flavor analysis, they were ushered into a room to evaluate samples for visual attributes. Visual analysis samples were randomly laid across a counter, displayed under fluorescent light, with approximately 2.5 ft between each sample. Panelists were reminded to evaluate each sample individually and not to compare between samples.

6.3.6 Statistical Analysis

Statistical analysis was performed on the shelf-life measurements by factorial design of 3 treatments x 6 storage applications (temperature and time) using SAS (SAS Institute Inc., Cary, NC, USA) GLM ANOVA. Statistical analysis was performed on the sensory attributes by factorial design of 3 treatments x 9 attributes using SAS (SAS Institute Inc., Cary, NC, USA) GLM ANOVA. Significant differences were found using least mean squares.

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6.4 Results and Discussion

6.4.1 Shelf-life Attributes

6.4.1.1 Color. It is common for a consumer to evaluate color of a food as indication of freshness and safety, making minimal color change desirable when applying an antimicrobial coating. The Minolta provides measurement of lightness (L*), redness (a*) and yellowness (b*). There were no significant differences observed in L* values within storage between no coating, SPI and SPI+ hop treatments (Fig 6.2). The only significant difference in L* values found was within the control treatment, between Day 7/0 inside the vacuum package and the following three storage days (Day 3, 7 and 10).

The redness values (a*) did not indicate substantial variation, however, the control samples were found to be significantly more red than the coated treatments. The treatments were not significantly different over the storage periods. The loss of redness could be due to the off-white color of the soy protein isolate solution used to produce the coatings. The b* values were found to be significantly higher in the SPI+ hop treatments and there were no significant differences found within treatments over the different days of storage. The L*, a* and b* values held the same trend over vacuum, fluorescent storage and resealable storage bag, dark storage.

6.4.1.2 Lipid oxidation. Oxidation of lipids can affect food quality by limiting shelf-life and altering flavor and mouthfeel. Lipid oxidation was evaluated with the measurement of TBARS. No significant differences were found in TBARS levels between all three treatments within storage or day. There were also no significant differences (P > 0.05) within each treatment over the storage periods (Fig 6.3). Biswas
and others (2004) found no significant difference in pH, TBARS and Warner-Bratzler shear force between uncoated precooked pork patties containing BHA or BHT, and those enrobed in a flour batter.

6.4.1.3 Moisture. Moisture loss is a critical factor effecting RTE meat quality. The SPI coated and SPI+hop coated bologna had significantly higher ($P \leq 0.05$) moisture levels than the control over all storage days (Fig 6.4). This is most likely due to the added moisture of the coating which was still tacky upon packaging.

6.4.1.4 Spoilage organisms. Overall, there were only one to two growths on the lowest dilution of 10x1 found on random plates over all three treatments (Table 6.1). There were no significant differences ($P > 0.05$) found between or within treatments. The few counts observed were thought to be due to pre-packaging contamination by ubiquitous organisms found in the laboratory.

6.4.2 Sensory Attributes

6.4.2.1 Palatability attributes. The spider charts (Fig 6.8) provide a visual representation of the mean response values to the treatments (no coating, SPI and SPI+hop) over the evaluation period of 0, 2 and 4 days. When observing the spider charts, it was apparent that the bitter taste, although only slightly detectable, accompanying the inclusion of hop extract into the coating correlates with the decrease in overall acceptance. There were also a few comments associated with the SPI+hop treatment that indicates other tastes picked up, such as oniony taste and sweet taste. By Day 2 and 4, the SPI+hop treatment had significantly higher ($P \leq 0.05$) mean values for atypical aroma (Table 6.2). The bitter taste attribute was found to be significantly higher
(\(P \leq 0.05\)) in the SPI+hop coated samples versus the no coating and SPI coated bologna. These attributes seemed to correlate with mean scores of overall acceptance since the SPI+hop treatment had significantly lower (\(P \leq 0.05\)) scores than the other two treatments on all three sampling days. There were no significant differences (\(P > 0.05\)) within or between treatments in texture, juiciness and chewiness.

6.4.2.2 Visual attributes. The visual attributes evaluated were kept to a minimal with only moisture, color and overall acceptance on the sensory card. By using spider charts to plot mean response values, a general overview of treatment effect on visual attributes is shown (Fig 6.9). The overall acceptance scores for the no coating treatment are significantly higher (\(P \leq 0.05\)) than the coated treatments on Days 0 and 4, but not on Day 2. This could be due to the visual moisture difference found. Visual moisture observed was significantly higher (\(P \leq 0.05\)) in both the coated treatments than the no coating treatment, which correlates with the moisture analysis in the shelf-life study. There were no significant differences (\(P > 0.05\)) found in color between the no coating and coated treatments over the entire storage period. This suggests that although the Minolta can detect extremely subtle color changes, the human eye cannot (Table 6.3).

6.5 Conclusion

Overall, the SPI+hop coating is very promising as an antimicrobial coating, as observed in previous chapters, which when applied to commercial bologna, does not contribute large effects to shelf-life and sensory attributes. Even with significant differences in a* and b* values observed by the Minolta, the sensory analysis of color
suggested there was no color difference between the coated treatments and the no coating bologna. There were no differences in TBARS values between treatments, meaning there was no difference in lipid oxidation, and there were no comments suggesting rancidity issues. Hence, the SPI and SPI+hop coatings did not alter typical oxidation of the bologna. Both SPI and SPI+hop coating treatments significantly increased percent moisture of the bologna. Significant differences found in mean values of atypical aroma and bitter taste were thought to be the cause of the decrease in overall acceptance on the palatability sensory analysis; however, these differences were only slightly noticeable to the panelists.
Prepare bologna at 12 slices per treatment (no coating, SPI and SPI+hop)

Vacuum package all slices (one slice per vacuum bag)

Measure Baseline values for Measurement groups 2 and 3 using samples labeled for baseline

Measure Baseline values for Measurement group 1 using samples labeled for color on the outside of vacuum bag

Place remaining packages in display cooler at 7 ± 1°C with fluorescent lighting

On Day 7 of vacuum/fluorescent storage, all bags were opened and sampled for Measurement groups 1, 2 and 3. Measurement group 1 was also measured on the outside of the vacuum bag

All opened vacuum bags were sealed in resealable storage bags and stored in dark conditions at 7 ± 1°C until further sampling days

<table>
<thead>
<tr>
<th>No Coating</th>
<th>SPI Coating</th>
<th>SPI+hop Coating</th>
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<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidation and Moisture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spoilage Organisms</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6.1:** Experimental design and flowchart for the shelf-life study on coated bologna.
Figure 6.2: Comparison of color values (L*, a*, b*) between three soy protein isolate (SPI) coating treatments (no coating, SPI and SPI+hop) over storage. Day 7/0 outside is representative of color measurements taken on the outside of the vacuum package and Day 7/0 inside is representative of color measurements directly on bologna surface.

* abc within storage between treatments, different letters differ significantly ($P < 0.05$).
* xyz within treatment between baseline and Day 7/0 outside, different letters differ significantly ($P < 0.05$).
* ABC within treatment between Day 7/0 inside and Day 3-10, different letter differ significantly ($P < 0.05$).
* Standard error was 0.4259 for all L* observations according to least squares mean.
* Standard error was 0.2517 for all a* observations according to least squares mean.
* Standard error was 0.0956 for all b* observations according to least squares mean.
Figure 6.3: Comparison of TBARS values between the three treatments (no coating, SPI and SPI+hop) over storage periods at 7 ± 1°C.

*abc within storage between treatments, different letters differ significantly ($P < 0.05$).
*ABC within treatment between storage, different letters differ significantly ($P < 0.05$).
*Standard error was 0.0121 for all observations according to least squares mean.
Figure 6.4: Comparison of moisture between the three treatments (no coating, SPI and SPI+hop) over storage periods at 7 ± 1°C.

*abc within storage between treatments, different letters differ significantly ($P < 0.05$).
*ABC within treatment between storage, different letter differ significantly ($P < 0.05$).
*Standard error was 0.1569 for all observations according to least squares mean.
Table 6.1: Colony forming unit (CFU/mL) counts for no coating, soy protein isolate (SPI) and SPI+hop coating treatments on bologna over storage days 0, 7/0, 3, 7 and 10 at 7 ± 1°C.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>No coating</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SPI</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SPI+hop</td>
<td>0.833</td>
</tr>
<tr>
<td>Day 7/0</td>
<td>No coating</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SPI</td>
<td>2.500</td>
</tr>
<tr>
<td></td>
<td>SPI+hop</td>
<td>0.833</td>
</tr>
<tr>
<td>Day 3</td>
<td>No coating</td>
<td>2.500</td>
</tr>
<tr>
<td></td>
<td>SPI</td>
<td>0.833</td>
</tr>
<tr>
<td></td>
<td>SPI+hop</td>
<td>0.00</td>
</tr>
<tr>
<td>Day 7</td>
<td>No coating</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SPI</td>
<td>1.667</td>
</tr>
<tr>
<td></td>
<td>SPI+hop</td>
<td>0.000</td>
</tr>
<tr>
<td>Day 10</td>
<td>No coating</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SPI</td>
<td>1.667</td>
</tr>
<tr>
<td></td>
<td>SPI+hop</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Prepare bologna at 28 slices per treatment (no coating, SPI and SPI+hop)

Vacuum package all slices at 3 slices per bag leaving 1 slice per treatment for microbial analysis

Place all vacuum packaged slices in sensory refrigerator at 7 ± 1°C

On Day 0, open all vacuum bags and remove 1 slice per bag

Per treatment, stack 6 slices to cut into pie shapes for taste/aroma sensory analysis so that each panelist samples each treatment 3 times, 3 different randomly generated numbers

Place the remaining 3 slices on individual plates with each having a randomly generated number for visual sensory analysis

Place all opened vacuum bags into sealed resealable storage bags until further sampling on Days 2 and 4

Figure 6.5: Experimental design for the sensory study on coated bologna.
<table>
<thead>
<tr>
<th>Atypical Bologna Aroma</th>
<th>Non-detectable</th>
<th>Moderately Mild</th>
<th>Slightly Intense</th>
<th>Moderately Intense</th>
<th>Extremely Intense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bitter Taste</td>
<td>Non-detectable</td>
<td>Moderately Mild</td>
<td>Slightly Intense</td>
<td>Moderately Intense</td>
<td>Extremely Intense</td>
</tr>
<tr>
<td>Texture</td>
<td>Extremely Gritty</td>
<td>Moderately Gritty</td>
<td>Slightly Smooth</td>
<td>Moderately Smooth</td>
<td>Extremely Smooth</td>
</tr>
<tr>
<td>Chewiness</td>
<td>Extremely Chewy</td>
<td>Moderately Chewy</td>
<td>Slightly Tender</td>
<td>Moderately Tender</td>
<td>Extremely Tender</td>
</tr>
<tr>
<td>Juiciness</td>
<td>Extremely Dry</td>
<td>Moderately Dry</td>
<td>Slightly Juicy</td>
<td>Moderately Juicy</td>
<td>Extremely Juicy</td>
</tr>
<tr>
<td>Overall Acceptability</td>
<td>Extreme Dislike</td>
<td>Moderately Dislike</td>
<td>Slightly Like</td>
<td>Moderately Like</td>
<td>Extreme Like</td>
</tr>
</tbody>
</table>

**Figure 6.6:** The texture/aroma score card used to evaluate the bologna samples with edible coating treatments (no coating, SPI and SPI+hop).

<table>
<thead>
<tr>
<th>Moisture</th>
<th>Extremely Dry</th>
<th>Moderately Dry</th>
<th>Slightly Moist</th>
<th>Moderately Moist</th>
<th>Extremely Moist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atypical Bologna Color</td>
<td>Non-detectable</td>
<td>Moderately Mild</td>
<td>Slightly Intense</td>
<td>Moderately Intense</td>
<td>Extremely Intense</td>
</tr>
<tr>
<td>Overall Acceptability</td>
<td>Extreme Dislike</td>
<td>Moderately Dislike</td>
<td>Slightly Like</td>
<td>Moderately Like</td>
<td>Extreme Like</td>
</tr>
</tbody>
</table>

**Figure 6.7:** The visual score card used to evaluate the bologna samples with edible coating treatments (no coating, SPI and SPI+hop).
Figure 6.8: Comparison of palatability sensory attributes between bologna samples with edible coating treatments (no coating, SPI and SPI+hop) over 0, 2 and 4 days of storage at 7 ± 1°C.
Table 6.2: Palatability sensory attribute scores of bologna samples with edible coating treatments (no coating, SPI and SPI+hop) over 0, 2 and 4 days of storage at 7 ± 1°C.

*abc within storage between treatments, different letters differ significantly ($P < 0.05$).  
*ABC within treatment between storage, different letter differ significantly ($P < 0.05$).  
*Std error was 0.0973 for atypical aroma observations according to least squares mean  
*Std error was 0.1151 for bitter taste observations according to least squares mean  
*Std error was 0.0613 for chewiness observations according to least squares mean  
*Std error was 0.0485 for texture observations according to least squares mean  
*Std error was 0.0973 for juiciness observations according to least squares mean  
*Std error was 0.0973 for overall acceptance observations according to least squares mean

<table>
<thead>
<tr>
<th></th>
<th>Atypical Aroma</th>
<th>Bitter Taste</th>
<th>Chewiness</th>
<th>Texture</th>
<th>Juiciness</th>
<th>Overall Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.56 aA</td>
<td>1.36 bA</td>
<td>3.99 aA</td>
<td>3.58 aA</td>
<td>3.96 aA</td>
<td>4.31 aA</td>
</tr>
<tr>
<td>SPI</td>
<td>1.56 aA</td>
<td>1.26 bA</td>
<td>3.94 aA</td>
<td>3.61 aA</td>
<td>4.13 aA</td>
<td>4.52 aA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.72 aA</td>
<td>1.98 aB</td>
<td>3.96 aA</td>
<td>3.63 aA</td>
<td>4.11 aA</td>
<td>3.63 bA</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.19 bB</td>
<td>1.33 bA</td>
<td>3.92 aA</td>
<td>3.57 aA</td>
<td>4.00 abA</td>
<td>4.22 aA</td>
</tr>
<tr>
<td>SPI</td>
<td>1.28 bB</td>
<td>1.37 bA</td>
<td>4.02 aA</td>
<td>3.54 aA</td>
<td>4.09 aA</td>
<td>4.24 aA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.91 aA</td>
<td>2.46 aA</td>
<td>3.91 aA</td>
<td>3.61 aA</td>
<td>4.22 aA</td>
<td>3.44 bA</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.22 bB</td>
<td>1.30 bA</td>
<td>3.87 aA</td>
<td>3.48 aA</td>
<td>3.98 bA</td>
<td>4.44 aA</td>
</tr>
<tr>
<td>SPI</td>
<td>1.35 bAB</td>
<td>1.41 bA</td>
<td>3.94 aA</td>
<td>3.52 aA</td>
<td>4.22 aA</td>
<td>4.27 aA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.72 aA</td>
<td>2.19 aAB</td>
<td>3.87 aA</td>
<td>3.58 aA</td>
<td>4.20 aA</td>
<td>3.68 bA</td>
</tr>
</tbody>
</table>
Figure 6.9: Comparison visual sensory attributes of moisture, color and overall acceptance between three treatments, no coating, soy protein isolate (SPI) and SPI+hop, over 0, 2 and 4 days of storage at 7 ± 1°C.
Table 6.3: Data from sensory attributes of visual analysis between three treatments, no coating, soy protein isolate (SPI) and SPI+hop, over 0, 2 and 4 days of storage at 7 ± 1°C.

*abc within storage between treatments, different letters differ significantly ($P < 0.05$).
*ABC within treatment between storage, different letter differ significantly ($P < 0.05$).
*Standard error was 0.1139 for all observations according to least squares mean
*Std error was 0.0973 for atypical color observations according to least squares mean
*Std error was 0.0973 for visual moisture observations according to least squares mean
*Std error was 0.0973 for overall acceptance observations according to least squares mean

<table>
<thead>
<tr>
<th></th>
<th>Atypical Color</th>
<th>Visual Moisture</th>
<th>Overall Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.44 bA</td>
<td>3.46 bAB</td>
<td>4.11 aAB</td>
</tr>
<tr>
<td>SPI</td>
<td>1.59 abA</td>
<td>5.20 aA</td>
<td>3.76 bA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.67 aA</td>
<td>4.83 aA</td>
<td>3.83 bAB</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.31 aA</td>
<td>3.13 bB</td>
<td>3.93 aB</td>
</tr>
<tr>
<td>SPI</td>
<td>1.24 aB</td>
<td>5.00 aA</td>
<td>3.98 aA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.29 aB</td>
<td>4.80 aA</td>
<td>4.02 aA</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coating</td>
<td>1.39 aA</td>
<td>3.81 bA</td>
<td>4.26 aA</td>
</tr>
<tr>
<td>SPI</td>
<td>1.37 aB</td>
<td>5.04 aA</td>
<td>3.81 bA</td>
</tr>
<tr>
<td>SPI+hop</td>
<td>1.33 aB</td>
<td>5.22 aA</td>
<td>3.70 bB</td>
</tr>
</tbody>
</table>
CHAPTER 7
OVERALL CONCLUSIONS

The hop ethanol extract produced elicited antimicrobial activity against the Gram-positive bacterium, *Listeria monocytogenes*. All suspected key antimicrobial components of the hop ethanol extract were positively identified, i.e., various forms of humulone, lupulone, and xanthohumol. By identifying these compounds, conclusions can be drawn about the mode of action or actions the hop ethanol extract may utilize to inhibit the growth of the Gram-positive bacterium, *Listeria monocytogenes*. The weak acids (humulones and lupulones) are thought to disrupt the bacterial cell membrane via hydrophobic interactions, mainly via distinct prenyl side groups (Keukeleire 2000). Organic acids such as lactic, citric and sorbic acids have been linked to the disruption of the ion gradient in bacterial cells by entry of the undissociated form into the bacterial cells, further disrupting proton motive force and inhibiting production of ATP (Jay and others 2005), suggesting an alternate mode of action for the hop acids.

These data suggested the antimicrobial edible coating, produced by incorporating hop ethanol extract into edible SPI solution, was highly bacteriostatic. In this study, initial inoculation was high enough (10⁴) to produce quantifiable results; however, in everyday circumstances, most food contamination would be at a much lower level (only about 10 cells required to cause illness). Based on the data presented herein, he tested antimicrobial edible coating would act as an additional hurdle and possibly eradicate normal, lesser levels of contaminating cells; thereby, preventing foodborne illness. Further research should involve better understanding of the mode of action of the hop ethanol extract, then combining the hop ethanol extract with substances that use different
modes of action to achieve a synergistic or additive antimicrobial affect. For example, nisin works by producing pores in the cell wall of bacteria and a combination of nisin with hop extract may result in a greater population reduction. The SPI+hop coating is very promising when considering the effects on shelf-life and sensory attributes. Even with significant differences in a* and b* values observed by the Minolta, the sensory analysis of color suggested there was no color difference between the coated treatments and the control bologna. There were no differences in TBARS between treatments and there were no comments suggesting rancidity issues. Significant differences found in mean values of atypical aroma and bitter taste were thought to be the cause of the decrease in overall acceptance on the taste/aroma sensory analysis; however, these attributes were found to be only slightly noticeable. Overall, the SPI+hop coating elicited a bacteriostatic effect with no changes in shelf-life and only slightly noticeable changes in sensory attributes associated with aroma and bitter taste.

Overall, the results showed that hop ethanol extract was inhibitory of *Listeria monocytogenes* strains ATCC 4644, UKADL and ATCC 49594. Key antimicrobial components, as mentioned above, were identified via mass spectrometry. The hop ethanol extract, when incorporated at 10 and 20% (v/v) into edible soy protein isolate (SPI) films, continued to exhibit antimicrobial action against the three *L. monocytogenes* strains. The SPI with 10% incorporated hop ethanol extract (SPI+10%hop) antimicrobial coating was applied to bologna samples, prepared without *L. monocytogenes* inhibitors, where it exhibited a significant (*P ≤ 0.05*) bacteriostatic effect against strain ATCC 4644. The SPI+10% hop coating was then applied to a commercial bologna to examine effects on shelf-life and sensory attributes. Significant differences (*P ≤ 0.05*) were found in
instrumental red and yellow colors, however not in sensory color. There was no significant difference ($P > 0.05$) found in measured lipid oxidation between the bologna with no coating, SPI coating or SPI+10%hop coating. The incorporation of hop did cause a slightly bitter taste. Overall, the findings indicate that the SPI+10%hop antimicrobial coating functioned as an inhibitor of *L. monocytogenes* while producing minimal effects on shelf-life and sensory attributes of bologna.
Research was performed to evaluate hop extracts as added antimicrobial ingredients in bologna. The first of three studies examined both hop ethanol extract and hop water extract. A large batch of bologna was made in the meat lab using a standard ingredient packet including nitrites. Common *Listeria monocytogenes* inhibitors such as sodium diacetate and sodium lactate were not added. The bologna was then portioned into 300 g aliquots into large stomacher bags. One aliquot was used per treatment (hop/ethanol, hop/water, and control). The extracts were added to the stomacher bags with the bologna batter, and the mixture was homogenized by hand massage outside of the bag. Each aliquot was then inoculated with *Bacillus cereus* spores, Difco Spores (Difco Laboratories, Detroit MI) and homogenized similarly. The tip of one corner of each bag was cut so that the aliquot could be piped into a 1 lb disposable aluminum mini loaf pan. The samples were covered with aluminum foil and cooked by floating in an 85°C water bath for approximately 2 h.

After the samples were cooked, they were held at refrigeration temperature (7 ± 1°C) for 24 h. The following day, each sample was cut into 8 (day 0, 1, 3 and 7 with two samples per day) approximately 25 g samples. Each sample was weighed into a stomacher bag and the weight was written on the bag for future dilution. Day 0 samples were massaged, serial diluted and plated on aerobic count petrifilm, then held in a 32°C
incubator for 24 hrs. Day 1, 3 and 7 samples were placed in the refrigerator until the specified sampling day. The results show no difference in treatments (Table 1).

A second bologna study was performed similar to the above but using *L. monocytogenes* ATCC 4644 inoculation post-cooking. The bologna was portioned, treated (hop ethanol extract at 3%, hop water extract at 3% and control), and cooked the same as above, only; it was not inoculated before cooking with *Bacillus* spores, it was inoculated after cooking with *Listeria monocytogenes* ATCC 4644. Each treated loaf was again cut into 8 approximately 25 g samples. The samples were weighed into stomacher bags. Once in the bags, the bologna was inoculated with 25 µg of *L. monocytogenes* ATCC 4644. The inoculum was spread onto one surface of the bologna using a sterile hockey stick. The samples were stored in refrigeration until day of sampling. The results show no difference in treatments (Table 2).

A third bologna study was performed similar to the above. *L. monocytogenes* ATCC 4644 inoculated study only with different treatments. The treatments examined included ground hops pellets, hop ethanol extract and hop water extract. These were all incorporated at 5%. After cooking, the loaves were portioned and inoculated as mentioned above. The results indicate no difference in treatments (Table 3).

Research interest moved to adding the hop extracts to processed cheese which did not require a cook step which was a suspicion as to why the bologna studies were unsuccessful. The most promising extract, hop ethanol extract, was added at 5 and 10% to store bought processed cheese previously weighed into a sterile stomacher bag. The extract and cheese were homogenized via hand massage through the stomacher bag. These treatments were compared to a control of 75% ethanol at the same inclusion levels.
The prepared cheese treatments were stored at 7 ± 1°C for a period of 7 days. Each cheese treatment was aseptically sampled on day 0, 3 and 7 by using an autoclaved spoon to scoop approximately 25 g into a sterile stomacher bag. Dilutions were made and plated on MOX. After 48 hr incubation at 37°C, the plates were counted. Although the hop ethanol extract at 10% almost completely killed off the bacteria, so did the control of 75% ethanol.

These experiments were not duplicated and further research did not occur due to lack of promising results. Lack of promising results could be due to the concentration of the hop and hop extracts added to the foods, however, the foods would not have been functional or favored if these concentrations were to be increased. Statistical analysis was not performed on these data due to low sample number generated.
Table 1: Average plate counts over a 7 day storage period of bologna samples prepared with different ingredients (control, hop water extract and hop ethanol extract) and inoculated with *Bacillus* spores.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.10E+05</td>
<td>1.02E+05</td>
<td>8.10E+04</td>
<td>2.70E+04</td>
</tr>
<tr>
<td>hop water</td>
<td>9.50E+04</td>
<td>5.70E+04</td>
<td>7.50E+04</td>
<td>1.50E+04</td>
</tr>
<tr>
<td>hop ethanol</td>
<td>8.90E+05</td>
<td>3.20E+05</td>
<td>1.50E+05</td>
<td>1.27E+05</td>
</tr>
</tbody>
</table>
Table 2: Average plate counts over a 3 day period of bologna samples prepared with different ingredients (control, hop water extract and hop ethanol extract) and inoculated with *L. monocytogenes* ATCC 4644.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.10E+05</td>
<td>5.50E+04</td>
<td>5.15E+04</td>
</tr>
<tr>
<td>hop water</td>
<td>6.45E+04</td>
<td>5.80E+04</td>
<td>1.02E+05</td>
</tr>
<tr>
<td>hop ethanol</td>
<td>7.80E+04</td>
<td>4.45E+04</td>
<td>1.40E+05</td>
</tr>
</tbody>
</table>
Table 3: Average plate counts over a 3 day period of bologna samples prepared with different ingredients (control, hop water extract, hop ethanol extract and ground hops pellets) and inoculated with *L. monocytogenes* ATCC 4644.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.45E+04</td>
<td>4.90E+04</td>
<td>6.80E+04</td>
</tr>
<tr>
<td>hop water</td>
<td>7.45E+04</td>
<td>5.50E+04</td>
<td>5.80E+04</td>
</tr>
<tr>
<td>hop ethanol</td>
<td>8.30E+04</td>
<td>4.85E+04</td>
<td>4.80E+04</td>
</tr>
<tr>
<td>ground hops</td>
<td>5.90E+04</td>
<td>5.10E+04</td>
<td>5.30E+04</td>
</tr>
</tbody>
</table>

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Vanhoucke LC. 1964. De folklore van de hop in Vlaams-Belgie vroeger en nu (The folklore of hop in Flemish Belgium in the past and at present). Oudenaarde: Drukkeriji Sanderus.


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VITA

Jamie Renee Greene Skudlarek was born Jamie Renee Greene on September 15, 1980 in Boonville, Indiana. She graduated from Boonville High School in 1998 and the University of Southern Indiana with her Bachelor of Science in General Biology in 2004. She went on to earn her Master of Science in Aquaculture in 2009 from Kentucky State University and, began work and class at the University of Kentucky in the fall of 2010. She married Nicholas A. Skudlarek on June 5, 2011.

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