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Effect of Storage Conditions on Stability of Laminated Antimicrobial Films

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ABSTRACT

Foodborne pathogens, such as *Listeria monocytogenes* and pathogenic *Escherichia coli,* are a major concern to public health. Due to the rise in foods containing little to no preservatives, antimicrobial packaging has emerged as an innovative food packaging trend. Foodborne illnesses are often caused by the mishandling of foods at various temperatures, thereby promoting the growth of microorganisms. This project will evaluate the antimicrobial efficacy and film stability of laminated antimicrobial films (LAF) containing lauric arginate (LAE) at various storage temperatures (-20ºC, 4ºC, 25ºC, 35ºC, 55ºC) and humidity (15%, 20%, 25%, 30%, 90%) across an 8-week storage period. With the incorporation of LAE into the experimental films, the growth of microorganisms can be inhibited. The antimicrobial activity of the LAF will be evaluated through plate overlay assays, using approximately 8 $\log_{10}$ CFU of *L. innocua* and non-pathogenic *E. coli* K-12. After conducting two trials, the greatest inhibition against *L. innocua* and *E. coli* K-12 was observed during the first four weeks, specifically at lower storage temperatures (-20ºC, 4ºC, 25ºC), although inhibition varied throughout the course of the 8 weeks under most storage conditions. The study data demonstrated that lauric arginate exhibits inhibition against *Listeria* spp. and *E. coli* when stored at temperatures ranging between –20ºC and 55ºC and humidities between 15% and 90%, especially in the first four weeks of storage, although additional replicates should be conducted to verify these results. The results from this project will be useful to the food and packaging industries that are interested in the commercialization of the LAF.

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Chapter 1

Literature Review

Introduction

The presence of food spoilage and pathogenic microorganisms in food is of frequent concern as it relates to the shelf life of foods and the health and safety of humans, respectively. These concerns can be addressed within a food processing environment by the implementation of sanitation and hygienic procedures, as well as interventions such as heat treatments, proper cooling, or incorporation of antimicrobials into food formulations. These approaches address concerns during processing, although another crucial step could be the incorporation of antimicrobials onto food surfaces through packaging to minimize microorganisms that come from the production facility or the consumer's environment. Several antimicrobials are used in food formulations, such as benzoic acid, sulfites, nitrites, and propionic acid, among others, although many lack broad inhibition of microorganisms.

Benzoic acid and sulfites have been used in food products for many years and are proven to be effective against target microorganisms, including bacteria, yeast, or molds, although not all work as broad inhibitors. Benzoic acid, for example, is often found on ingredient labels as sodium benzoate. It works well at a lower pH or in high acid foods, preventing the growth of yeasts and molds, as well as some bacteria. It is allowed in foods at maximum levels of 0.1%, as concentrations beyond 0.1% have resulted in peppery and burnt tastes in food products (Jay, 2000). These compounds work by blocking glucose and pyruvate oxidation, encourage proton leakage, and inhibit substrate uptake in cells (Jay, 2000). Sorbic acid, only allowed at a maximum

level of 0.2%, is also more effective in acidic foods, losing its effect at pH levels above 6.5. It has a very similar effect to benzoic acid on microorganisms, affecting molds more commonly than yeasts. It can also inhibit dehydrogenase in yeasts (Jay, 2000). Sulfites, an ingredient often added to wine, is a broad antimicrobial and is bactericidal with increasing concentrations. It has a greater effect on bacteria, but can be effective against yeasts and molds, specifically aerobic species (Jay, 2000). Sulfites can lower oxygen tension below necessary requirements for aerobic species to grow and can inhibit enzymes, such as those responsible for enzymatic browning (Jay, 2000). Lastly, nitrites, primarily added to bacon and other cured meats, inhibit spoilage and pathogenic bacteria, specifically *Clostridium botulinum*. Like many other antimicrobials, effectiveness increases with decreasing pH (Jay, 2000). Nitrites are highly reactive and can serve as oxidizing or reducing agents, which can react with various compounds to inhibit bacterial growth (Jay, 2000). There are other antimicrobials, but these are some of the most effective and therefore, most common.

Lauric arginate (LAE)

Lauric arginate (LAE), an up-and-coming antimicrobial, can be applied to a variety of foods while maintaining broad inhibition of microorganisms and preserving food attributes (Becerril et al., 2013; Terjung et al., 2017). The use of LAE is applicable to a wide range of areas within food manufacturing and safety, from packaging to inhibition of bacterial biofilms. The compound has the potential to serve as a safe antimicrobial against pathogenic and spoilage microorganisms, such as pathogenic *Escherichia coli* and *Listeria monocytogenes* that can cause foodborne illness, as well as yeasts and molds.

LAE is an odorless and colorless antimicrobial and food additive approved for use by the Food and Drug Association (FDA) and USDA-Food Safety and Inspection Service (USDA-

FSIS), capable of maintaining effectiveness in a pH range of 3 to 7 (Becerril et al., 2013). It is synthesized from L-arginine, lauric acid, and ethanol (Figure 1; Ma et al., 2019; Sadekuzzaman et al., 2017).

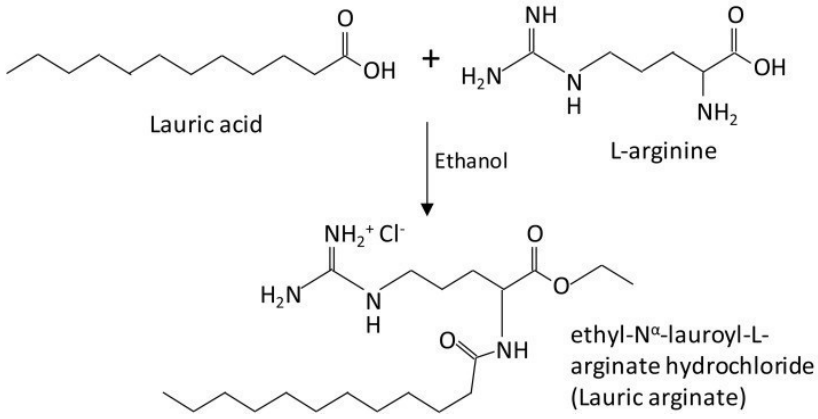

Figure 1: Synthesis of lauric arginate from lauric acid, L-arginine, and ethanol (Infante et al., 1984)

Once inside the body, LAE is hydrolyzed back into L-arginine and lauric acid, both of which are consumed as dietary components (Ma et al., 2019). *In vitro* assays have been performed, showing 50% hydrolysis of LAE to lauroyl arginine after a four-hour incubation period within human plasma (Ma et al., 2019). When analyzing the concentration within the plasma using preliminary dosages of 1.5-2.5 mg/kg, it was below detectable levels at 1 ng/ml (Hawkins et al., 2009). In the stomach, LAE is capable of being hydrolyzed approximately 90% after an hour (Ma et al., 2019). It has been shown to have low toxicity due to this hydrolysis, as both L- arginine and lauric acid are consumed in the human diet (Ruckman et al., 2004). Similarly, neurotoxicity studies have been performed on rats using commercial products containing 25% LAE and doses of 2000 mg/kg/day, which resulted in no signs of neurotoxicity after 13 hours (Ma et al., 2019; Ruckman et al., 2004).

LAE is considered GRAS or "generally recognized as safe" by the FDA, as these studies, along with many others, have found the compound to be safe for its intended uses (U.S. FDA, 2018). It was approved for use in 2005 to improve food quality and safety and has since been applied to meats, poultry, and cheeses in concentrations of 44 mg/kg as "sprayed lethality in container" (SLIC) without labeling requirements and up to 200 ppm as a surface treatment in the United States (Lavieri et al., 2014; Ma et al., 2019; Terjung et al., 2017). LAE is considered both an antimicrobial and food additive according to the FDA. As an antimicrobial, it inhibits growth of specific microorganisms. Antimicrobials added to food packaging materials are excluded from "pesticide chemical" definitions and are regulated by the FDA as food additives, no matter the effect of the antimicrobial on any part of the packaging. Any antimicrobials added directly to the food are also excluded from this definition and must be regulated as food additives as well (U.S. FDA, 1999). Being considered both, LAE has a variety of uses, from inhibiting growth of microorganisms such as *Staphylococcus aureus* and *Escherichia coli* and inactivating biofilms, that can be beneficial to food companies and consumers. (Sadekuzzaman et al., 2017; Becerril et al., 2013).

Mode of Action of LAE

Although the antimicrobial mechanism of LAE is still being characterized, it is suggested that its primary target is bacterial membranes, affecting cell viability without causing cell lysis (Becerril et al., 2013; Bonnaud et al., 2010). The effects of LAE on yeasts and molds are similar, due to its cationic characteristics. When in contact with cells, adherence to their membranes and diffusion into the cytoplasm occurs, where metabolic and other process disruption can occur (Hu et al., 2020). Figure 2 (B and C) demonstrated abnormalities in the cell structure following 3 to

5 minutes of exposure to minimum inhibitory concentrations of 12.5 to 25mg/L LAE, depending on the microorganism. These abnormalities include irregular structures, rougher surfaces, and development of pores, when compared to picture A, which shows healthy and unexposed *E. coli* cells. As the exposure time increases, cell structure weakens and changes become significantly more noticeable, resulting in some cell death (Bonnaud et al., 2010).

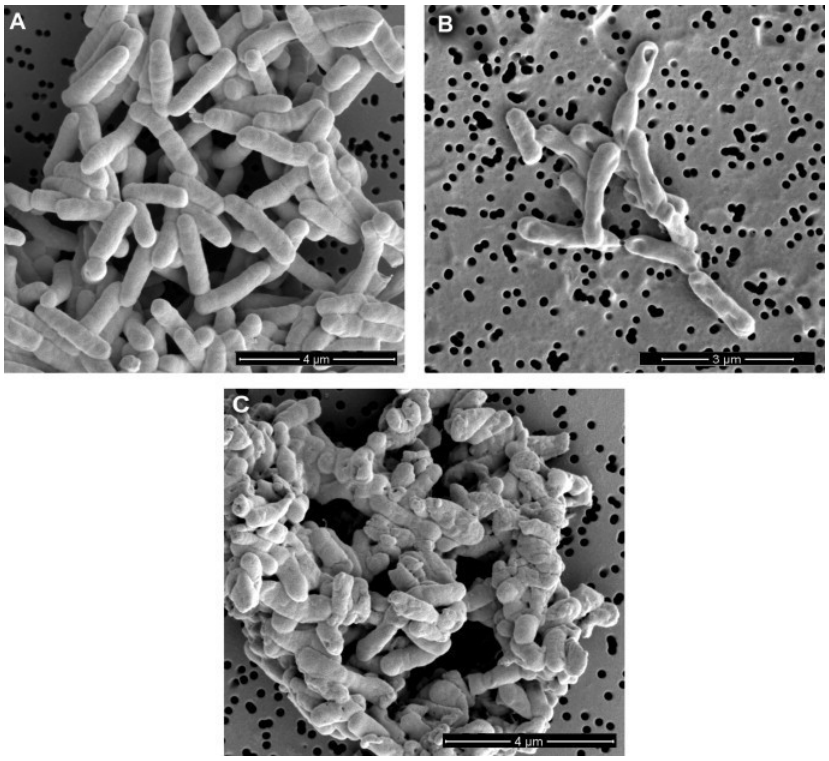

Figure 2: E. coli cell abnormalities following exposure to LAE after 3 to 5 minutes of exposure to MIC of LAE (Becerril et al., 2010).

Studies also were performed using fluorescence and transmission electron microscopy, ion-flux, and flow cytometry across cell membranes in *Salmonella* Typhimurium and *Staphylococcus aureus*. The results indicated outer membrane disruption of *Salmonella* Typhimurium, while the cytoplasm remained unaffected. In *S. aureus,* septation of cytoplasm and mesosome-like structure formation was observed. Using flow cytometry, the membrane

damage was able to be confirmed and identified as leakage of intracellular potassium. There was no cell lysis observed (Rodriguez et al., 2004). Another study using LAE reported deformed *E. coli* O157:H7 cells and abnormal division of cytoplasmic membranes in *L. monocytogenes* cells (Pattanayaiying et al., 2014). More generally, the antimicrobial affects plasma membrane lipid bilayer stability as well as inhibits cellular processes and metabolism (Pattanayaiying et al., 2014).

Packaging and LAE

Food Packaging: Active Packaging

Given the amount of food, water, and other nutrients available for microbial growth, food processing facilities provide favorable conditions for spoilage and pathogenic microorganisms to thrive. Therefore, it is important to minimize microbial growth associated with these organisms by integrating interventions (cooking, chilling, etc.), improving sanitation methods (ex. cleaning and sanitizing), and incorporating packaging to foods (Appendini and Hotchkiss, 2002). Antimicrobials added to food packaging materials are excluded from "pesticide chemical" definitions and are regulated by the FDA as food additives, no matter the effect of the antimicrobial on any part of the packaging (U.S. FDA, 1999).

The incorporation of LAE into various packaging materials to prevent microbial growth has been investigated. For example, researchers have begun developing active packaging which allows for the incorporation of antimicrobials into food packaging materials to create an extra layer of protection against microbes. Examples of active packaging include the use of absorbent pads containing the antimicrobial or incorporation of antimicrobials to a packaging surface

(Appendini and Hotchkiss, 2002). Previous attempts at the use of antimicrobial packaging using essential oils were unsuccessful due to strong aromas and a relatively narrow range of effectiveness against microbes associated with foods. Generally, an antimicrobial should minimize changes to the appearance, taste, smell, and structure of foods while maintaining strong antimicrobial properties (Appendini and Hotchkiss, 2002; Becerril et al., 2013). Because LAE is odorless, transparent, and can work alone or in combination with other antimicrobials, it can maintain food attributes while preventing the growth of microorganisms. A study analyzing the antimicrobial effectiveness of LAE against foodborne pathogens, such as *Staphylococcus aureus* and *Escherichia coli*, determined that it demonstrates rapid and significant antimicrobial activity, with approximately a 3.5 $\log_{10}$ CFU reduction in both genera, in 2 to 6 minutes, even following a heat treatment (Becerril et al., 2013). Similarly, when used against *Listeria monocytogenes* in commercially produced frankfurters, LAE increased initial lethality by 1.8 $\log_{10}$ CFU/package, and in combination with diacetate and lactate, by 2 log CFU/package (Lavieri et al., 2014). While effective, there are concerns about inhibition by LAE against surviving cells (Lavieri et al., 2014). As indicated above, adding antimicrobials to existing food packaging has been shown to be effective against pathogenic and spoilage microorganisms, but there is another, more sustainable yet effective, type of packaging that has been developed.

Food Packaging: Edible Films

Consumer demands and regulations have encouraged the development of environmentally friendly and less wasteful packaging to replace synthetic polymers and plastics (Sadekuzzaman et al., 2017). Edible films, which are either consumable or biodegradable, protect food products from the extrinsic environment and inhibit much of the surface bacteria

that can be found on most meats (Pattanayaiying et al., 2015). The incorporation of both LAE and nisin into ready-to-eat (RTE) packaging made of pullulan inhibited both Gram-positive and Gram-negative pathogenic bacteria, such as *Staphylococcus aureus, Listeria monocytogenes,* and *E. coli* O157:H7 following a 21-day challenge study. Films containing solely LAE also inhibited these organisms but were less effective than in combination (Pattanayaiying et al., 2015).

The recent development of biopolymer packaging: packaging composed of organic materials, has become a popular area of research (Babaremu et al., 2022). With the potential to provide a healthier substitute to plastic, biopolymer packaging uses abundant raw materials to mimic the convenience and appeal of plastic (Babaremu et al., 2022). This type of packaging can be made of a range of different polymers, some of which are biomass derived (Babaremu et al., 2022), including starches and recently, pullulan, a nontoxic and edible compound produced by *Aureobasidium pullulans* (Trinetta and Cutter, 2016). Also, the incorporation of antimicrobials, such as LAE, to inhibit microbial growth when incorporated into these films can further improve the safety and shelf life of foods.

These biopolymer films have become an area of interest for both raw and ready-to-eat (RTE) muscle foods. Research done by Hassan and Cutter (2020) and Cutter and Hassan (2023) consisted of the development of pullulan-based antimicrobial films containing lauric arginate to inhibit several pathogenic microorganisms: *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*. These films were paired with an outer polyethylene package to enhance durability and oxygen and water impermeability (Hassan and Cutter, 2020). Antimicrobial effectiveness of lauric arginate was analyzed using disc diffusion assays to observe resulting zones of inhibition,

as well as a challenge study to determine its inhibition against pathogenic bacteria (Hassan and Cutter, 2020). The minimum inhibitory concentration for all four organisms was determined to be 0.078% and the minimum bactericidal concentrations were 0.625% for *E. coli* and *Salmonella* spp., and 0.313% for *L. monocytogenes* and *S. aureus*, with greater inhibition against Gram-positive bacteria (Hassan and Cutter, 2020). For the disc diffusion assay, films containing strictly 2.5% LAE had zones of inhibition ranging from 8.6 ± 0.57 mm to 15.3 ± 0.57 mm, depending on the organism (Hassan and Cutter, 2020). When applied to raw and RTE meat products, films containing 0.5%, 1%, and 2.5% LAE significantly reduced microbial counts on these products after 28 days of refrigeration (Hassan and Cutter, 2020). Still, further studies need to be performed to better determine the efficacy of these films under various storage conditions.

There are concerns over the stability of biopolymer packaging when stored at various temperatures and humidities. When starch-base films are exposed to a higher humidity, the tensile strength of the film decreases, while the permeability of water vapor increases (Hazrati 2021). More generally, most biopolymer films will become brittle at low humidity and lose strength at higher humidity (Agarwal, 2021). The incorporation of plasticizers, such as glycerol, can decrease this risk by increasing film flexibility and lowering the glass transition temperature (Agarwal, 2021). In terms of temperature, the effects vary depending on the material. Pullulan decomposes at high temperatures (250-280ºC), which would not be impacted by normal or abused storage conditions (Trinetta and Cutter, 2016). The effect of temperature on LAE is much greater, with the potential to cause hydrolysis back into its original components (Ma et al., 2019). The antimicrobial effectiveness of LAE is also correlated with temperature; it has been shown that the antimicrobial effectiveness of LAE decreases at lower temperatures, potentially due to

more resilient organisms (Ma et al., 2019). In terms of microbial cell permeability, higher temperatures result in a faster release of LAE into the cell, which can be beneficial for the reduction of initial microbial counts but can decrease the longer-term shelf life (Kashiri et al., 2019). Although some bacteria can be inhibited by LAE, some may be able to survive after exposure.

Antimicrobial Activity Against Biofilms

Microorganisms can produce their own defense mechanisms, allowing them to be more resilient. An example of this defense mechanism is biofilm production. Biofilms are microcolonies that exist in an extracellular polymetric substance matrix, which provides an extra level of protection from environmental conditions, sanitation (cleaning, sanitization), and disinfectants (Fu et al., 2017). Some organisms, such as *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp., produce these biofilms in food processing environments, which can pose a threat to food and consumer safety due to their resistance to antimicrobial treatments. LAE may be a potential solution to this problem.

In one study, biofilms were grown on stainless steel, rubber, a biofilm eradication concentration device, or lettuce and then subjected to varying concentrations of LAE (50, 100, 200 ppm) for 2 hours. Results of this study demonstrated that strains of *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* were reduced up to 7 $\log_{10}$ CFU/cm^2 on stainless steel; 4 $\log_{10}$ CFU/cm^2 on rubber; and approximately 1.5 $\log_{10}$ CFU/cm^2 on lettuce when treated with 200 ppm LAE (Sadekuzzaman et al., 2017). When *Pseudomonas aeruginosa* samples were treated with varying concentrations of LAE (10, 50, and 100 μM LAE), biofilm formation was inhibited. The overall growth of the microorganism decreased as the concentration of LAE

increased. As discussed earlier, it has been hypothesized that LAE can alter the morphology and structure of the microorganisms by potentially interacting with the cell membrane to cause leakage and ultimately cell death (Kim et al., 2017; Sadekuzzaman et al., 2017). In food processing environments, LAE can be combined in aqueous solution with octanoic, lactic, octenyl succinic, glycolic acids, or EDTA to function as a disinfectant against target biofilms on food contact surfaces (Coughlin, 2010; Sadekuzzaman et al., 2017). Although the mode of inhibition of biofilms by these compounds has yet to be determined, these antimicrobials may provide potential solutions to biofilm formation and overall processing plant safety.

Concerns

The utilization of LAE within the food and beverage industry may be limited due to its ability to interact with other components within food matrices, specifically proteins and lipids (Bonnaud et al., 2010; Terjung et al., 2014). Because it is a cationic compound, it can react with anionic or hydrophobic molecular groups, altering its solubility within aqueous solutions and potentially decreasing the quality of the final product (Bonnaud et al., 2010). This observation is especially pertinent when LAE is used during meat processing. Delivery system solutions have been found, such as oil in water emulsions and solid lipid nanoparticles, where the antimicrobial is included in a carrier lipid. These solutions can decrease interactions and maintain the antimicrobial effectiveness of LAE (Terjung et al., 2014). When combined in a nano-emulsion with Tween 80, a nonionic surfactant, and cinnamon bark oil, there was no decrease in effectiveness when applied to milk samples (Hilbig et al., 2016). Although initial findings indicated potential setbacks for the application of LAE in various food products and packaging

materials, solutions have been identified, increasing the likelihood of using lauric arginate in food processing.

Conclusions

Foodborne illness is of frequent concern and although many attempts have been made to minimize this risk, issues always arise, and microbial growth can prevail. There are current and effective antimicrobials, although they are incredibly dependent on pH and are not applicable to a wider range of foods. LAE is a relatively new compound identified as a broad-spectrum antimicrobial with promising potential. Due to its hydrolysis into dietary components following consumption and its capability in inhibiting pathogenic microorganism growth and biofilm production, LAE can be used as a novel antimicrobial for a variety of foods, from RTE meats to cheeses. From active packaging to sanitation, LAE can be used alone or with other antimicrobials to minimize food spoilage and foodborne illness. There are some potential issues, such as inhibitors in food matrices, that may make the application and commercialization of LAE somewhat more difficult. With more research and a greater understanding of its modes of action and delivery methods using pullulan or LAFs, the use of LAE in foods could be increased.

Chapter 2

Project Introduction

Ready-to-eat (RTE) meats have become increasingly popular among consumers, due to their convenience. However, these meat products are susceptible to contamination with pathogenic microorganisms such as *Listeria monocytogenes,* that may increase foodborne illness among vulnerable populations. Pathogenic strains of *E. coli* have also been found in raw meats, which can be another cause of concern for consumers. These pathogens are also much more tolerant to refrigeration temperatures, meaning their growth will not be inhibited as effectively as other microorganisms by packaging or atmosphere alone (Ma et al., 2019). With the growing emphasis on preventing food waste, it is also crucial to limit the possibility of microbial growth on food products, as it decreases shelf life and causes rapid spoilage of food.

This project will determine the impacts of various storage temperatures and humidities on laminated antimicrobial films (LAFs) containing lauric arginate (LAE) to better understand their stability. This project also will enhance the knowledge about LAF and its shelf life, which is essential for making storage recommendations during the manufacturing process and for potential use by consumers.

The films used for this project consisted of a control LAF and an experimental film made with 2.5% LAE water and laminated onto EVOH (Bedford et al., 2023)..

The proposed project will examine the effectiveness of pullulan-based LAE films laminated onto EVOH and evaluated against nonpathogenic strains of *E. coli* or *Listeria* spp. at various temperatures and humidities using a plate overlay assay. Environmental temepratures and humidities will be collected and visual observations of the LAF will be made over the course of

eight weeks to observe any changes as a result of these storage conditions. It is hypothesized that these films will have varying levels of inhibition against *Listeria* spp. or *E. coli* depending on the temperature and humidity exposure; with films exposed to higher humidities and temperatures being less effective against these microorganisms during plate overlay assays.

Chapter 3

Materials and Methods

1.1 Film Preparation and Storage

Control and experimental films were prepared prior to storage and experimentation. For the control films, a mixture of 135 g pullulan (HPPE, Columbus, GA), 30 g gelatin (VWR; Radnor, PA), and 70 ml glycerol (VWR; Radnor, PA) in 1000 ml of water was prepared, while an experimental film was made from 135 g pullulan, 30 g gelatin, 70 ml glycerol, and 2.5% LAE (Cytoguard; A&B Ingredients, Fairfield, NJ) in 1000 ml of water. Resulting films were applied onto an EVOH film (Transcontinental Films, Chicago, IL) using a Microm II adjustable micrometer film applicator (Paul N Gardener Co. Inc.; Pompano Beach, FL) to a thickness of 1000 µm and left to dry overnight in a biosafety cabinet, allowing the water to evaporate completely, and resulting in a laminated antimicrobial film (LAF).

Prepared LAFs were stored in square petri dishes (VWR; Radnor, PA) in the freezer, refrigerator, desiccator, and incubator at corresponding temperatures: 2^{o}C, 5^{o}C, 25^{o}C, 35^{o}C, and 55^{o}C, and relative humidities: 15% to 90%, for a duration of time, ranging from 0 to 8 weeks. Small magnets or stones were sterilized and placed at the corners of the LAF inside the petri dishes to ensure the films remained flat until sampled on the desired day. These films were stored in temperature–controlled incubators in which the humidity was monitored using a handheld traceable hygrometer (Model 35519-050; VWR; Radnor, PA). Experiments were performed in duplicate. During the experiments, the weather was noted, as the humidity of each location varied with outdoor temperature and humidity.

1.2 Agar Preparation

Tryptic soy agar (TSA; Becton, Dickinson and Sparks, MD) was prepared according to manufacturer's instructions and poured into sterile petri dishes. TSA plates were stored in a refrigerator at 4°C until needed for the plate overlay assays. Tubes of sterile 9 ml tempered (50°C) TSA soft agar were prepared using 2% of the original TSA agar formulation in distilled water. Once autoclaved, tubes were stored in at 4°C until needed.

1.3 Stock Preparation from Freezer Stocks

Freezer stocks (-80°C) of non-pathogenic *E. coli* K-12 and *Listeria innocua* were obtained from the Food Microbiology Culture Collection (Erickson Food Science Building, Penn State, University Park, PA) The stocks were thawed until the consistency resembled that of a slurry. Using a sterile loop, one loopful of stock culture was transferred onto prepared TSA agar plates and incubated at 37ºC overnight. Following incubation, 9 ml of tryptic soy broth (TSB) was inoculated aseptically using an isolated colony from the TSA agar and incubated at 37ºC for 24 hours. Serial dilutions were done in sterile buffered peptone water (BPW; Difco) to obtain specific cell concentrations needed for the soft agar inoculation. The prepared TSA plates were overlaid with approximately 10 ml of tempered (50°C) TSA soft agar and allowed to set at room temperature. The resulting overlaid layer contained approximately 8 $\log_{10}$ CFU/ml of *E. coli* or *L. innocua*.

1.4 Plate Overlay Assay

Once the LAF was subjected to the desired temperature and humidity and held until a specified day, hole punches were made by using a sterilized hole punch (OfficeMate; Edison NJ), with a 6.3 mm diameter hole. Each punched piece of LAF was flipped aseptically using sterile tweezers film-side down onto the soft agar overlay surface on TSA plates and placed in an

incubator for 48 hours at 37ºC. An example of this can be seen in Figure 3. After incubation, zones of inhibition for each organism were measured using a caliper (General Tools & Instruments LLC., Secaucus, NJ; recorded to 0.01mm). Two trials were performed and resulting data was recorded in Excel spreadsheets.

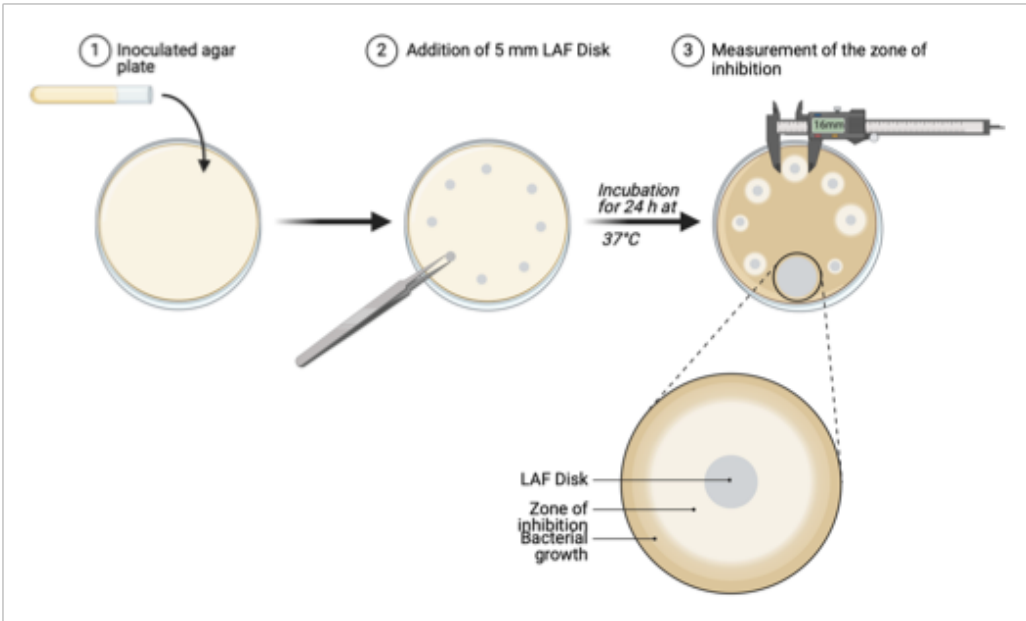

Figure 3: Plate overlay assay

1.5 Statistical Analyses

Data generated from replications 1 and 2, alone or in combination, were entered into Excel (Microsoft, Redmond, WA) and graphs generated. Due to inhomogeneity of variance issues, the resulting data from the combination of replications 1 and 2 were analyzed using a Welch ANOVA in SPSS (IBM, Armonk, NY) to illustrate significant differences between treatments, including week, temperature, and time.

Chapter 4

Results and Discussion

For plate overlay experiments, lawns were made of either *E. coli* K-12 or *Listeria innocua* on TSA plates. Films subjected to various temperatures were obtained by punching a hole into the LAF, placing the hole punch onto the lawns, and zones of inhibition (ZOI; mm) measured with calipers after incubation to allow for the target organisms to grow. In most instances, LAFs stored at 55°C became too sticky and were difficult to handle during plate overlay assays, thereby making data collection difficult for this treatment.

Figure 4 demonstrates the average ZOI from hole punches on lawns of *E coli* after LAF was stored at temperatures of -20ºC, 4ºC, 25ºC, 35ºC, or 55ºC for up to 8 weeks during replication 1. Inhibition was greater by 1 mm at lower storage temperatures, especially at –20°C, with a peak of inhibition (4.4 mm) at week 4. While not unexpected, there was a decrease in inhibition over time for the other treatments.

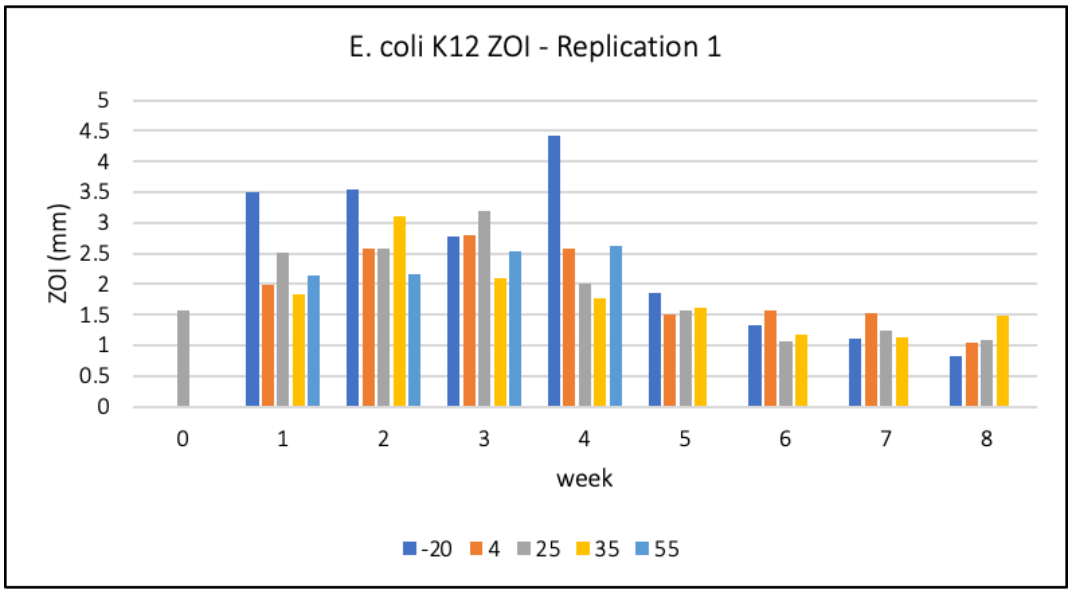

Figure 4: *E. coli* K-12 zones of inhibition, replication 1

Figure 5 depicts the average ZOI from hole punches on lawns of *E coli* K-12 after LAF was stored at temperatures of -20ºC, 4ºC, 25ºC, 35ºC, or 55ºC for up to 8 weeks during replication 1. Inhibition was greatest (3.7-5.7 mm) at lower temperatures, specifically with films stored at –20ºC, again peaking (5.7 mm) around week 4. There was decreased inhibition over time after 4 weeks for the other storage temperatures.

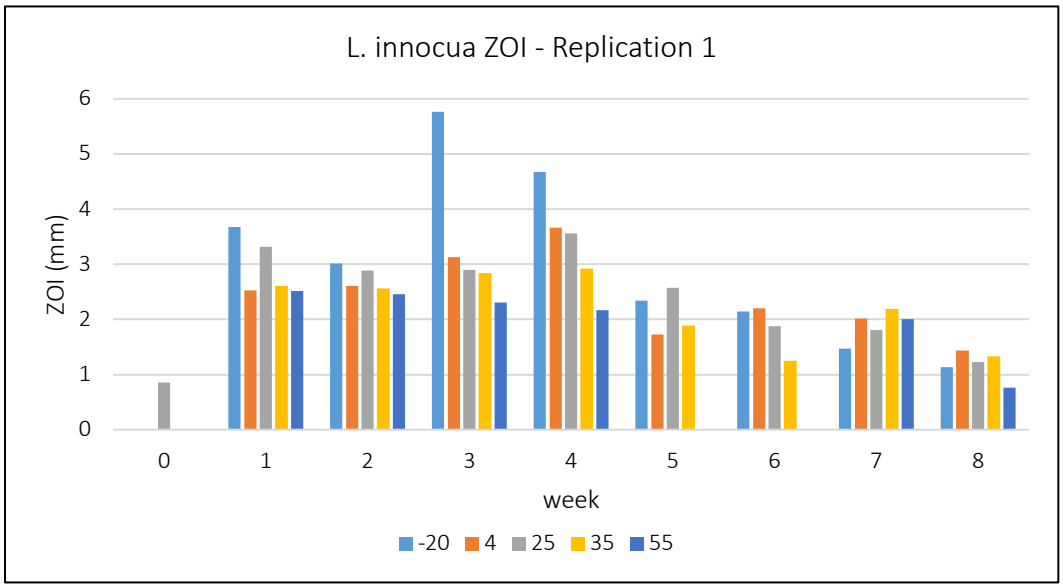

Figure 5: *L. innocua* zones of inhibition, replication 1

Figure 6 demonstrates the average ZOI for *E. coli* K-12 under storage conditions of -20ºC, 4ºC, 25ºC, 35ºC, and 55ºC during replication 2. Inhibition was relatively high (1.25-2.5 mm) throughout the entire 8-week storage period, although inhibition peaked (2.1-2.5 mm) around weeks 4 and 5 of the study, especially when stored at treatments of 4ºC, 25ºC, or 35ºC.

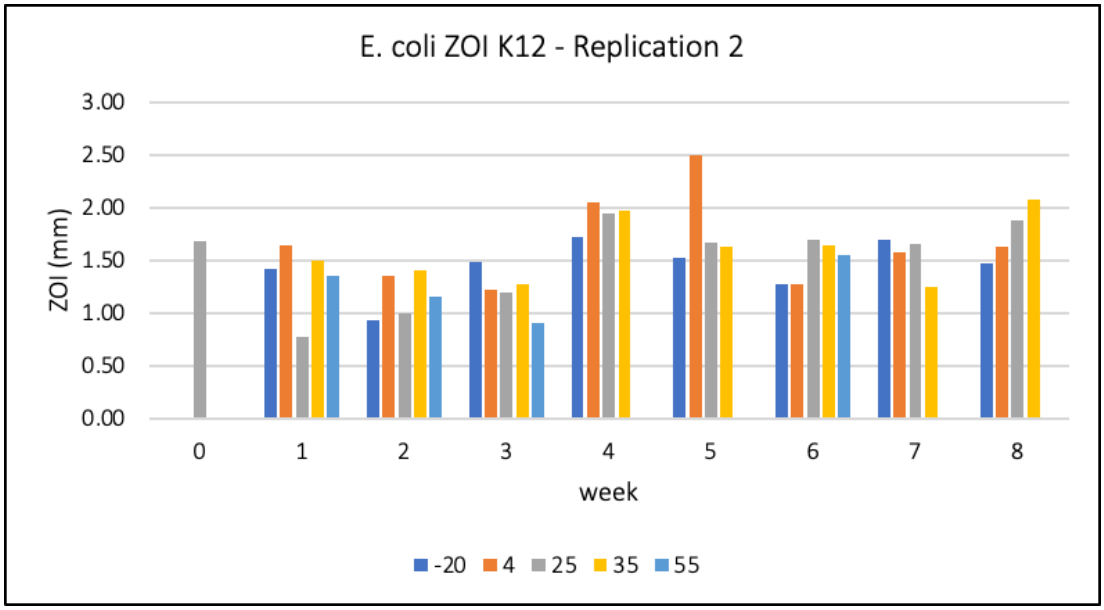

Figure 6: *E. coli* K-12 zones of inhibition, replication 2.

Figure 7 depicts the average ZOI for replication 2 against *L. innocua.* Inhibition peaked (2.4-2.7 mm) around weeks 4 and 5, especially at storage temperatures of -20ºC, 4ºC, and 35ºC. There was a significant peak (3.25 mm) in ZOI for the films held at 55°C by week 7. Overall, inhibition fluctuated significantly from around 1.25 mm to 3.25 mm over the 8 weeks.

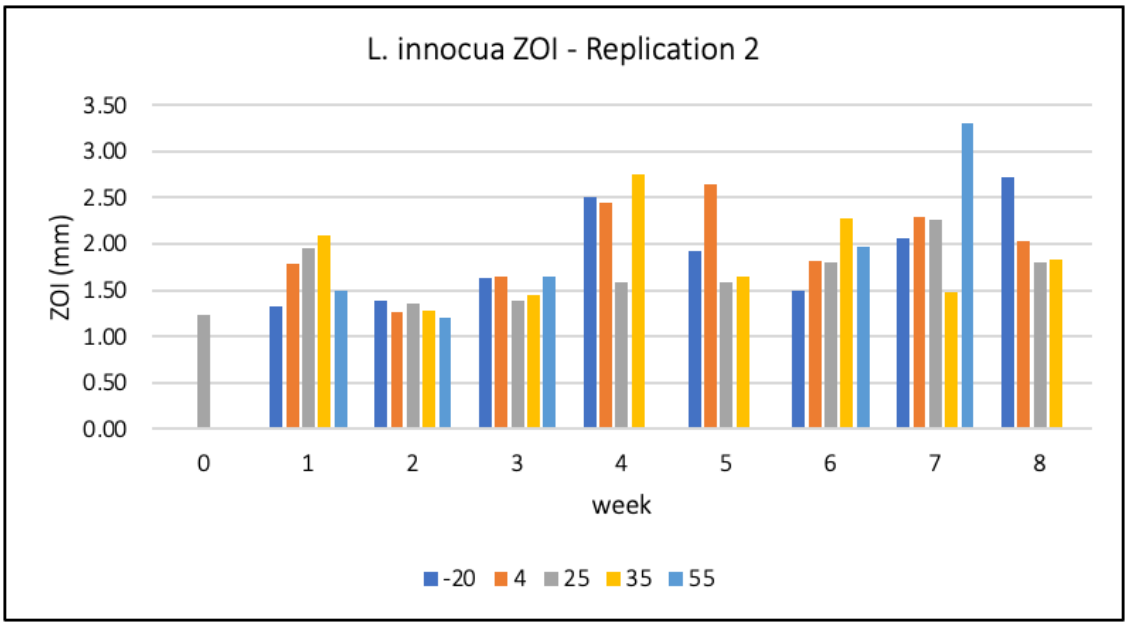

Figure 7: *L. innocua* zones of inhibition, replication 2.

Figure 8 demonstrates the average ZOI for both replications against *E. coli* K-12. Inhibition was greatest (1.5-3.1 mm) in the first four weeks for all storage temperatures, with the greatest inhibition observed at -20ºC, followed by 4ºC and 25ºC. The inhibition (1.0-2.25 mm) was similar between storage treatments at 4ºC, 25ºC, and 35ºC throughout the entire study. After week 4, inhibition decreased (1.25-1.5 mm) for all storage temperatures, with data missing for films stored at 55ºC due to issues described previously.

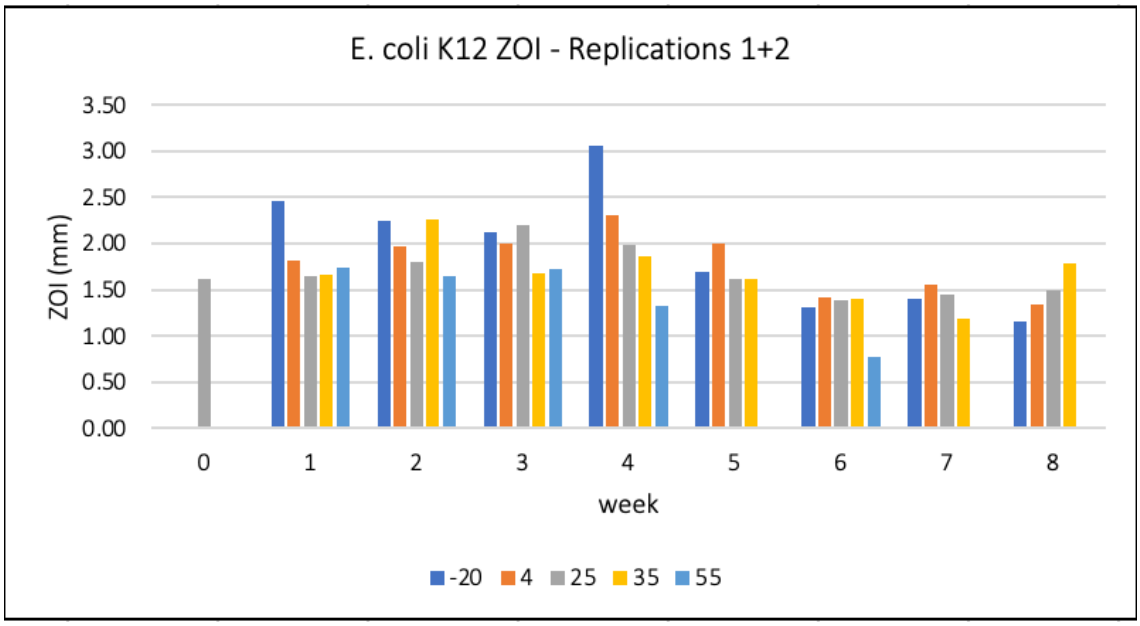

Figure 8: *E. coli* K-12 zones of inhibition, replications 1 and 2 combined.

Figure 9 depicts data for average ZOI against *L. innocua* for both replications. Inhibition against *L. innocua* was strongest (3.5-3.6 mm) at weeks 3 and 4, when stored at -20ºC. Films stored at 4ºC, 25ºC, and 35ºC showed the most inhibition (2.5-3 mm) at week 4. In general, inhibition was rather constant for all storage temperatures except 55ºC. Films stored at 55ºC decreased in effectiveness after week 3, though there was a peak at week 7.

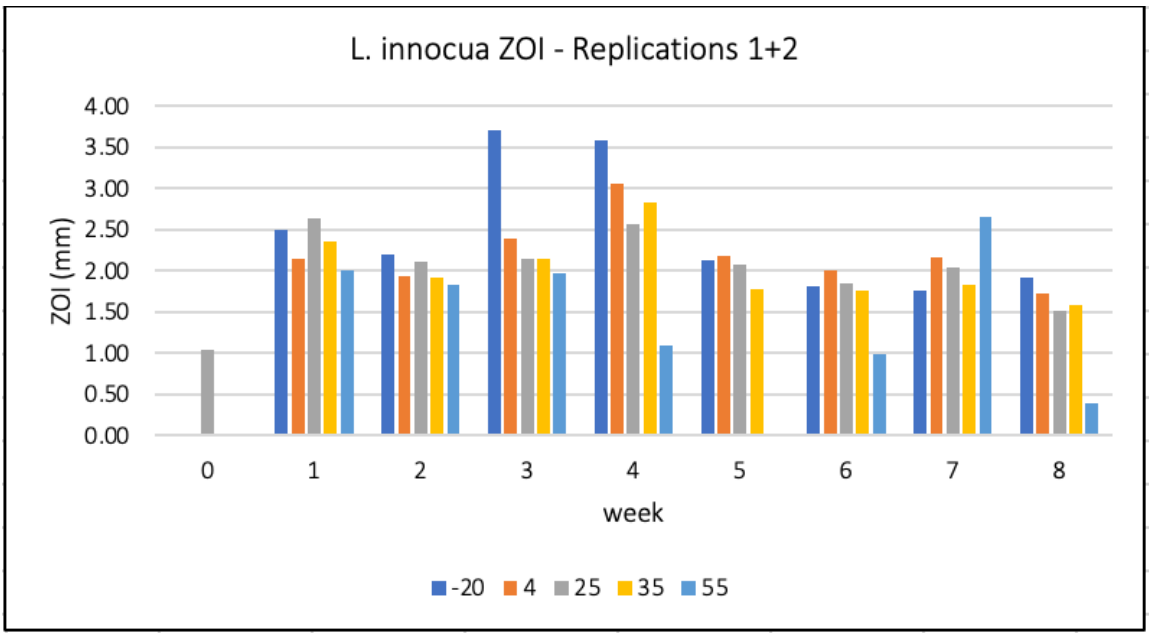

Figure 9: *L. innocua* zones of inhibition, replications 1 and 2 combined.

Figure 10 demonstrates the percent relative humidity for each of the storage periods during replication 1, measured each day that samples were tested. Storage humidities fluctuated greatly, due to the lack of ability to keep humidity constant, though most averaged between 15 % RH and 40 % RH. Films at 55ºC were stored in humidities between 75 and 95 % RH.

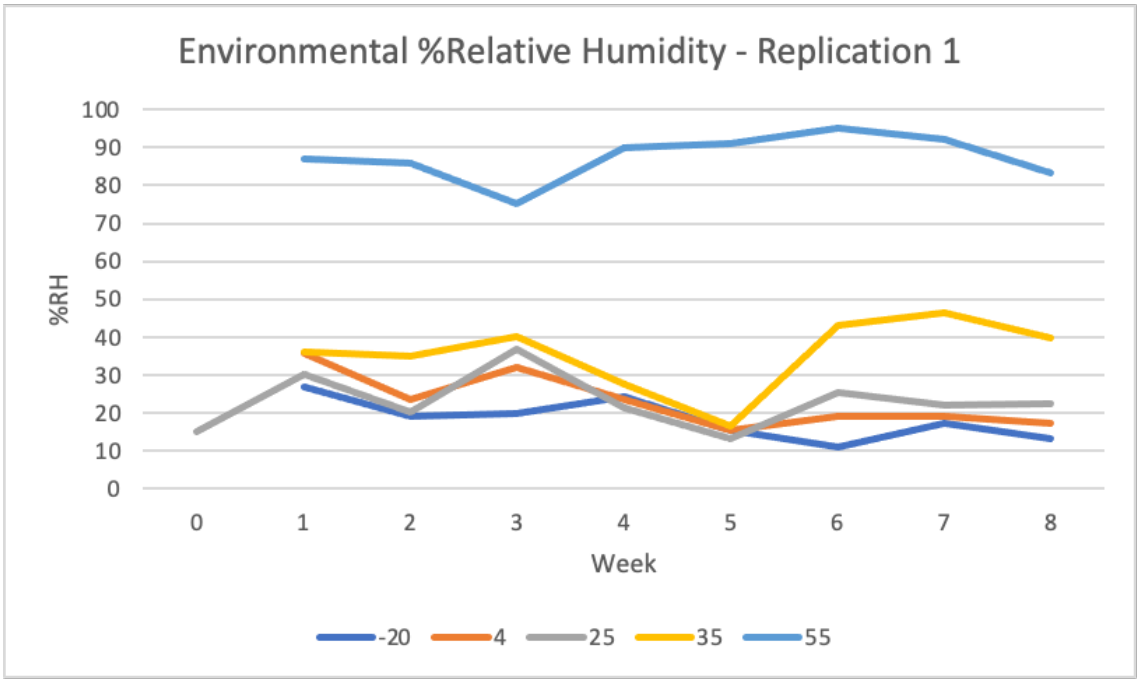

Figure 10: Environmental relative humidity (%), replication 1.

Figure 11 depicts the percent relative humidity for each of the storage areas during replication 2, measured each day that samples were tested. Storage humidities fluctuated weekly, especially at week 5, where the humidity was recorded at 75 % RH for 35ºC, likely due to weather conditions outside. Films at 55ºC were stored in humidities between 85 and 98 % RH.

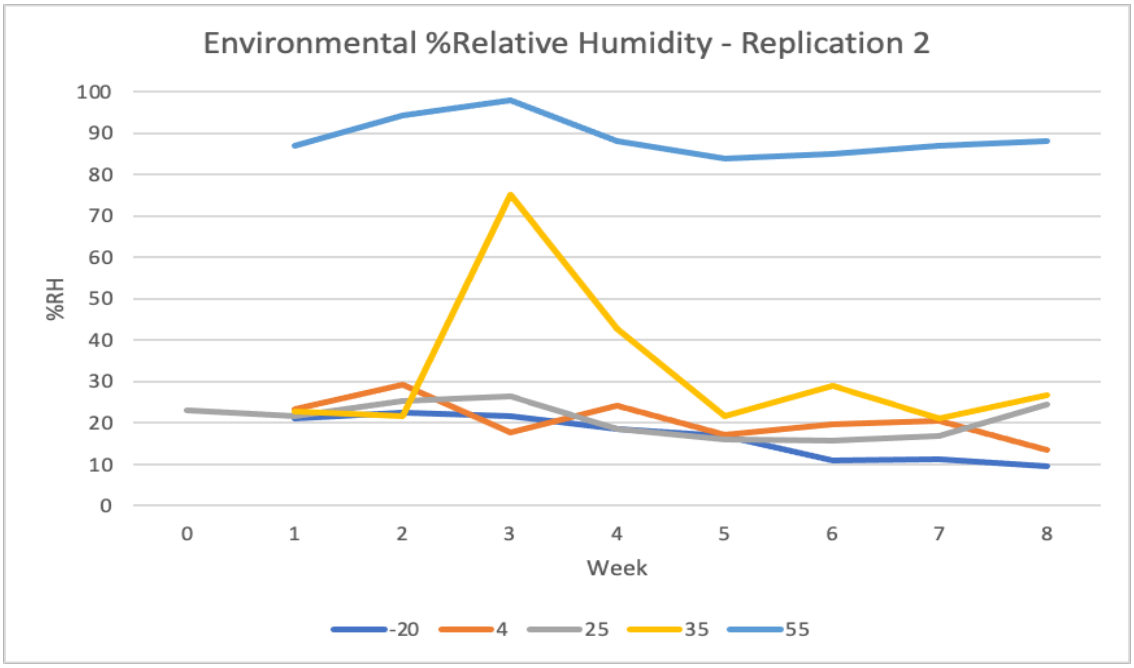

Figure 11: Environmental relative humidity (%), replication 2

Figure 12 demonstrates average temperatures recorded in State College, PA during the time of the first replication of the study. Temperatures fluctuated greatly during the 8-week study, ranging from 8ºC to 30ºC.

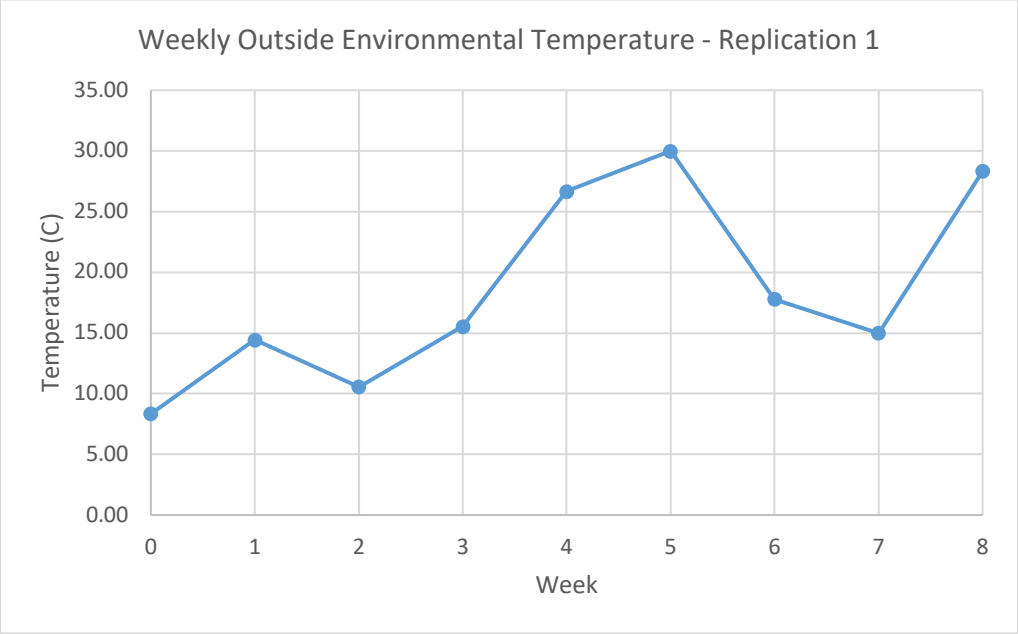

Figure 12: Weekly outside environmental temperature (°C) replication 1.

Figure 13 demonstrates average temperatures recorded in State College, PA during the time of the second replication of the study. Temperatures ranged from around 5°C to 30°C during the 8 weeks.

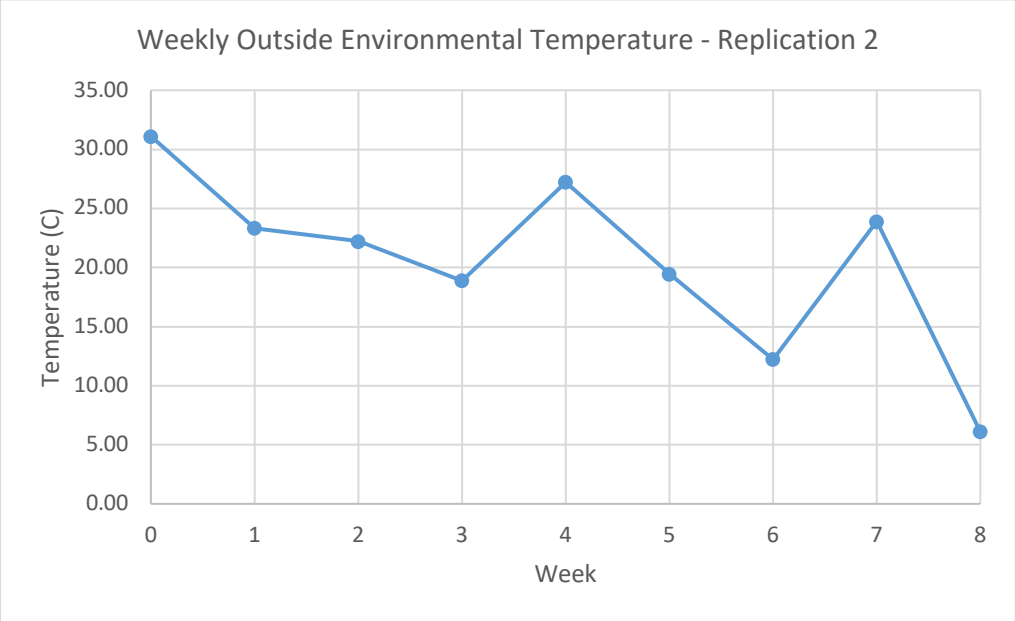

Figure 13: Weekly outside environmental temperature (°C), replication 2.

ANOVA analyses were performed on the data from the combined replications and the significance of the effects of time (week), temperature, and the interaction (as it relates to zones of inhibition) were determined following long term storage of the LAF for *E. coli* K-12 and *L. innocua*, respectively. Figure 14 demonstrates that week, temperature, and the interaction of week-temperature are significant ($p < 0.001$) for the inhibition of *E. coli* K-12 in plate overlay assays. Figure 15 demonstrates that none of the variables (week, temperature) or the internation of week-temperature are significant ($p > 0.001$) for the inhibition of *L. innocua* in plate overlay assays.

Dependent Variable: mmZOIE.c

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	112.719[a]	40	2.818	5.715	<.001	.457
Intercept	683.908	1	683.908	1387.024	<.001	.836
TempE.c	24.441	4	6.110	12.392	<.001	.154
WeekE.c	58.196	8	7.275	14.753	<.001	.303
TempE.c * WeekE.c	31.877	28	1.138	2.309	<.001	.192
Error	134.117	272	.493			
Total	1154.383	313				
Corrected Total	246.836	312				

a. R Squared = .457 (Adjusted R Squared = .377)

Figure 14: ANOVA results for *E. coli* K-12 (combined data from replications 1 and 2).

Dependent Variable: mmZOIL.i

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	319.314[a]	40	7.983	1.320	.102	.141
Intercept	1344.276	1	1344.276	222.213	<.001	.409
WeekL.i	58.235	8	7.279	1.203	.296	.029
TempL.i	101.332	4	25.333	4.188	.003	.050
WeekL.i * TempL.i	159.337	28	5.691	.941	.555	.076
Error	1941.889	321	6.049			
Total	4042.688	362				
Corrected Total	2261.203	361				

a. R Squared = .141 (Adjusted R Squared = .034)

Figure 15: ANOVA results for *L. innocua* (combined data from replications 1 and 2).

Chapter 5

Discussion

Throughout the entirety of the experiment, the control films exhibited no inhibition against *E. coli* K-12 or *L. innocua*, as expected. Therefore, the data were not included in the results since this study focused primarily on the effect of lauric arginate against these two microorganisms in plate overlay assays following storage under various temperatures and humidities.

The films exhibited greater inhibition against *L. innocua* than *E. coli* K-12, especially at lower temperatures, which was observed in Figures 3, 4, 5, 6, 7, and 8. Looking specifically at Figures 7 and 8, films stored at –20°C exhibited greater inhibition against *E. coli* K-12 and *L. innocua*, especially within the first four weeks of the study. This finding is most likely the result of the structural differences between the two microorganisms, as *L. innocua* is Gram-positive, whereas *E. coli* K-12 is Gram-negative. The double membrane structure of Gram-negative microorganisms provides a less permeable protective layer, which could signify that the LAE present in the LAF was unable to diffuse through the membranes as easily. Films stored at higher temperature (37°C, 55°C) and higher humidities, had significantly lower antimicrobial effectiveness, especially as the weeks progressed. Given that the efficacy of LAE decreases with an increase in temperature, it is likely that towards the end of the experiment, the LAE began hydrolyzing back into its original components, L-arginine and lauric acid, resulting in less inhibition towards *E. coli* K-12 and *L. innocua* (Ma et al., 2019). It is also possible that the higher storage temperatures and humidities contributed to a faster release of LAE, so films that were stored for longer periods of time and were losing structure, released some LAE and therefore, could not inhibit microbial growth well, if at all (Kashiri et al., 2019).

The results also indicate a greater effect of humidity on film degradation, though the efficacy generally decreased with an increase in temperature as well. As the films remained at higher humidities for longer, they began curling and became sticky and stringy. As the strength of the film decreased, the permeability of water vapor increased (Hazrati 2021), making the films difficult to handle.

Managing the humidity proved to be more difficult than initially anticipated, as the humidity in each storage compartment fluctuated with the external weather conditions. Similarly, due to the equipment available at the time of the study, it was impossible to ensure a constant humidity within each area, so the majority of the humidities, with the exception of the 55^{o}C incubator, fluctuated between 15 and 30% relative humidity. This finding may have resulted in greater discrepancies between the observed data points.

There were several factors that also may have contributed to variable data between weeks and replications. Bacterial lawns grown in replication 2 were often thicker, resulting in a lower zone of inhibition due to a greater concentration of microbes within the given area. This observation is likely due to leaving plates in the incubator for longer than the recommended 24 hours. Discs were not always uniformly round due to issues with the hole punch (sticking and rusting), which impacted hole punch functionality as well as uniformity of bacterial zones of inhibition. Similarly, due to the translucent nature of the films, discs may have been placed upside down, resulting in less data points per plate.

Chapter 6

Conclusions and Future Directions

Laminated antimicrobial films (LAFs) have the potential to serve as a type of food packaging designed to keep food safe for consumers. This project was designed to study the antimicrobial effectiveness of LAFs containing LAE against *L. innocua* and *E. coli* K-12 under varying temperature and humidity conditions over 8 weeks. Results from the study demonstrate that the films exhibited strong antimicrobial effectiveness against both microorganisms, especially at –20C°, 4°C, and 25°C, which peaked at 4 weeks and then substantially decreased. The films stored at higher temperatures and humidities lacked a cohesive structure and antimicrobial ability.

Future studies will need to be conducted to ensure that LAFs and LAE under controlled environmental conditions will inhibit foodborne pathogens to ensure consumer safety, while reducing microbial spoilage and improving food quality and shelf-life. Since the films decreased efficacy at higher temperatures, further studies analyzing the effects of higher temperature increments (between 35°C and 55°C) could be done to determine more closely at what temperature the films begin showing signs of degradation. Similarly, as the humidity could not be adequately controlled and was influenced by external factors, another study could be done under manipulated humidity conditions to prevent great fluctuations in humidity. Though many of the findings from this project still need to be verified, the current study provides a good basis for potential storage recommendations to ensure LAFs against foodborne pathogens affecting the food system.

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