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Evaluation of a Flexographically Applied Antimicrobial Coating for Ready-to-Eat Turkey Deli Meat Packaging

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EVALUATION OF A FLEXOGRAPHICALLY APPLIED ANTIMICROBIAL COATING FOR READY-TO-EAT TURKEY DELI MEAT PACKAGING

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ABSTRACT

With an increase in demand for fresh, ready-to-eat meats, the use of antimicrobial food packaging could help reduce food waste by delaying and decreasing growth of spoilage bacteria, thereby extending the shelf-life and enhancing food quality and safety. Nisaplin® is a commercially available antimicrobial peptide that is approved by the FDA and is designated GRAS as a food additive. This additive has the potential to be commercially added into antimicrobial packaging applications, specifically, ready-to-eat meat packaging. This study was conducted to determine if a coating containing Nisaplin® could be commercially applied to a polymer film substrate using large scale production equipment while still maintaining antimicrobial efficacy. A flexographic printing press was used to apply a liquid coating containing Nisaplin® to a PET/LLDPE laminate, a non-forming web film substrate. pH and viscosity testing of the liquid coatings was conducted prior to applying the coating. Heat seal tests were conducted to determine if the coated film could be commercially converted into a package. Basis weight tests were conducted to determine the weight of the coating applied, and for determining the total cost of the converted package. Tests were also conducted using a colorimeter to determine if the liquid coatings caused a change in the overall appearance and clarity of the coated films. Antimicrobial efficacy and tests to determine inhibition of aerobic bacteria, coliform bacteria, and staph growth were performed. Preservative and additive free sliced turkey deli meat was inserted into the treated and untreated converted pouches over an 11- day trial period. Inhibition testing was conducted on dry coated films

to determine the maximum length of time in days that Nisaplin® proved effective in the inhibition of aerobic bacteria growth. Control films did not contain Nisaplin®. Uncoated films containing no coating were used as a second control set of pouches. The pH of the antimicrobial coating was found to be 7.27 with a viscosity of 19.53 when tested with a #3 Zahn cup. Peel-able seals for all three tested films (nisin coated, coating only, and uncoated film) were achieved at 300˚F, with seal properties for the treatment containing Nisaplin® showing an increase in strength as temperature increased. Coated films without Nisaplin® also achieved inhibition against the growth of aerobic bacteria with statistical difference between the coating with Nisaplin® and the two control groups (the coating without Nisaplin® and the uncoated film) on Day 5 (2-log reduction) and Day 9 (1-log reduction), 45 days after the coating was applied to the film. No statistical difference was found in the inhibition of coliform colony growth between the two coatings (with and without Nisaplin®) throughout the sample periods. Thus, the application of the coating, as well as converting and efficacy of the antimicrobial coating process suggest that it is possible to commercially produce an effective antimicrobial coating contain Nisaplin®. The work showed that the antimicrobial coating containing Nisaplin® reduced the overall aerobic microbial population of the refrigerated ready-to-eat turkey deli-meat. Further work needs to be conducted to validate shelf-life extension and improved safety of ready-to-eat food products.

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INTRODUCTION

In 2015, approximately 133 billion pounds of America's overall food supply went to waste, resulting in 40% of food wasted each year in the United States alone. Not only does this impact the U.S. food security and resource conservation, it also contributes to approximately 18% of the total U.S. methane emissions from landfills (EPA, 2016). 46% of food waste in the US occurs in the household and 13% of total waste occurs at the retail and grocery distribution level. 30% of food waste is composed of meat, poultry, and fish (NRDC).

Active packaging, more specifically, antimicrobial packaging has been an area of growth in research due to its suggested ability to decrease spoilage microorganism and bacteria growth, thus extending the average shelf life of food products and reducing food waste. It is estimated that the demand for active packaging in the US will expand 5.4 percent annually to $2.5 billion in 2019 (Freedonia, 2015). Active packaging is a packaging system or technology that changes its own properties or attributes once it senses a change in the internal or external environment Forms of active packaging include pouches, sachets, desiccants, labels, integrated films and coatings to perform functions such as gas-permeability control, odor removers, oxygen scavengers, moisture control, as well as antimicrobial functions (Brody, Strupinsky, & Kline, 2002).

Recently, there has been a consumer drive towards convenient, fresh, high-quality, ready-to-eat foods. These foods require little to no cooking or handling once removed from the package, such as sliced deli meats (Freedonia, 2015). Ready-to-eat

meat products, such as sliced deli meat that is typically served and consumed cold, is susceptible to post-process contamination such as Listeria monocytogenes. *L. Monocytogenes* is a gram-positive, nonsporeforming, anaerobic bacteria that contributes to approximately 260 deaths per year world-wide (CDC, 2018). 83% of ready-to-eat deli meat contamination occurs when the meat is being sliced at the deli ((FSIS, 2013; Morgan, 2014).

The main focus of the research to be discussed is to commercially apply an antimicrobial coating to the surface of a polymer film to reduce or retard the growth of spoilage microorganisms and bacteria, which could extend the shelf life of food products, improve food safety, and also reduce waste. The research discussed could also have the potential to reduce or prevent the growth of L. monocytogenes, which is the strian of bacterium that causes the foodborne infection listeriosis (listeria). Past research (Morgan 2014; Perna, 2016) has shown success in using nisin in the form of commercially available Nisaplin® (2.5% concentration) to inhibit the growth of gram-positive spoilage microorganisms in a laboratory environment. The objective of this research will be to determine if an antimicrobial coating containing Nisaplin® can be commercially applied to a polymer web substrate while still maintaining antimicrobial efficacy.

LITERATURE REVIEW

1.1 Active Packaging

Active packaging, also referred to as "smart" packaging, is a packaging system or technology that senses a change in the internal or external environment of the package and responds by changing its own properties or attributes. Active packaging for food packaging is commonly used to ensure the microbial safety and/ or enhance the preservation of a food product while maintaining the quality of the product (Fang, Zhao, Warner, & Johnson, 2017) (Brody, Strupinsky, & Kline, 2002). In 2019, the demand for active packaging in the US is forecasted to expand 5.4 percent annually to $2.5 billion. The interest and increasing need for active packaging in the upcoming years is attributed to the increase in food safety and food waste concerns globally (Freedonia, 2015).

For several decades, active packaging has been an added component to food packaging in the form of desiccants or sachets in dry food packaging. Other forms of active packaging include pouches, patches, labels, coatings, and integrated films. Not all forms of active packaging perform the same function. Active packaging applications can function as oxygen scavenging systems, odor removers, gas-permeability controllers, ethylene controllers, carbon dioxide controllers, moisture controllers, and antimicrobial packaging systems (Brody, Strupinsky, & Kline, 2002). Past active packaging applications for meat packaging have been in the form of oxygen scavengers or sachets. Recently, active packaging in relation to ready-to-eat meat packaging, such as deli-meat or hotdogs, has focused on antimicrobial active packaging systems and the inhibition and

reduction of microbial growth within the meat package (Fang, Zhao, Warner, & Johnson, 2017).

1.2 Food Waste

In 2017, it was reported that, in America, 40% of food goes to waste each year. It was estimated that the average American throws out more than 400 pounds of food annually. Food waste goes beyond wasting the food product itself. It was estimated that annually food waste contributes to 21% of the U.S. agricultural water usage, 21% of U.S. landfill content (the number one contributor by weight), 19% of all U.S. croplands, and 2.6% of all U.S. greenhouse gas emissions (NRDC, 2017). The USDA found that top three food groups that contributed to food waste each year are: meat, poultry, and seafood (30%, $27 billion), vegetables (19%, $30 billion), and dairy products (17%, $27 billion). Of that only 4.5% of the 30% of fresh meat, poultry, and seafood was wasted at the retail level. Thus, majority of poultry, seafood, and meat food waste occurs at the consumer level (Buzby, Farah-Wells, & Hyman, 2014). Food waste throughout the distribution channel is attributed to poor quality appearance at the consumer, food damage, spoilage microorganisms, oxidation, pest and insect contamination (FAO, 2011). The use of antimicrobial packaging to reduce food spoilage could help reduce the amount of food waste American's contribute to each year.

1.3 Ready-to-Eat Meat Product

Ready-to-eat (RTE) food products are defined by the FDA as any food that is normally consumed its raw state or any food, including those that are processed, that will be eaten without further processing. Further processing includes methods that would

significantly minimize biological hazards, such as appling heat to reduce harmful microbial bacteria (FDA, 2017). The global market for RTE food products is expected to expand at a compound annual growth rate of 7.2% from 2016 to 2026, with an estimated value of $195.3 billion by the end of 2026. The meat and poultry segment of RTE foods has the largest value share during this forecast period, with 45.7% value share in 2016. In 2016, the US accounted for 40.1% of the valued market share of RTE foods, making it largest consumer of RTE foods (FMI, 2016).

Convenience and desire for fresh, healthier foods on the go have driven the increase in ready-to-eat foods such as fresh sliced deli meat. The freshness and quality of RTE deli meat is highly dependent upon the packaging to protect the consumer against harmful contamination, delay spoilage, reduce weight loss of the meat product, retain color and aroma, and allow some enzymatic activity to help promote meat tenderness (Fang, Zhao, Warner, & Johnson, 2017). RTE deli meats are typically not cooked or further processed before consumption, making RTE products more susceptible to harmful microbial and bacteria that can cause food borne illness such as *Listeria monocytogenes*. The average shelf life of uncured RTE fresh sliced deli meat is three to five days, depending on the amount of added preservatives, such as sodium nitrate and sodium nitrite. Consumption of fresh sliced deli meat past three to five days can attribute to food borne illnesses.

Spoilage microorganisms, commonly found in ready-to-eat deli meats often include aerobic bacteria, coliform bacteria, and staph. Aerobic bacterium requires oxygen for to microbial growth to occur. Aerobic bacterium includes both gram-positive and

gram-negative bacteria, such as *Bacillus* (gram positive) and *Pseudomonas* spp. (gram negative) (Gram, et al., 2002). Coliforms include gram-negative, nonspore-forming bacteria such as *Escherichia coli* (*E.coli*)*, Enterobacter aerogenes, Citrobacter*, and *Klebsiella* (Fung, 2009). Lastly, *Staphylococcus aureus* (staph) is a pathogenic gram-positive, nonspore-forming coccal bacterium that is commonly found in RTE and dairy products. Food products are contaminated with Staph through unsanitary worksites, as well as the workers coughing, sneezing, and improper washing of hands (Wirtanen & Salo, 2016). One method to reduce and inhibit the growth of harmful bacteria and spoilage microorganisms is through the use of antimicrobial packaging.

1.4 Listeriosis

According to the CDC, it is estimated that listeriosis is the third leading cause of death from foodborne illness with approximately 260 deaths per year around the world (CDC, 2018). Over that past 7 years, 13 outbreaks of listeriosis have been reported in the US. The most recent case (2017) spanning across four states, resulting in 8 hospitalizations and 2 deaths (CDC, 2018).

Listeria spp. outbreaks in food occur from post processing contamination of *Listeria monocytogenes*. *L. monocytogenes* is a gram-positive, nonspore-forming, anaerobic bacteria which grows between 31.28 to 122˚F (-0.4 to 50˚C) (Farber & Peterkin, 1991). Nonspore-forming anaerobic bacteria do not form spores, and they live and grow in an environment with little to no oxygen (A.D.A.M, 2018). Numerous studies have shown that the majority of listeriosis linked outbreaks have been caused by consumption of RTE meat products. *L. monocytogenes* is reported to be to the leading

cause of death associated with RTE deli meat (Morgan, 2014). Refrigerated conditions permit growth of *L. monocytogenes* in RTE meat products after processing although *L. monocytogenes* can not survive the high heat exposure that occurs during processing (Hoelzer, et al., 2011).

The Food Safety and Inspection Service upholds a zero tolerance policy for *L. Monocytogenes* in RTE food products (FSIS, 2000). Risk assessment data performed by the FSIS in 2011 indicated that 83% of *L.monocytogenes* contamination of RTE sliced deli meat occurs due to contamination of the deli slicer (FSIS, 2013; Morgan, 2014). Zero tolerance is often hard to achieve due to limited success in training and post-process contamination. To properly clean a deli case and slicer, the slice and deli case must be taken apart after each product is sliced. Properly cleaning a deli meat slicer and case is not practical due to the limited amount of working hours in a day and the high volume of customer orders. In 2013, Oliver conducted a study testing thirty retail deli meat stores for *L.monocytogenes* before and after proper employee training on cleaning and proper prevention practices. The study found that there was only a 0.5% decrease in the presence of *L.monocytogenes* after the training (from 4.5% to 4.0%), and that eleven of the thirty samples showed signs of *L. monocytogenes* on the surface of the deli meat (Oliver 2013; Morgan, 2014).

1.5 Antimicrobial Packaging

Ready-to-eat deli meat with minimal preservatives provides an excellent environment for potential microbial growth. Thus, active packaging, specifically antimicrobial packaging is an important packaging concept when packaging meat. The

use of antimicrobial packaging for ready-to-eat deli meat products could minimize bacterial growth and provide safe and longer lasting product to consumers (Fang, Zhao, Warner, & Johnson, 2017).

The growth of microorganisms such as mold, yeast, and bacteria including, *Pseudomonas*, *Listeria* monocytogenes, *Lactobacillus* spp., *Staphylococcus aureus*, *Escherichia coli* (E. coli), and *Enterobacter* are all associated with the spoilage of meat. The growth of these microorganisms can lead to off-flavors, off-odors, illness, and sometimes death (Jayasena, D. D., & Jo, C. 2013). The use of an active meat package to reduce or inhibit the growth of such microorganisms in RTE meat packaging is critical. An antimicrobial RTE meat package could extend the shelf life and prolong the safety and quality of the food product.

Antimicrobial packaging is a form of active packaging that can be classified into two types of package materials. The first type is a packaging material that contains antimicrobial agents that migrate to the surface of the material and come in direct contact with the food product. The second type is a packaging material where the active agents do not migrate to the food, yet are still effective against food surface microbial growth by working as a carrier for the antimicrobial compound (Cooksey, 2001) (Brody, Strupinsky, & Kline, 2002).

Antimicrobial compounds can be incorporated into packaging to control undesirable microorganisms in various methods (Morgan, 2014). Antimicrobial compounds can be incorporated into the package through the use of a sachet or pad. Sachets are used to absorb and release antimicrobial compounds into the headspace of a

packaged product. The antimicrobial compounds released in the headspace then migrate to the surface of the packaged food. Pads come in direct contact with the food product. For example, pads are used in commercial meat packaging to soak up meat secretions while also releasing antimicrobial compounds to delay the growth of spoilage microorganisms (Fang, Zhao, Warner, & Johnson, 2017). Another common method is through the extrusion of the antimicrobial so that it is directly incorporated into the package material using high amounts of heat and pressure. This method is not always suitable for active compounds derived from natural sources (such as plant extract or bacteriocins) because the active ingredient often becomes inactive at high temperatures (Morgan, 2014; Perna, 2015; Fang, Zhao, Warner, & Johnson, 2017). Lastly, antimicrobial compounds can be applied to the internal surface of the package by coating the material with a matrix. The matrix acts as a carrier for the antimicrobial agents, allowing them to be released into the headspace of the product though evaporation or migrate on to the surface of the food through diffusion (Cooksey, 2001; Fang, Zhao, Warner, & Johnson, 2017).

The US Food and Drug Administration (FDA) requires that antimicrobial agents that are applied as a coating or that are come in direct contact with the surface of meat products must be classified as "generally recognized as safe" (GRAS) for consumption within regulatory limitations based upon the consumer's daily intake (Hyldgaard, Mygind, & Meyer 2012). It is also important that the antimicrobial agent is cost effective and commercially available when considering commercial production (Morgan, 2014). One antimicrobial agent on its own cannot inhibit and protect against all forms of

microbial growth (Perna, 2015; Mauriello, 2016). For example, some antimicrobial compounds only inhibit the growth of gram-positive bacteria and not gram-negative bacteria, such as Nisin (Cooksey 2001). The identification of which microorganisms the antimicrobial package is designed to inhibit is essential in selecting the proper antimicrobial substances. Antimicrobial substances used in active packaging materials can be derived from the following: metals, chemicals, plant extracts, enzymes, and bacteriocins (Mauriello, 2016; Brody, Strupinsky, & Kline, 2002). Each antimicrobial substance and compound contains properties and performs functions that make them a more suitable choice as an inhibitor than others depending on the desired application method and food product that is packaged.

Recently, consumer demand has pushed the market towards ready-to-eat meat products with less salt and more all-natural preservatives. Active packaging reduces the need for added salts and preservatives, such as sodium nitrate and sodium nitrite, through the use of plant extracts and bacteriocins (Jayasena & Jo 2013). The use of antimicrobial compounds that are naturally derived from plants in the form of oils or spices is widely accepted because they have high volatility, they are all-natural preservatives, and they are often biodegradable. Plant extract compounds include spices and oils such as cinnamon, allspice, oregano, thymol, clove, onion, garlic, radish, mustard, horseradish, carvacrol, rhubarb, thyme, and rosemary (Mauriello, 2016) (Brody, Strupinsky, & Kline, 2002).

Some antimicrobial compounds derived from plant extracts and animal sources bond with organisms in the food product in order to perform properly. Most function by interfering with the cell wall membrane (or structure) to interrupt the metabolic pathway.

A bacteriocin is a proteinaceous (protein) compound that is produced by bacteria that exhibits a bactericidal or bacteriostatic mode of action against a sensitive bacteria species (Nettles & Barefoot, 1993). For example, the bacitracin compound nisin, interacts with sulfur containing compounds in the bacterial membrane, disrupting the cell structure and permeability, causing the cell to undergo lysis (Brody, Strupinsky, & Kline, 2002).

1.6 Nisin

Nisin is a class I bacteriocin polypeptide (a molecular chain of amino acids) antibacterial substance, containing 34 amino acids, produced by particular strains of *Lactococcus lactis* from fermented milk (Mitra, Chakrabartty, & Biswas, 2005; Morgan, 2014). Nisin inhibits bacteria growth, including pathogenic foodborne bacteria such as *Listeria monocytogenes* and other gram-positive microorganisms. Pathogenic bacteria are those that are capable of causing disease. 13 of nisin's 34 amino acids are involved in post-translational modification, including the formation of five thio-ether bonds that form internal rings. Post-translational modification is a biochemical modification that occurs to one or more amino acids on a protein following protein biosynthesis (Newsome, 2015). Additionally, nisin possesses both amino and a carboxyl end groups. Overall, nisin has a positive charge but also has amphipathic properties, meaning it has both a hydrophilic and hydrophobic tail. The molecules have a polar water soluble group attached to a water insoluble hydrocarbon chain (Delves-Broughton, 1990; Modugno, et al., 2018; Perna, 2016). The molecular structure of Nisin A can be seen in Figure 1.

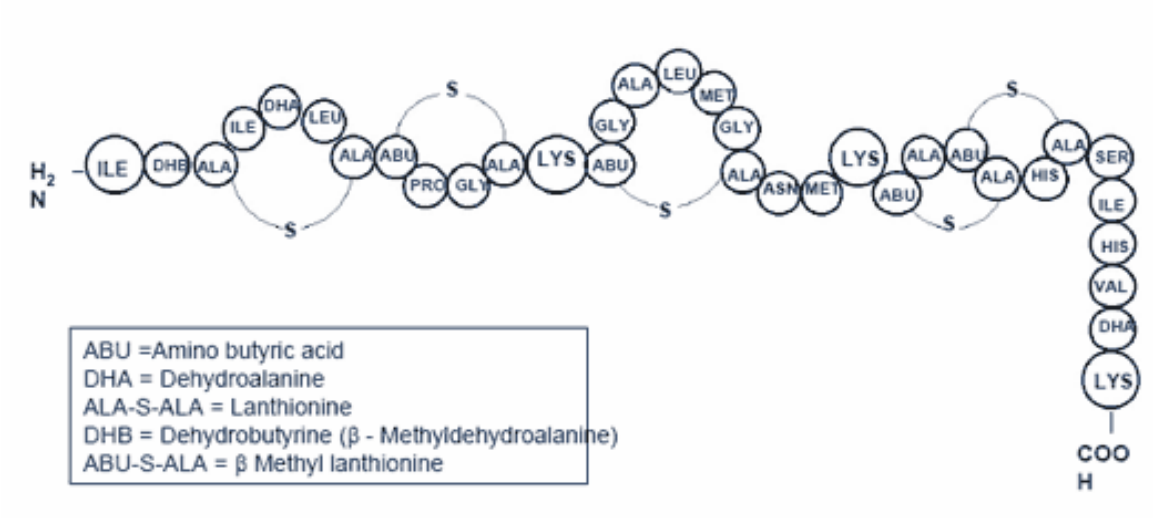

Figure 1. Molecular structure of nisin A (Delves-Broughton, 1990)

Nisin was introduced 40 years ago as a commercially available food preservative in the UK. Nisin is the only GRAS approved bacteriocin food preservative, and is commonly used in dairy products and meats. In 1988, the FDA approved nisin as a food preservative in concentrations lower than 250 ppm (10,000 IU/ml) in cheeses to prevent the growth of *Clostridium botulinum* (Gharsallaoui, Oulahal, Joly, & Degraeve, 2015; Food and Drugs, 2017). Nisin is commercially available as Nisaplin® (2.5% concentration). Nisaplin® is produced with an antimicrobial activity of 1 x 10^6 IU/g, meaning, Nisaplin® has an activity of 10^6 IU (activity containing 1µg of International Reference Preparation). 1 gram of pure nisin contains 40 x 10^6 IU, thus the biological activity of 40 IU corresponds to 1µg of pure nisin (Vandamme & Vuyst 1994). The properties of nisin have made it an ideal antimicrobial in the food industry. Nisin has no odor or flavor, it is easily digestible and non-toxic, stable at a low pH, mostly colorless, water soluble, and heat stable (Integrated Ingredients 1993).

1.6.1 Nisin Stability

Nisin is acidic in nature and has its greatest stability under acidic conditions. In pH ranges from 3 to 7, the nisin molecule becomes more unstable to the effect of heat. Effect of pH on the activity of nisin can be seen in Table 1.

Table 1. The effect of pH on nisin activity (Delves-Broughton, 1990)

pH Value	% Retention of Nisin Activity
3.0	100
4.0	71
5.0	35
6.0	14.5
7.0	0.5

Heat stability of the nisin is critical to proper activity of the antimicrobial. Nisin is stable in temperatures below 141˚C (285.8˚F). Excessive heat can cause the antimicrobial agents to deactivate and loose antimicrobial efficacy. Nisin is able to withstand heat treatment in order to ensure sterile conditions in laboratory testing that occur from autoclaving nisin at 121˚C (249.8) (Perna, 2016). However, the antimicrobial activity of nisin could become an issue during commercial application due to heat which is used for drying a coating on a press.

1.6.2 Nisin Effectiveness as an Antimicrobial in RTE Meat.

In a study conducted by Morgan (2014), the effectiveness of a nisin in a liquid coating was tested. Morgan developed both a nisin pectin coating to identify the effectiveness of the coating formulation for inhibition of *L. monocytogenes* over the

shelf-life of ready-to-eat turkey bologna. The developed coating was applied to a wax paper and a polymer laminate. The coating developed was food safe, colorless, and in the commercial flexographic printing range for proper viscosity. The results of the study were compared to a standard nisin curve. The turkey bologna was surface-inoculated (0.1ml) with a 10^7 CFU/ml suspension of *L. monocytogenes* cocktail. The samples were then serially diluted in buffered peptone water (BPW), then 0.1ml spread plated in duplicate onto a modified oxford agar (MOX), and then incubated for up to 48 hours at 37˚C to determine the initial inoculum level. The remaining inoculated turkey bologna was placed inside a low density polyethylene pouch with the inoculated side of the bologna in contact with the coated substrate. Un-inoculated turkey bologna was plated onto TSA plates to enumerate the background microflora that was naturally present on the meat. All of the pouches were then packaged and stored at refrigeration temperature. The duration of the study took place over 63 days. After testing, it was determined that the film on lawn studies proved that the coatings maintained antimicrobial efficacy in controlling the growth of L. monocytogenes. There was also no noticeable difference in the substrate used. Morgan also found that the approximately 50% of the nisin remained trapped in the nisin coating after the the trial period was completed based on a HPLC analysis.

Perna (2016) conducted a study to modify the nisin coating created by Morgan (2014) to formulate a coating that could be used for large scale production equipment while maintaining antimicrobial efficacy. The focus of this work was to create a coating that could be commercially applied to a polymer substrate using a flexographic or

gravure printing press. Perna determined a grade of polyvinyl alcohol (PVOH) and compatible plasticizer, while developing a coating with percent solids appropriate for coating in gravure or flexography. She developed a coating with a percent solids of 20.53% which was well within the range (15%-50%) required for printing on a flexographic or gravure printing press (later defined in Section 1.8). The formulated coating was also able to pass through a Zahn #3 cup in an average of 24.47 seconds (approximate viscosity measurement of 175-200cP). Perna conducted a film on lawn study of the coated samples determining that the coated films did show inhibitory effects against *L. monocytogenes*, while showing no inhibitory effects against the control. After creating a coating that was able to be run on large scale production equipment, Perna conducted a press run. The nisin coating was applied to a LLDPE co-extrusion film that was corona discharge treated prior to the application of the coating. Perna also applied a Polyethylene imine primer to the film prior to applying each of the coatings. Both a gravure and a flexographic press were used to apply the coating in separate press runs to compare application methods. After each application another film on lawn test was conducted. Perna's flexographic control group (without Nisaplin®) had inhibition zones of 0.0±0.0 and the treatment group (with Nisaplin®) had inhibition zones of 3.6±1.36. After the experiment was conducted, it was determined that the coating could be applied using either a flexographic or gravure printing press maintaining efficacy against *M. luteus*. Perna concluded that using a flexographic printing press is the best method for applying the coating in future research. The low pH (high acidity) of the coating could

cause erosion of the metal rollers on a gravure printing press. Also, gravure printing can be more expensive compared to flexographic printing.

1.7 Coating Formulation

The formulation for the antimicrobial coating containing Nisaplin® was adapted from previous formulations conducted by Perna, Morgan, Franklin (Perna, 2016; Morgan 2014; Franklin, 2004). Each ingredient and its purpose along with the amount used for both creating a small batch (Perna, 2016) and the amount needed for a proper press run is listed in Table 2 (Perna, 2016).

Table 2. Formulation of nisin coating for commercialization (Perna, 2016).

Ingredient	Amount for small batch	Amount for Press Run	Purpose
4-88 Mowiol PVOH resin	10g	249.48 g	Adhesive. Water soluble, synthetic polymer.
50/50 (v/v) mixture of 95% ethanol and distilled water.	30ml/30ml	750ml/750ml	Solvent. Ethanol evaporates out upon drying.
Glycerin	3.2ml	80ml	Plasticizer for Polyvinyl Alcohol
Tween® 80 (0.25% v/v)	185µL	4.63ml	Surfactant. Decrease overall surface tension of liquid coating, and emulsifying component.
Nisaplin® (2.5% - 12,500 IU/mL in solution)	1g	49.98g	Active antimicrobial
0.02 M acetic acid solution	2ml	50ml	Dissolve Nisaplin® prior to mixing.

1.8 Application Method: Flexography

Flexography is a direct, rotary printing method used for commercial application of liquid coatings or inks to a web substrate. This method of printing is very common in the flexible packaging industry for printing on substrates such as films, paper, and foils. Flexography utilizes either a photopolymer plate or a rubber roller to transfer inks or coatings from a cell-structured "anilox" roller to a substrate. The ink or liquid coating is metered out through the use of a doctor blade. Doctor blades are placed on top of the anilox roller to remove excess liquid coating or ink from the roller before coming in contact with the rubber roller or plate cylinder. This process commonly uses low viscosity inks or coatings in order to ensure the liquid coating or ink properly "lays down" across the substrate during the fast paced printing process (Cusdin, 1999; Peter, Engledow, Freye, & Kershner, 1999). When applying a liquid coating to a substrate, a rubber roller is typically used in place of a photopolymer plate. The use of photopolymer plates while coating a substrate using a flexographic press is typically done to create a pattern or texture when laying down the coating. However, using printing plates results in an area of missing coating due to a plate gap cause by wrapping the photopolymer plate around the plate roll. If no pattern or texture is necessary while coating, rubber rollers or coating sleeves are used. Rubber rollers or coating sleeves are seamless rollers that are used to distribute a coating across the entire surface of a substrate (Cusdin, 1999).

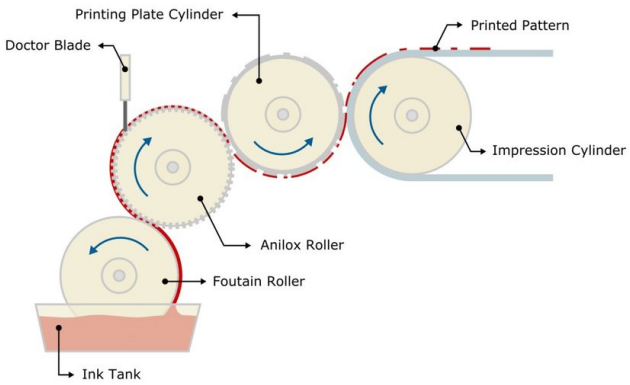

Figure 2. Flexographic printing process (Kipphan, 2001)

Note: The plate cylinder located in this diagram is often replaced with a rubber roller (with no indentions) for coating applications

Typically, commercial flexographic presses run at speeds higher than 2000 ft/min. Faster run speeds require fast drying inks and coatings. Solvent based coatings dry through evaporation. Dryers are used after each coating/ink station to improve the dry time of the solutions applied to the substrate. Solvent based coating drier temperatures and the amount of time the coating takes to dry are dependent upon the amount of coating being applied to the substrate and the run speed on the substrate. Nisin is a heat sensitive antimicrobial that can not exceed temperatures above above 141˚C (285.8˚F) (Perna, 2016). Thus, the dryers on the press can not exceed temperatures above 141˚C (285.8˚F). If the dry time, press speed, and coating weight are not optimized, the coating may become only partially dry, causing the material to adhere to internal rollers in the press or to the back side of the substrate inhibiting proper application of the coating.

1.8.1 Coating Characteristics

Maintaining pH and viscosity are critical when commercially applying nisin coatings on a flexographic press. The viscosity of a solution is defined as a solution's resistance to flow (Encyclopedia Britannica, 2018). In flexography, it is important that the coating maintain a low viscosity. Anilox rollers are cylinders containing a pattern of engraved cells with a particular cell width, cell angle, and cell depth. A coating with too high of a viscosity could become trapped in the cells of the anilox roller. When the coating becomes trapped in the cells of the anilox roller, is inhibited from properly flowing out of each cell and transferring on to the substrate. Also, when doctor blades are used, high viscosity coatings will not allow the cells of the anilox roller to reload properly, causing ink starvation and thus resulting in an undesired coat weight (Peter, Engledow, Freye, & Kershner, 1999). According to TAPPI, the standard viscosity range for an ink or coating being applied using the flexographic printing process should be between 12 to 60 seconds (64-596 cP) using a Zahn Cup #3 (TAPPI, 2010). Thus, it is important that flexographic coatings have a low viscosity that is best optimized for the press specifications.

Maintaining proper pH is also important when running solvent coatings on a flexographic plate. The pH value is related to the degree of acidity of the substance being measured on a scale from 0.0 to 14.0. A substance with a pH value from 0 to 7 is acidic, 7 is considered neutral, and from 7 to 14 is alkaline (Argent, Field, Gilbert, & Sickinger, 1999). Coatings that are highly acidic (low pH value) and highly basic (high pH value) would require flexographic press equipment, such as doctor blades, rubber rollers, and

coating stations that are corrosion resistant. Typically, commercial flexographic presses perform well when inks have a pH value between 6 and 9.5 to ensure longer press runs without needing to stop and clean the press or replace equipment caused by corrosion. Because nisin is most stable at a low pH value of 2.0, changing the pH to commercially stable pH value could cause the nisin to have a loss in activity.

1.9 Substrate

The substrate selected for this research is a linear low density polyethylene (LLDPE)/polyethylene terephthalate laminate donated by Sealed Air Corporation for this work. This material was selected based on previous research conducted Perna, who used meyer rods to apply coatings containing Nisaplin® to a LLDPE/PET co-extrusion film (Perna, 2016). For this research, the nisin coating will be "commercially" applied to the LLDPE side of the laminated film, since the LLDPE on the inside of the package will come in contact with the RTE deli meat.

LLDPE is a common polymer used for make films in the food packaging industry. As of 2016, LLDPE accounts for over 30% of the global polyethylene market and 31% of the U.S. polyethylene market and is steadily increasing with an anticipated average annual rate of 5% from 2017-2024 in sales (Intratec Solutions, 2013). Today, LLDPE is commonly used for films, plastic bags, food packaging, and in blow molding, injection molding, and rotomolding applications.

The properties of LLDPE are defined by the production and structure of the material. LLDPE is commercially manufactured and produced at lower temperatures and pressures than those used for LDPE. This process occurs by copolymerizing ethylene, at

a low pressure, with other alpha olefinic monomers, such as butene-1, hexene-1, or octene-1 (Mirabella & Ford, 1987) which has the following structure below.

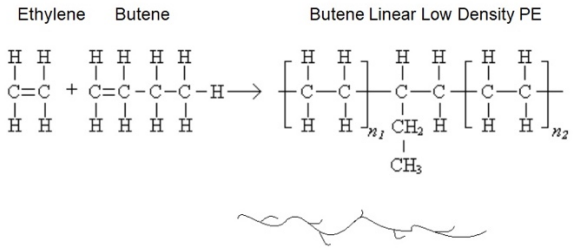

Figure 3. Copolymerization of ethylene and butene-1 monomers to form butene linear low density polyethylene

LLDPE includes short-chained, uniform branches that prevent the polymer chains from closely packing together (Encyclopedia Britannica, 2013). These short branches give LLDPE characteristics that set it apart from LDPE and HDPE. The short chains do not let the individual polymer structures stack on top of each other as compactly as HDPE, therefore giving it a lower density than HDPE (seen in Figure 4). The density of LLDPE ranges from 0.89 to 0.93 g/cm^3. The lower density of the LLDPE compared to the density of HDPE (equal or higher than 0.941 g/cm3) allows for increased flexibility of the structure. The short chain branches also allow for higher chemical stability, increased operation features at both high and low temperatures, increased puncture and tearing stability, and a higher stability against cracking (Polidon, 2011).

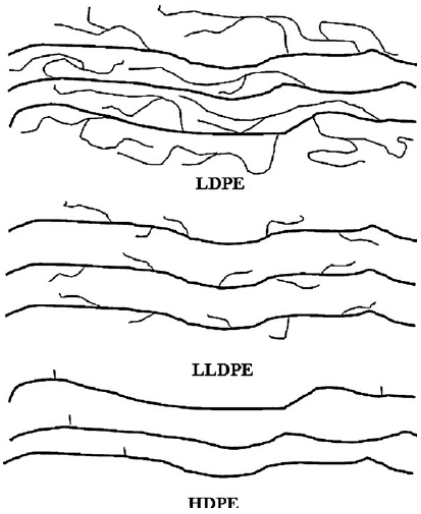

Figure 4. Comparison of the stacking of individual polymer structures for LDPE, LLDPE, and HDPE (Omar, 2012)

Polyethylene terephthalate (PET) is known in the polymer packaging industry for its excellent physical properties, being that it is tough and flexible to temperatures exceeding 200˚C (392˚F). The density of the film for all gauges, which range from 25 to 750 gauge, is 1.38 grams per cubic centimeter (Amborski & Flierl, 1953). Along with toughness, PET has excellent tensile strength, tear resistance, flexibility, and impact strength. However, its high melting point and resistance to high temperatures makes it very difficult to achieve a good seal at relatively low temperatures compared to the melt and seal temperatures of LLDPE. In fact, PET is often used for its thermal resistance. PET has good barrier properties with oxygen, carbon dioxide and water vapor permeation rates in the ranges of 0.6-0.8, 3-5, and 2.5-5.0 mol/(m • s • Pa) x 10^{-17} at 23˚C, 50% RH respectively (Massey, 2004). PET is typically used in semi-rigid packages, such as

bottling carbonated beverages and thermoformed containers for prepackaged RTE deli meat.

1.10 Surface Treatment

When applying liquid coatings to a substrate, it is necessary to have compatible surface energies and critical surface tensions to ensure proper wetting and adhesion. Wettability is the interaction between a liquid and a solid. In terms of coating, it is how well the liquid coating spreads across the surface of the substrate. Wettability is measured by the contact angle (θ) of the liquid to the solid (Berg, 1993). A low contact angle means high wettability ($\theta < 90^{\circ}$), contact angle equal to zero means perfect spreading or wetting ($\theta = 0^{\circ}$), and high contact angle means low wettability ($\theta > 90^{\circ}$) (Berg, 1993). The contact angle in relation to a single droplet of a coating can been seen in Figure 5 below.

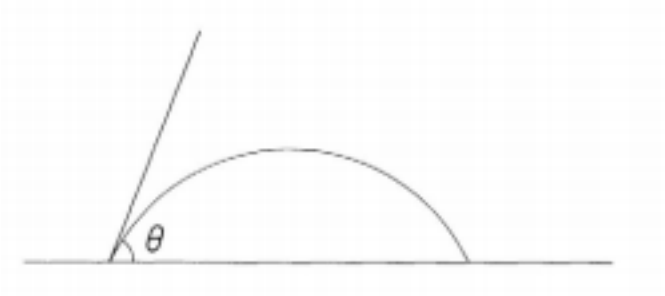

Figure 5. Contact angle and wettability (Berg, 1993)

As stated previously, in order to have good wettability, the critical surface energy and surface tension must be compatible. Liquid droplets contain interior cohesive forces that cause the droplet to create a perfect spherical shape in a zero-gravity environment. The solid surface exerts are forces that cause a liquid droplet to spread across the surface.

These two forces working against each other result in a contact angle (Figure 5) (Berg, 1993). The cohesive forces that are between molecules in a liquid coating are shared with all molecules nearby. When a liquid is applied to a solid surface, there are no neighboring molecules above, thus the liquid exhibits stronger adhesive forces on the neighboring molecules below the solid surface. Surface tension is defined as the amount of force required to increase the surface area and can be measured in dynes per cm (dynes/cm). When a drop of liquid coating is applied to a substrate, the wettability of the droplet depends on the amount of work put into the system to break the attraction between molecules in the droplet (Thompson, 1998). If a substrate has a low critical surface energy and the coating has a high surface tension, the contact angle will be greater than 90˚ causing low wettability. If the substrate has a high critical surface energy, the attraction forces exerted on the surface tension of the liquid coating droplet will be stronger than the internal cohesion forces of the droplet, resulting in a high wettability (Berg, 1993).

Surface treatments can be performed to raise the the critical surface energy of the substrate, allowing higher wettability. These surface treatments include the use of surfactants, primers, and corona treatment systems. In a previous study (Perna, 2016), the application of a primer and corona treating the surface of the LLDPE film was performed in order to create greater wettability and adhesion. However, the use of both surface treatments were found to adversely effect the seal strength of the final product. Proper seals and good seal strength are essential to the commercialization of the nisin coated substrate. In this research, the use of a primer was eliminated from the commercialization

process, in hopes of increasing the seal strength of the antimicrobial coated pouch. The surface of the LLDPE was treated through the use of a corona discharge treatment system.

1.10.1 Corona Discharge Treatment

One method of increasing the critical surface energy of a substrate is through corona discharge treatment. Corona discharge treatments are typically used in high speed operations such as commercial printing or coating. Corona treatment units can be installed in-line on a flexographic printing press so that the treatment can occur directly prior to applying the coating (Tracton, 2005). Corona discharge treatments apply a high voltage to the surface of the film which creates a strong electric field. The air between the two electrodes on a corona treater becomes ionized. The process accelerates electrons towards the positive electrodes, continuously colliding with gas molecules as they move. The collision of electrons and gas molecules creates additional positive ions, free electrons, and excited molecules. This excited mixture bombards the film surface, causing polar groups to be introduced into the polymer surface, leading to a higher surface energy (Zhang, Sun and Wadsworth 1998).

The effectiveness of a corona treatment or the overall change in the surface energy can be measured through the use of dyne pens. Dyne pens contain a liquid test solution with a known surface tension. When applied to a substrate, the liquid will either wet the surface of the substrate or bead up. If beading occurs, the surface energy of the substrate is too low compared to the surface tension of the dyne liquid. The dyne level of the substrate surface must exceed the dyne level of the solution's critical surface tension

by at least 2 dyne/cm for adhesion to occur. Typically, polymer films have critical surface tensions between 20 to 32 dyne/cm and conventional coatings have a dyne levels of 42 to 44 dyne/cm, thus the dyne level of the films must be raised to 44 to 46 dyne/cm to ensure proper wetting at the commercial level (Pierce, Downing, & Ladwig, 2008).

1.11 Heat Seal

A heat seal is the process of joining polymer films together by pressing two films together between two heated plates, for a set dwell time, to achieve fusion at the interface between the films (Meka & Stehling, 1994). The purpose of a seal is to protect the product throughout the distribution channels until the product's time of intended use by the sonsumer. Seal strength in plain language is the force required to pull apart the two substrates that are sealed using energy (heat), pressure and time. It is measured as the maximum force/width value obtained during a T-peel test. According to ASTM F88, "Seal strength of the package, which is defined as the measure of the ability of a package seal to resist separation". Minimum seal temperature (MST) or seal initiation temperature (SIT) is the minimum temperature required to obtain an acceptable seal strength (which is commonly 200 g/in). The strength of a seal is dependent upon the material, temperature, pressure, and time. T-peel tests are used to determine the seal strength of a polymer film (Figure 6). During a T-peel test, two "legs" of the polymer test sample are pulled a part a constant rate after being heat seal to achieve a force vs. extension (distance) curve (Meka & Stehling, 1994).

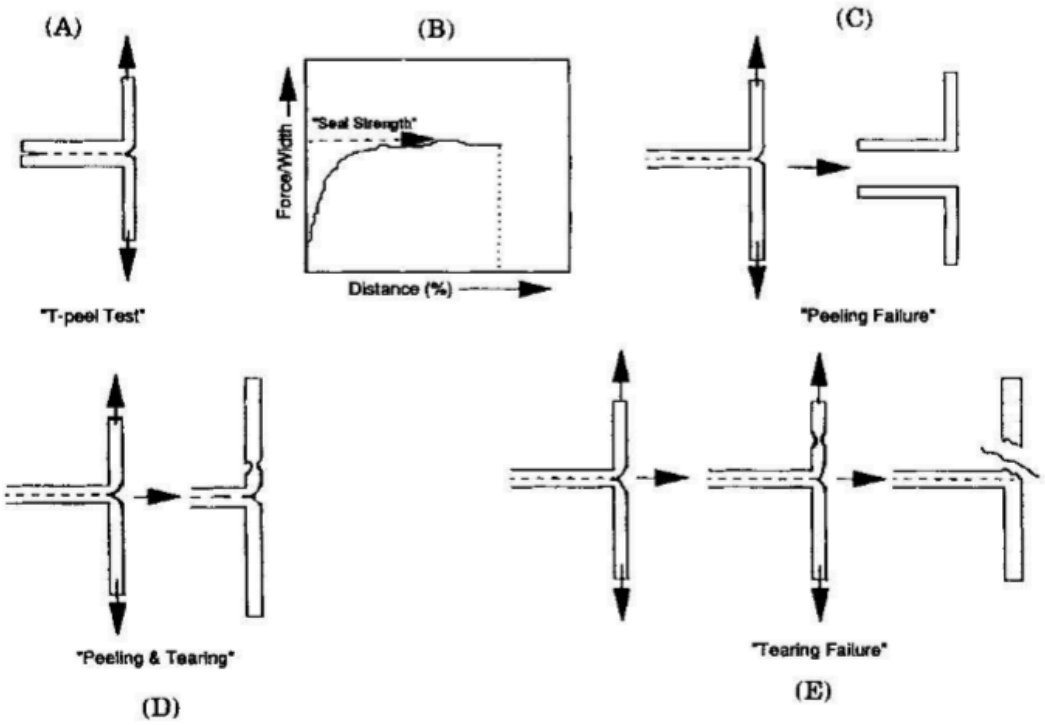

Figure 6. T-Peel test and types of possible failures (Meka & Stehling, 1994).

Heat seal curves are used to indicate the heat seal properties of the film. The curve plots the seal strength of the film (g/in) vs. the heat seal temperature (Figure 7). The heat seal curve seen in Figure 7. indicates at which point in time the seal "failed" and how failure occurred. These types of failures can be seen above (C-E) in Figure 6. above. This curve is individualized for each T-peel test that occurs.

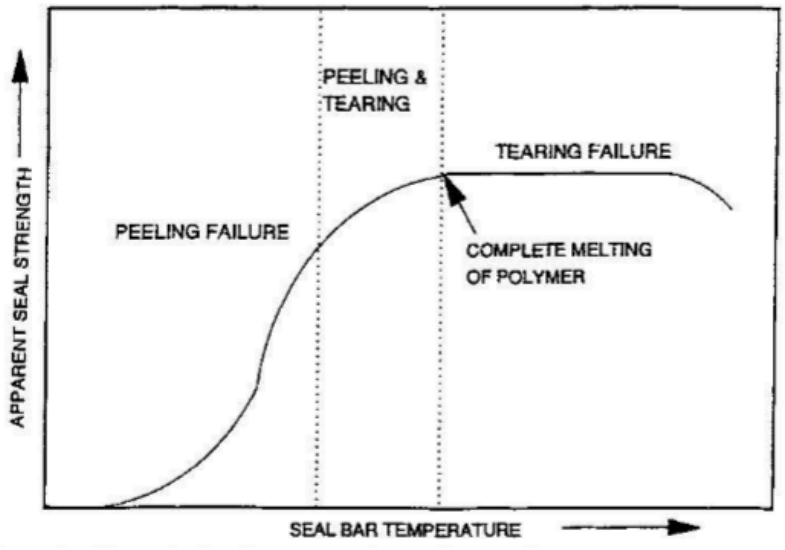

Figure 7. Heat Seal Curve (seal strength g/in vs. temperature)

(Meka & Stehling, 1994)

In a study (Farley and Meka 1994) found that the use of corona treatment has the potential to decrease the seal strength from a tear to a peel due to the cross-linking of the polymer surface. The cross-linking reduced the mobility of the chains and reduced chains diffusions at the interface of the seal. It was found that polymer films that underwent corona discharge surface treatment required a higher temperature heat seal than the control with no corona discharge surface treatment. In a study conducted by Perna (2016) it was suggested that the use of both a PEI primer and corona discharge surface treatment could eliminate the possibility of an achievable seal. Corona discharge surface treatment is required to achieve proper wetting and thus adhesion of a coating to the films surface. Therefore, for the purpose of this research, the use of a PEI primer was removed from the commercial application process. This may have an effect on the overall adhesion of the

nisin coating to the LLDPE film, however, the importance of a "good seal" is critical to the commercialization of the nisin coated film.

1.13 Justification

Commercialization of an antimicrobial coating containing Nisaplin® could be beneficial in inhibiting the growth of spoilage microorganisms and bacteria. Past research conducted (Perna, 2016 & Morgan, 2014) has shown that Nisin (Nisaplin®) is effective in inhibiting the growth of some spoilage microorganisms and different strands of bacteria in a small batch, laboratory setting. Commercialization of an antimicrobial coating containing Nisaplin®, using the Nisaplin® coating formulated by Perna (2016), could be effective on the inhibition of spoilage microorganisms and potentially harmful bacteria in ready-to-eat deli meat, if the antimicrobial maintains its efficacy. The results of this experiment could lead to a possible reduction of spoilage microorganisms and enhanced food safety and quality of ready-to-eat food.

METHODS AND MATERIALS

Film Coating Materials

One batch of each coating (coating with Nisaplin® and coating without Nisaplin®) was created using the materials provided in Table 3.

Table 3. Coating materials for antimicrobial coating with Nisaplin® and coating without Nisaplin®

Coating Materials	**Amount for small batch**	**Amount for Press Run**	**Purpose**
4-88 Mowiol PVOH resin (Kurary Poval 4-88, Kurary Amercia Inc, Huston, TX)	10g	249.48 g	Adhesive. Water soluble, synthetic polymer. Carries Nisaplin
50/50 (v/v) mixture of 95% ethanol and distilled water.	30ml/30ml	750ml/750ml	Solvent. Ethanol evaporates upon drying.
Glycerin (Vegetable Glycerin- Natural Oil. Streetsboro, OH)	3.2ml	80ml	Plasticizer for Polyvinyl Alcohol
Tween® 80 (0.25% v/v) (Polysorbate 80- Spectrum, New Brunswick, NY)	185µL	4.63ml	Surfactant. Decrease overall surface tension of liquid coating, and emulsifying component.
Nisaplin® (2.5% - 12,500 IU/mL in solution)* (Danisco, Inc. Madison, Wisconsin, USA)	1g	49.98g	Active antimicrobial
0.02 M acetic acid solution (Glacial-Fisher Chemical Company, Fair Lawn, NJ)	2ml	50ml	Dissolve Nisaplin® prior to mixing.

*For the control (Coating without Nisaplin®) the Nisaplin® was removed from the preparation.

** "Small batch" was the amount necessary for Perna to conduct lab research (Perna, 2016). For this experiment 1 gallon of each coating was required by the Clemson Sonoco Institute to properly run the flexographic printing press (Appendix C).

Coating Cost Analysis

Cost analysis was conducted for the antimicrobial coating created. The cost analysis was adapted from the Perna (2016) study and converted from the 671cm^2 used to calculate cost to 735cm^2. This was done because the calculations for this work are based of the area of the package used to package the sliced deli turkey meat used for this experiment. Also, the total cost per package is estimated to be higher for this work than if produced commercially due to the higher cost of buying ingredients at a smaller volume for laboratory use. Note, the cost of operating equipment and the cost of the material has been extracted from this equation, only focusing on the cost of the coating containing Nisaplin®.

Table 4. Coating cost calculation for 1 #/ream of coating to cover 735cm^2 area of a turkey deli meat package

Ingredient	Unit Cost ($)	Unit Volume	Amount Used per Pkg	AMT PKG/Unit Volume	Cost ($ per package)
Distilled Water	3	1 Gallon (3785.41 mL)	0.054355	1.43E-05	4.30492E-05
95% Ethanol	28.5	4000 mL	0.0510937	1.28E-05	0.000364179
Tween 80®	87.08	4000 mL	3.12E-04	1.28E-08	6.79438E-06
Glycerin (Food/USP grade)	13.49	32 oz (907.184 mL)	0.0054355	5.99E-05	8.07715E-05
Nisaplin®	80	1000 g	0.001685005	1.69E-06	0.000145671
Acetic Acid	99.11	4000 mL	0.000391356	9.78E-08	9.69693E-06
PVOH	12	1000 g	0.01685005	5.98E-04	0.000205027
			****1 #/ream**	**Cost Per Package ($)**	**0.000855189**

Coating and Film Preparation

Treatment: Coating Containing Nisaplin®. The coating was adapted from a previous formulation created by Perna (2016). The amount of materials used to prepare the coating in Perna's small batch study was scaled in order to ensure there was enough coating to operate a commercial flexographic printing press. The coating was prepared by heating and stirring 249.48 grams of 4-88 Mowiol PVOH resin in 750 mL of distilled water to 160°C for approximately 45-60 minutes until the resin dissolved into solution. For this step, 250 mL of distilled water was added, followed by 83.26 grams of 4-88 Mowiol PVOH resin. After the PVOH resin had completely dissolved into the distilled

water, this step was repeated until all of the resin had been dissolved into the distilled water. Once the resin was dissolved, 80 mL of glycerin (40 parts per 100 grams of PVOH resin) and 4.63 mL of Tween® 80 (0.25% v/v) were added to the cooling resin solution. In a separate beaker, 49.98 grams of Nisaplin® (2.5% - 12,500 IU/mL in solution) was dissolved in 50 mL of 0.02 M acetic acid solution. 750 mL of 95% ethanol was added, to the Nisaplin/acetic acid solution, which was then covered and stirred. After the resin solution had cooled and the Nisaplin® solution had completely dissolved, the two solutions were combined and mixed slowly until completely combined. In a previous study conducted by Franklin et al (2004), it was determined that 1 gram of Nisaplin® is equal to 12,500 IU/mL. This coating preparation created 1 batch of coating which is equal to 1750 mL.

Control: Coating without Nisaplin®. The same steps used to produce the coating contain Nisaplin® were used to create the coating without Nisaplin®, with the execption of the step where the Nisaplin® was added. No Nisaplin® was added to create this coating, however, the acetic acid was still added.

An uncoated film was also used as second control set, however, no coating was created or applied to the material.

pH and Viscosity

After producing the coating, the pH and viscosities of the coating without Nisaplin® and coating with Nisaplin® were measured and recorded. A Hanna pH tester pHEP 5 (Hanna Instruments, Woonsocket, RI, USA) with HI73127 pH electrode and a

standard buffer set of of 4.01, 7.01 and 10.0 was used to determine the pH of the coating before printing. The viscosity of both the coating with Nisaplin® and the coating without Nisaplin® were measured using a Zahn Cup #3 the results were given in seconds and then converted using a conversion table (Northcross Corporation, 2004).

Substrate

A 2.5 mil (0.0635 mm), PET/LLDPE laminate (Sealed Air, Duncan SC, USA) was used as the substrate to which the coating was be applied. The material has a heat seal range of 248-356˚F (120-180˚C). According to the datasheet supplied by Sealed Air, the OTR @ 23˚C, 0% R.H. is 5 cc/m^2/day (0.3 cc/100 in^2/day) in compliance with ASTM D-3985 standards. Additionally, in compliance with ASTM F-1249 standards, the WVTR @ 38˚C, 100% R.H. is 8 g/m^2/day (0.5 g/100 in^2/day). The coating was applied to the LLDPE side of the film.

Coating Trials

Two press runs using a flexographic printing press were conducted in order to replicate the experiment. The press runs are listed and described in Table 5.

Table 5. Press runs and material/coating identification

Press Run #	Name	Description	Code
1	No Nisaplin® Coated Film	Coating without Nisaplin®	C
2	Nisaplin® Coated Film	Coating containing Nisaplin®	N
**	Uncoated Film	LLDPE/PET Material, No Coating, No Corona Treatment	P

**The LLDPE/PET was never run through the press. However, the material was used during testing and experimentation; therefore, it was coded and named accordingly.

Coated films were produced by running a LLDPE/PET laminate (Sealed Air, Duncan SC, USA) on a flexographic OMET VaryFlex 530 printing press (Sonoco Institute, Clemson SC, USA). Both, the coating without Nisaplin® and coating containing Nisaplin®, were run on the 7th print station. Printing on the 7th station prevented the coated film from coming into contact with unnecessary nips and decreased the amount of heat applied to the coating when passing though the drying tunnels. The conditions of the OMET VaryFlex 530 press are listed below in Table 5. The press was set up to coat the LLDPE side of the laminate film. Prior to coating, the surface tension of the LLDPE side of the film was raised by corona discharge treating at 1660 watts to a

dyne level of 44 dynes/cm. Before corona treating, the dyne level of the LLDPE side of the laminate was measured at a dyne level of 32 dynes. The dyne level was determined through the use of a dyne pen test following ASTM D2578-09. The press was run at the lowest press speed of 32fpm. To avoid the nisin from becoming inactive due to excessive heat, the drying tunnels were set to 200˚F. The coating was applied using a 15.2 BCM anilox roller and a coating sleeve. The control coating was applied first to prevent contamination of Nisaplin®. Perna (2016) conducted a press run at similar conditions, however, the dry temperature was set at an average of 155˚F. The press run was held 11 days after the coatings had been produced. When the press, run at a higher speed (100 fpm), using a 30 BCM anilox roller, the applied coating was too thick and did not dry enough before passing through the press. This caused the coating to build up on accessory rollers in the press, resulting in an uneven coating with some of the coating areas removed form the film roller onto the press rollers. This required a press shut down to clean the rollers affected. From this, it was determined that the coating weight was too high in order to properly dry. This resulted in a second press run performed 11 days later, conducted at the press conditions listed below in Table 6, with a lower BCM and press speed.

Table 6. Conditions of OMET VaryFlex 530 Flexographic Press in Sonoco Institute of Packaging Design and Graphics for control and treatment of antimicrobial coatings

Sample	Control	Treatment (Nisaplin®)
Primary unwind material	2.5 mil LLDPE/PET laminate	
Material Core (in)	6	6
Coat Side	In	In
Web Width (in)	19	19
Corona Discharge Treatment Side	Coat Side	Coat Side
Corona Discharge Treatment Watts	1660	1660
Dyne Level Before Treatment (dyne/cm)	32	32
Dyne Level After Treatment (dyne/cm)	44	44
Rewind Coat Side	Out	Out
Coater Anilox	15.2 BCM, 160 CPI, 60˚	15.2 BCM, 160 CPI, 60˚
Coating	Control Coating (No Nisaplin®)	Treatment Coating (Nisaplin®)
Station Dryer Temperature (˚F)	200	200
Line Speed (ft/min)	32	32

Basis Weight Testing

The coating weight (basis weight of the coating) on the substrate was determined using ASTM 2217 (Standard Practice for Coating/Adhesive Weight Determination). The basis weight of both the control (C) and treatment (N) film were determined through the wash method.

Coating basis weight tests were conducted separately for the control (C) and treatment (T) films. The 18.3 inch sheet was marked with the locations Operator (O) Center (C) Drive (D) seen in Figure 8.

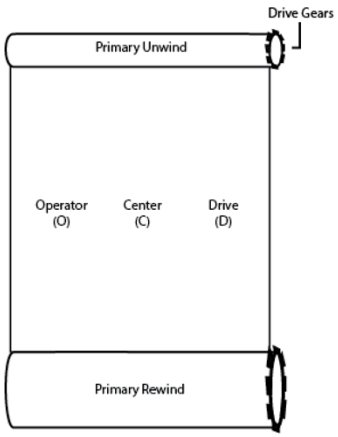

Figure 8. Diagram of Operator (O) Center (C) Drive (D) sides of web film

Using a 3.08 sq/in stencil, four squares were cut in a column for each of the control, operator, and drive sides leaving approximately 1.2 inches (30.48 mm) of space between the top and bottom of square, totaling in twelve labeled sample squares (ex. C1, C2, C3, C4, O1, O2, O3, O4, etc.).

The initial weight of each square was measured and recorded. Using ethyl acetate and a paper towel, each square was washed to remove all of the coating from the surface. The coated surface of the material was washed and the material was weighed until the

lowest number was achieved. The lowest measured weight of the washed film was recorded.

The following calculation was used to determine the basis weight of the coatings:

*Coating Basis Weight (BW)(#/ream) = (Sample Weight with Pre-wash – Sample Weight Post-wash) * 100 #/ream*

Adhesion Tape Testing

A tape test was conducted according to standard practices listed in ASTM D3359 – Standard Test Methods for Rating Adhesion by Tape Test. Two 6 in x 19 in (152.4 mm x 482.6 mm) in strips of both the coated films (coating with Nisaplin® and coating without Nispalin®) were cut to be tested. Using a clipboard, the operator side of each film was held down. Clear, 3M 610-tape (3M, Maplewood, MN) was placed down on the film moving in the direction from operator to the drive side of the film. The tape was pressed down with firm pressure and left on for a 90 second dwell time. After 90 seconds, the tape was removed by lifting and rapidly pulling the operator side of the tape back upon its self, with as close to a 180˚ angle as possible. Visual results were observed and recorded.

Color (ΔE)

The color (ΔE) of the films of the coating with Nisaplin® and coating without Nisaplin® was tested using a Minolta La*b* colorimeter (CR-400 Chromameter). Prior to testing the colorimeter was calibrated to standard white. Seven measurements were

taken from each of the three films. The ΔE of the films of the coating with Nisaplin® and coating without Nisaplin® were calculated using the uncoated material. ΔE was the calculated using the following The International Commission on Illumination (CIE) ΔE76 formula (Mclaren, 2008):

$$\Delta E: \sqrt{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2}$$

ΔE measurements are given in a range from 0 to 100. ΔE of 0 represents that there is no difference in color, where as a ΔE of 100 represents there is a drastic difference in color. A ΔE of 1 is considered the minimal level a trained observer can perceive a color difference (Hunter, 2009). The ΔE of the two coated films was calculated from the difference between each coated film and the uncoated film.

Heat Seal Strength

Preliminary heat seal tests were conducted on the Sentinel Heat Sealer (Packaging Industries Inc, Hyannis, MA) to determine the initial seal pressure, time, and temperature required to run a heat seal test. Strips (6 in by 1 in) (152.4 mm by 25.4 mm) were cut from the Treated (N) film. The initial temperature was set at 200˚F/206˚F (top bar/bottom bar) with a dwell time of 0.5 seconds, and a pressure set to 30 psi (206.84 kPa). Three heat seals were tested, all resulting in a tack and no seal. The temperature, dwell time, and pressure all increased until the seal resulted in a peel. The final recorded settings to retrieve a failed peel heat seal were 298˚F/299˚F (147.78˚C/1.48.33˚C), with a dwell time of 2 seconds, and a pressure of 35 psi (241.32 kPa).

After the range for a peel seal was established a heat seal profile was created for each of the three films; film with coating containing Nisaplin®, film with continuing without Nisaplin®, and the uncoated film, a SL-10 Laboratory Hot Tack/ Seal Tester (The TMI Group of Companies, New Castle, DE). Starting at 300˚F (148.89˚C), eleven test groups were conducted, ending in a temperature at 400˚F (204.44˚C). The dwell time and pressure remained constant at 2 seconds and 35 psi (241.32 kPa). With each seal test group, a 1-inch (25.4 mm) seal was created on five samples (13 in x 0.752 in) (330.2 mm x 19.1 mm), resulting in a total of 55 samples at the end of the entire heat seal profile test. Between each test group, the heat seal temperature increased by 10˚F (12.2˚C). For every seal test conducted, the LLDPE side of the film was sealed together. Seal curves, failure descriptions and additional data were recorded.

Inhibition Testing- Establishment of Maximum Effective Storage Time

Experiment 1. A study was performed to determine the maximum length of time (days) the antimicrobial coating containing Nisaplin® would show inhibition in the growth of aerobic bacteria, coliform bacteria, and staph, using ready-to-eat turkey deli meat. The three films (films with and without Nisaplin® coating, and uncoated film) were cut in 18.0 inch by 6.3 inch (457.2 mm by 160.0 mm)(1/3rd of the total 19-inch width) strips with LLDPE (sealant side) up. Each strip was labeled Operator (O), Center (C), and Drive (D) according to where the strip was cut. The film strips were folded in half converted into a 3 sided sealed pouch with a 1-inch (25.4 mm) seal at a temperature of 350˚F (176.67˚C), with a dwell time of 2 seconds and a pressure of 35 psi (241.32

kPa). The top seal was left unsealed for the deli meat to be inserted. After the deli meat was inserted, the top seal was created using the same time, temperature and pressure. The maximum days of storage were established as, 10 days, with sampling intervals testing on days 1, 6, and 10. One pouch was created and labeled for each side (O,C,D) of each sample film (C,N,P) for each trial day, resulting in a total of 27 pouches (ex. "Day 1, C, O", "Day 1, N, C", etc.). Pouches were created 11 days after the coating was applied to the substrate using the flexographic printing press.

After each pouch was created, each pouch was filled with one slice (approximately 75 to 120g) of "Boar's Head, Simplicity® All Natural Roasted Turkey Breast" deli meat (Publix Deli, Clemson, SC). This brand and type of turkey was selected because it contains no nitrates/nitrites added (except those naturally occurring in sea salt) no preservatives, and no added hormones/antibiotics. The turkey deli meat was sliced 30 minutes after store opening, 120 minutes prior to being packaged in pouches. The deli meat was first packed in all the uncoated film packages, followed by the packages with the film coated without Nisaplin®, and ending with the pouches created from the film coated with Nisaplin® to avoid any possible Nisaplin® contamination in the two control group pouches. The meat was placed into the pouch using sterilized forceps. The deli meat was placed into each pouch flat, with no fold, to maximize the amount of contact between the meat on the film. Immediately after packaging, a top seal was created and the samples were stored in a refrigerator with at, 41 ± 4˚F (5 ± 2˚C) and 90 ± 5% R.H. in compliance with ASTM Standards D4332–Standard Practice for Conditioning Containers, Packages, or Packaging Components for Testing.

Inhibition testing was performed using three different Petrifilms (3M, Maplewood, MN) Aerobic Count Plates, Staph Express Count Plates, and Coliform Count Plates. These were used to determine the effectiveness of the Nisaplin® coating. Prior to each sampling period, 0.1% peptone water solution was created using 1 gram of peptone (Difco™ Peptone Water, Becton Dickinson and Company, Sparks, MD) per 1000 ml of distilled water. Test tubes (22mL volume) were filled with 9 ml of 0.1% peptone water and covered with a cap. The remaining 0.1% peptone water solution and test tubes were autoclaved at 124˚C for 20 minutes (Primus Sterilizer, Sterilelink Inc, Greensboro, NC) then set aside until completely cooled.

On each sampling period, 9 samples (one for each film C,N,P per side O,C,D) were tested. Each turkey deli meat sample was removed individually from the film pouch and weighed on a Mettler Toledo scale (PG203-S Mettler Toldeo, Switzerland) in a sterile sampling bag (Nasco, Whirl-Pak®). Using the USDA rinse method, the turkey deli meat slice was rinsed in a 1:10 mL solution of the previously created 0.1% peptone water solution (10 mL of 0.1% peptone water per 1 g of deli turkey meat). The turkey was rinsed for approximately 60 seconds before plating. 1 mL of rinsed 1:10 turkey to peptone water solution was diluted to the 5^{th} dilution in 9 mL sterilized peptone water. 1 mL of 1:10 turkey to peptone water solution and 1 mL of each dilution was plated in duplicate onto the three types of Petrifilm plates (aerobic, coliform, and staph). The aerobic and staph Petrifilm plates were incubated for 48 ± 3 hours at 37˚C, and the coliform Petrifilm plates were incubated for 24 ± 3 hours at 37˚C. After incubation, plates were counted, plates with over 200 colonies were labeled as “Too Numerous To Count”

(TNTC). The colony count for each side of the film in duplicate (O,C,D) was analyzed as an average and as each individual side.

Experiment 2. A second experiment was conducted to determine the effectiveness of the Nisaplin® coating on the inhibition of aerobic bacteria, coliform bacteria, and staph microbial growth in a focused 11-day trial period, using the same materials that were previously created and stored for 45 days. Over an 11-day sample period, inhibition tests were conducted on day 0, 3, 5, 9, and 11. Note, sampling was suppose to be conducted on day 0,3,5,7, 9, and 11, however a power outage on campus inhibited the ability to properly test on day 7. Results from day 9 and day 11 were not affected from the power outage. A temperature probe placed in the refrigerator stated that the temperatures inside the refrigerator did not reach temperatures above required temperature conditions. The steps utilized in first experimental test section above were repeated in order to conduct the second experimental test.

Data Analysis for Inhibition Testing. The duplicate plate count measurements from each side of the sample films (O,C,D) were averaged to create the sample size (n=6). The web locations (O,C,D) were briefly analyzed separately as well (Appendix A). Prior to statistical testing, all data entries were transformed into logarithmic functions. ANOVA single factor statistic analysis tests (Excel 2015, RealStats Analysis ToolPak) for day 6 and 10 for Experiment 1 and day 3, 5, 9, and 11 for Experiment 2 were conducted to determine if the averages of the colony counts were different between the treatments (C,N,P). If the ANOVA concluded that the samples were different, a t-test: two-sample assuming equal variances (Excel 2015, RealStats Analysis ToolPak) was

conducted for each of the samples, (each film against the other two). The data for the inhibition was analyzed with the null hypothesis that all averages were equivalent, if an average were statistically different, the null hypothesis would be rejected. The level of significance used was $\alpha = 0.05$ (95% level of confidence).

RESULTS

The viscosity of the coating without Nisaplin® was measured at an average of 18.32 seconds and the viscosity of the coating with Nisaplin® was measured at an average of 19.53 seconds. The pH of the coating without Nisaplin® was measured at 7.27, where as the pH of the Nisaplin® coating was measured at 7.02.

The average coating basis weight of the coating containing no Nisaplin® was 0.478 #/ream (.708 GSM/ream). The average coating basis weigh of the coating containing Nisaplin® was 0.575 #/ream (.851 GSM/ream). The calculated basis weights for each film shown below in Table 7.

Table 7. Calculated coating basis weights

	Coating without Nisaplin® (#/ream)			Coating with Nisaplin® (#/ream)		
SAMPLE	O	C	D	O	C	D
1	0.090	0.470	0.460	0.550	0.540	0.610
2	0.700	0.460	0.490	0.490	0.550	0.600
3	0.400	0.440	0.510	0.620	0.580	0.590
4	0.730	0.470	0.520	0.600	0.600	0.570
Average	0.480±0.3	0.460 ±0.01	0.495±0.03	0.565±0.06	0.567±0.03	0.593±0.02
Total Average BW	**0.478±0.16**			**0.575±0.04**		

After the basis weights of each of the coatings were determined, a tape test was conducted on both the coating with Nisaplin® and coating without Nisaplin® film. The results of the tape test showed that there was no visible coating removal from the surface of the material.

The color test conducted with the colorimeter (seen in Table 8), provided a ΔE of 0.165 (ΔE < 1.0) for the coating without Nisaplin® and a ΔE of 0.987 (ΔE <= 1.0) for the coating with Nisaplin®. Both the coatings with and without Nisaplin® had minimal color perception.

Table 8. ΔE of coatings with and without Nisaplin®

	ΔE Calculation	ΔE Perception
Coating without Nisaplin®	0.165	< 1.0
Coating with Nisaplin®	0.987	<= 1.0

The results of the heat seal profile test of the three treatments, concluded that all three had an achievable seal that met minimum ASTM standards of 200 g/in (200 g/25.4 mm). Coating with Nisaplin® achieved a peel seal at 300˚F (148.89˚C) with with an average force of 328.7 g/in (g/25.4 mm)increasing in strength (force g/in) as temperature increased (Figure 9). Both the coating without Nisaplin® (Figure 10) and uncoated film (Figure 11) achieved peel seals at 300˚F (148.89˚C) with the coating without Nisaplin® having a seal strength average of 854 g/in (g/25.4 mm) and the uncoated film having an

average seal strength of 4254 g/in (g/25.4 mm). Both the coating without Nisaplin® and the uncoated film had slight increases in seal strength (force g/in) as temperature increased but did not achieve a change in failure. All three of the films tested maintained a seal peel throughout each of the heat seal profile tests.

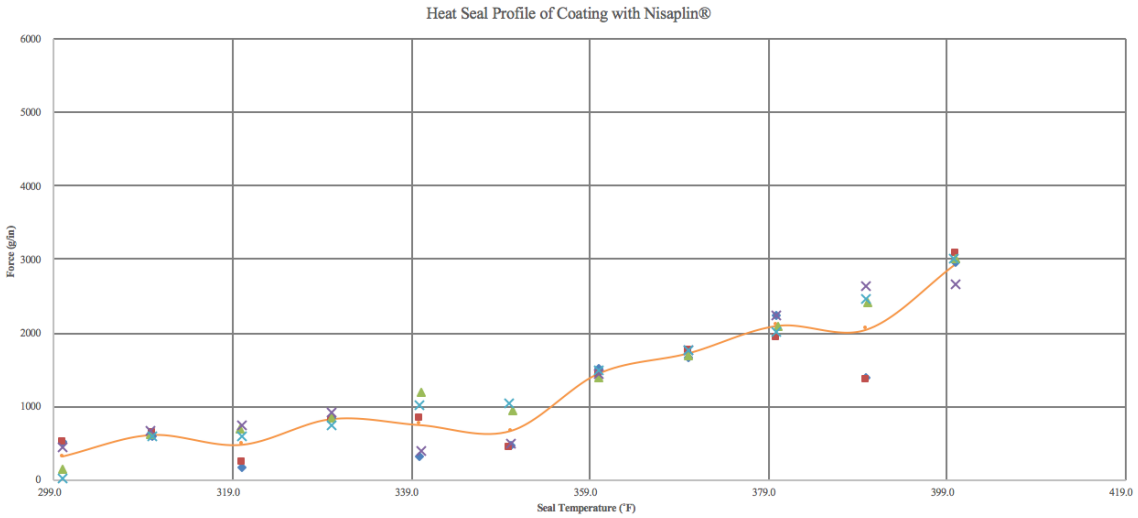

Figure 9. Heat seal profile of film with coating with Nisaplin®

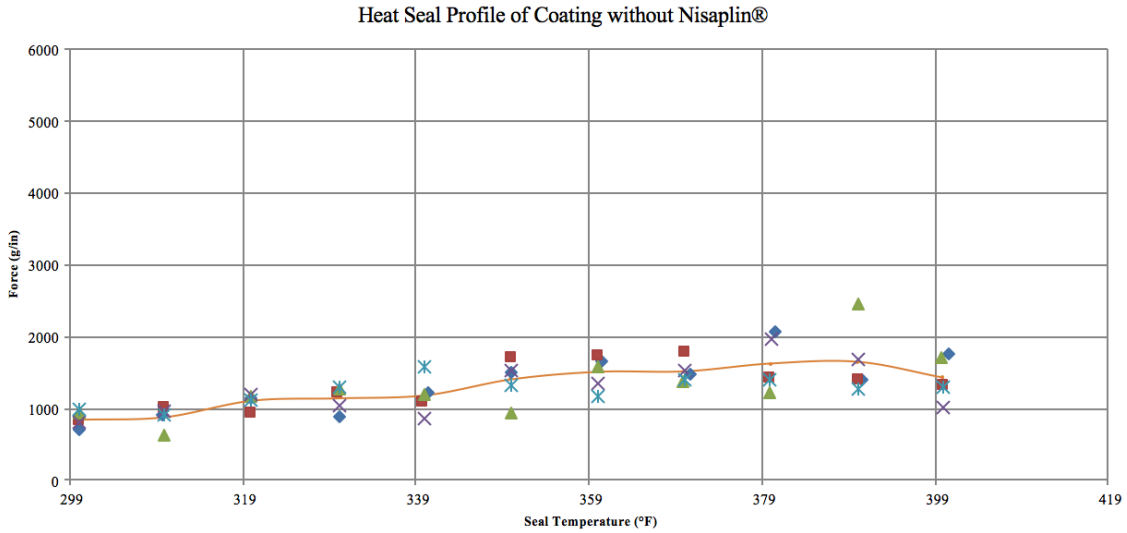

Figure 10. Heat seal profile of film with coating without Nisaplin®

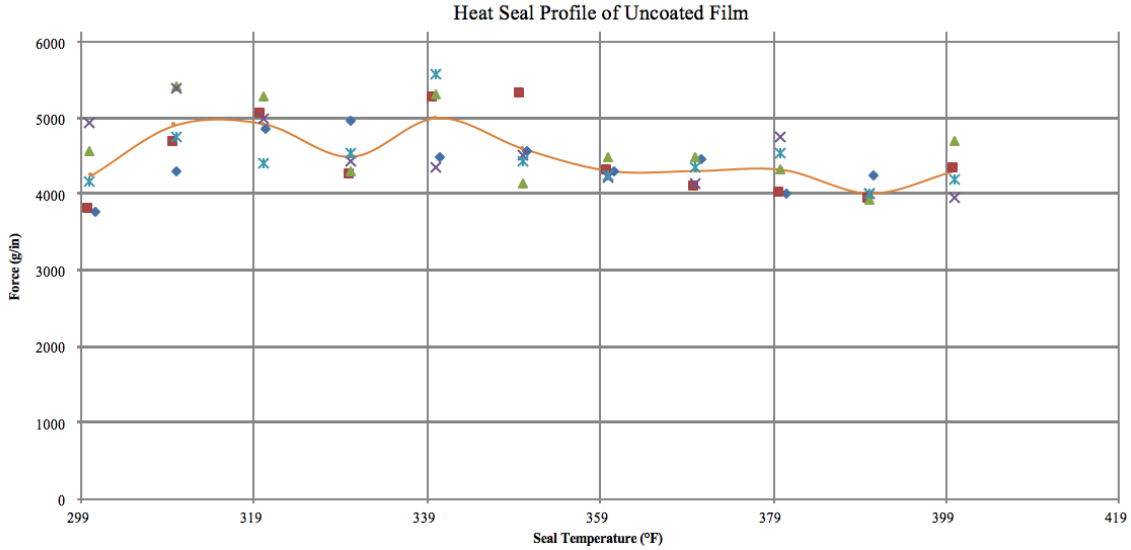

Figure 11. Heat seal profile of uncoated film

Inhibition of Staph Microbial Growth

At the end of the inhibition test for both Experiment 1 and Experiment 2, Staph countable colonies were not formed. Also, on Experiment 2 – Day 10, the Staph 3M Petrfilim's formed green colored colonies that are unspecified by the 3M Staph Petrifilm data sheet. The formation of these green colonies are suspected to be the growth of an unidentified yeast. Due an uncountable number of colony formations and the formation of unknown colonies, the results of the Staph Express 3M Petri film plates were not further analyzed.

Inhibition of Coliform Microbial Growth

ANOVA testing was conducted for the coliform plate counts for both Experiment 1 and Experiment 2 (Appendix B). There was statistical difference between the three treatments on Day 6 of Experiment 1. There was statistical difference between the

uncoated film vs. the coating with Nisaplin® and between the coating with Nisaplin® vs. the coating without Nisaplin®. There was no statistical difference between any of the treatments during Experiment 2. The plate counts of the coating with Nisaplin® on Experiment 1, Day 6 was considered "too few to count" (TFTC), which could have been caused by experimental error.

Inhibition of Aerobic Microbial Growth

The ANOVA tests conducted on the 3M Petrifilm Aerobic Count Plates suggested that for Experiment 1 and Experiment 2 there was a statistical difference between both the control groups (the uncoated film and the coating without Nisaplin®) and the coating with Nisaplin® (Appendix A). With the exception of a few outliers, there was no significant difference between three film sides (Operator, Center, Drive) for Experiment 1 or Experiment 2 (Appendix A).

Experiment 1. The results of the ANOVA test conducted for Experiment 1, suggested that there is a statistical difference between the three treatments for Day 6 (Table 9) and Day 10 (Table 10). On Day 6, there was a 2-log reduction of the aerobic colony counts (cfu/mL) between the uncoated film vs. the coating with Nisaplin® and the coating without Nisaplin® vs. the coating with Nisaplin® (Figure 12) There was only a 0.5-log reduction between the two control groups (the uncoated film vs. the coating with Nisaplin®). The t-test conducted for the three treatments on Day 6 suggested that there was a statistical difference between all three treatments (Table 11). The t-test conducted for Day-10 suggested that there was no statistical difference between the coating with

Nisaplin® and the two control groups, but there was a statistical difference between the two control groups (uncoated film vs. coating without Nisaplin®) (Table 12).

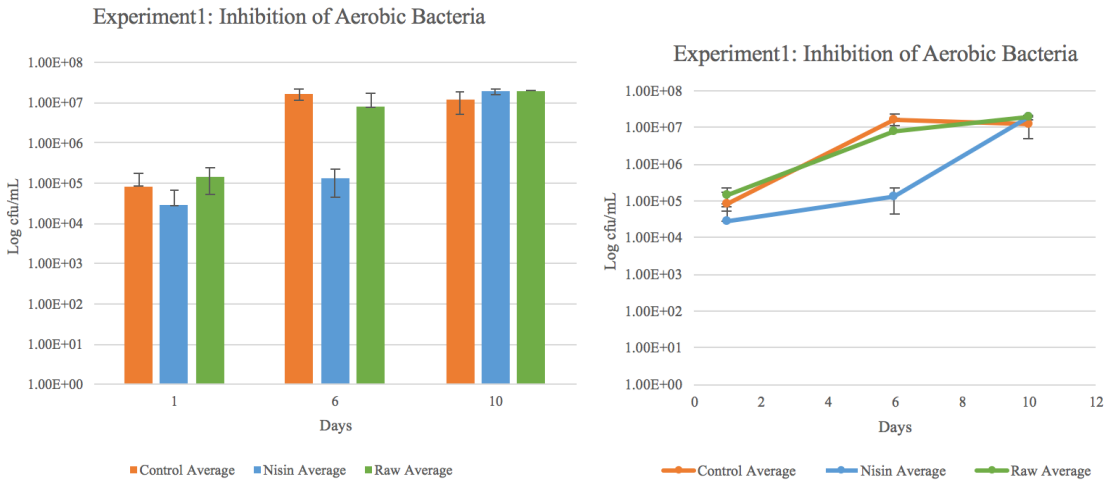

Figure 12. Treatment effect on the inhibition of aerobic microbial growth: Experiment 1

Table 9. ANOVA test for inhibition of aerobic microbial growth: Experiment 1, Day 6

ANOVA: Single Factor
Aerobic Count Experiment 1, Day 6
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	43.156428	7.192738	0.03028052
V2 With Nisaplin®	6	29.6877073	4.94795121	0.26094482
V3 Uncoated	6	39.6742363	6.61237272	0.28558321

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16.2923811	2	8.14619053	42.368602	6.7493E-07	3.68232034
Within Groups	2.88404271	15	0.19226951			
Total	19.1764238	17				

Reject The Null

Table 10. ANOVA test for inhibition of aerobic microbial growth: Experiment 1, Day 10

ANOVA: Single Factor
Aerobic Count Experiment 1, Day 10
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	42.0414634	7.00691056	0.07908365
V2 With Nisaplin®	6	43.6157397	7.26928995	0.00604458
V3 Uncoated	6	43.80618	7.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.3127132	2	0.15635661	5.5101558	0.01606352	3.68232034
Within Groups	0.4256412	15	0.02837608			
Total	0.7383544	17				

Reject The Null

Table 11. t-Test for inhibition of aerobic microbial growth: Experiment 1, Day 6

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V3 Uncoated	*V2 With Nisaplin®*
Mean	6.612372723	4.947951213
Variance	0.285583208	0.260944819
Observations	6	6
Pooled Variance	0.273264013	
Hypothesized Mean Difference	0	
df	10	
t Stat	5.514837574	
P(T<=t) one-tail	0.0001282	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.0002564	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V3 Uncoated	*V1 Without Nisaplin®*
Mean	6.612372723	7.192737999
Variance	0.285583208	0.030280516
Observations	6	6
Pooled Variance	0.157931862	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.529456237	
P(T<=t) one-tail	0.014949379	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.029898758	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V1 Without Nisaplin®	*V2 With Nisaplin®*
Mean	7.192737999	4.947951213
Variance	0.030280516	0.260944819
Observations	6	6
Pooled Variance	0.145612667	
Hypothesized Mean Difference	0	
df	10	
t Stat	10.18910753	
P(T<=t) one-tail	6.69202E-07	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	1.34E-06	
t Critical two-tail	2.228138852	

Reject the Null

Table 12. t-Test for inhibition of aerobic microbial growth: Experiment 1, Day 10

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V3 Uncoated	*V2 With Nisaplin®*
Mean	7.301029996	7.269289948
Variance	0	0.006044584
Observations	6	6
Pooled Variance	0.003022292	
Hypothesized Mean Difference	0	
df	10	
t Stat	1	
P(T<=t) one-tail	0.170446566	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.340893132	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V1 Without Nisaplin®	*V2 With Nisaplin®*
Mean	7.006910564	7.269289948
Variance	0.079083651	0.006044584
Observations	6	6
Pooled Variance	0.042564117	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.202766789	
P(T<=t) one-tail	0.026098022	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.052196043	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V3 Uncoated	*V1 Without Nisaplin®*
Mean	7.301029996	7.006910564
Variance	0	0.079083651
Observations	6	6
Pooled Variance	0.039541826	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.561863512	
P(T<=t) one-tail	0.014141316	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.028282633	
t Critical two-tail	2.228138852	

Reject the Null

Experiment 2. The results of the ANOVA test conducted for Experiment 2, suggested that there is a statistical difference between the three treatments for Day 5 (Table 13) and Day 9 (Table 14). The ANOVA test also suggested that there was no statistical difference between the three treatments on Day 3 or Day 11 (Appendix A). On Day 6, there was a 2-log reduction of the aerobic colony counts (cfu/mL) between the both of the control groups vs. the coating with Nisaplin® (uncoated film vs. coating with Nisaplin® and coating without Nisaplin® vs coating with Nisaplin®) (Figure 13). On Day 9, there was a 1-log reduction of the aerobic colony counts (cfu/mL) between the both of the control groups vs. the coating with Nisaplin® (uncoated film vs. coating with Nisaplin® and coating without Nisaplin® vs coating with Nisaplin®) (Figure 13). On Day 5, the T-test conducted suggested that there was a statistical difference between the

two control groups and the coating with Nisaplin®, but no statistical difference between the two control groups (Table 15). This result was the same for the t-Test conducted for Day 9 of this experiment (Table 16). One Day 11, the coating with Nisaplin reached a colony count that was “too numerous to count” (TNTC). Thus, it was considered no longer edible, and the coating with Nisaplin® matched the colony count numbers of both the control groups.

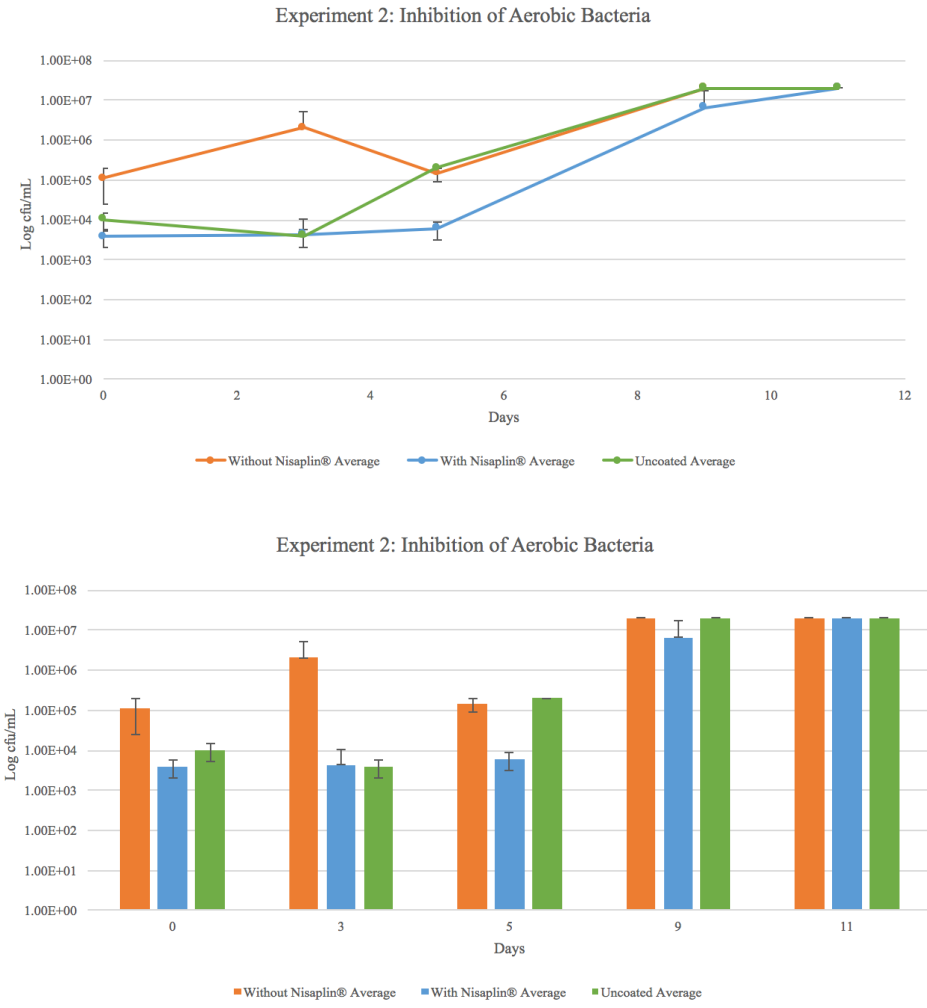

Figure 13. Treatment effect on the inhibition of aerobic microbial growth: Experiment 2

Table 13. ANOVA test for inhibition of aerobic microbial growth: Experiment 2, Day 5

ANOVA: Single Factor
Aerobic Count: Experiment 2: Day 5
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Wihtout Nisaplin®	6	30.7963967	5.13273278	0.04970489
V2 With Nisaplin®	6	22.3429958	3.72383264	0.08637535
V3 Uncoated	6	31.80618	5.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.00175017	2	4.50087508	99.2254716	2.2436E-09	3.68232034
Within Groups	0.68040116	15	0.04536008			
Total	9.68215133	17				

Reject the Null

Table 14. ANOVA test for inhibition of aerobic microbial growth: Experiment 2, Day 9

ANOVA: Single Factor
Aerobic Count: Experiment 2: Day 9
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Wihtout Nisaplin®	6	43.80618	7.30103	0
V2 With Nisaplin®	6	31.322386	5.22039767	3.09607089
V3 Uncoated	6	43.80618	7.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.3161235	2	8.65806173	8.38940261	0.00358635	3.68232034
Within Groups	15.4803544	15	1.03202363			
Total	32.7964779	17				

Reject the Null

Table 15. t-Test for inhibition of aerobic microbial growth: Experiment 2, Day 5

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V3 Uncoated	*V1 Wihtout Nisaplin®*
Mean	5.301029996	5.132732782
Variance	0	0.049704886
Observations	6	6
Pooled Variance	0.024852443	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.849068545	
P(T<=t) one-tail	0.047094413	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.094188826	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V3 Uncoated	*V2 With Nisaplin®*
Mean	5.301029996	3.723832636
Variance	0	0.086375347
Observations	6	6
Pooled Variance	0.043187673	
Hypothesized Mean Difference	0	
df	10	
t Stat	13.14518686	
P(T<=t) one-tail	6.17135E-08	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	1.23427E-07	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V1 Wihtout Nisaplin®	*V2 With Nisaplin®*
Mean	5.132732782	3.723832636
Variance	0.049704886	0.086375347
Observations	6	6
Pooled Variance	0.068040116	
Hypothesized Mean Difference	0	
df	10	
t Stat	9.355312908	
P(T<=t) one-tail	1.4588E-06	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	2.91761E-06	
t Critical two-tail	2.228138852	

Reject the Null

Table 16. t-Test test for inhibition of aerobic microbial growth: Experiment 2, Day 9

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V3 Uncoated	*V1 Wihtout Nisaplin®*
Mean	7.301029996	7.301029996
Variance	0	0
Observations	6	6
Pooled Variance	0	
Hypothesized Mean Difference	0	
df	10	
t Stat	65535	
P(T<=t) one-tail	0.00E+00	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.00E+00	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V3 Uncoated	*V2 With Nisaplin®*
Mean	7.301029996	5.220397672
Variance	0	3.096070889
Observations	6	6
Pooled Variance	1.548035445	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.896446549	
P(T<=t) one-tail	0.007965135	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.01593027	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V1 Wihtout Nisaplin®	*V2 With Nisaplin®*
Mean	7.301029996	5.220397672
Variance	0	3.096070889
Observations	6	6
Pooled Variance	1.548035445	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.896446549	
P(T<=t) one-tail	0.007965135	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.01593027	
t Critical two-tail	2.228138852	

Reject the Null

DISCUSSION

The viscosity of both the coating with Nisaplin® (19.35 seconds) and without Nisaplin® (18.32 seconds) had an approximant viscosity measure between 100-125cp. In the Perna (2016) study, the coating with Nisaplin® had viscosity of 25.27 seconds and the coating without Nisaplin® had a viscosity of 23.49 seconds, both with an approximant viscosity measure between 175-200 cp. The viscosity of the coatings created in this experiment were lower than the coating with Nisaplin® in Perna's study. However, the viscosity of the coatings for this experiment still was in an acceptable range for flexographic printing between 64-596 cp (TAPPI, 2010).

The coating with Nisaplin® has a pH of 7.02 and the pH of the coating without Nisaplin® was measured at 7.27. Both of these coating pH's are considered neutral, which is desirable for flexographic printing. However, previous research suggests that Nisaplin® is found to be most effective at lower pH values (Delves-Broughton, 1990). The coating with Nisaplin® in Perna's (2016) research had a pH value of 5.61. Perna stated that the low pH could have been attributed to dissolving the Nisaplin® in the .02M of acetic acid solution. Perna's coating without Nisaplin®, more closely matched the pH results of this experiment with a pH of 6.42. Although the pH was different, a natural pH value (7 pH) is more desirable in the printing industry due to the issues that highly acidic or basic coatings could cause, such as press corrosion. The concern of a neutral to high pH prior to testing was that it would decrease the antimicrobial efficacy of the Nisaplin®

in the coating. However, the coating with Nisaplin® at a pH of 7.02 still proved to effective in inhibiting aerobic microbial growth during the 11-day trial period.

The coating basis weight of the coating applied to the coating with Nisaplin® film (0.575±0.04 #/ream) was only slightly higher (0.097 #/ream) than the basis weight of the coating applied to the coating without Nisaplin® (0.478±0.16 #/ream). For the most part, the variation in coating basis weight between the O, D, C sides of both the coating without Nisaplin® and the coating with Nisaplin® were relatively small, resulting in a relatively even coat weight across the film surface. The coating basis weight results of the Perna (2016) study were slightly higher than the coating basis weights discovered as a result of this experiment. Perna received a coating basis weight of 0.64±0.07 #/ream for the coating without Nisaplin® and a coating basis weight of 0.74±0.08 #/ream for the coating with Nisaplin. The coating basis weights for this experiment are slightly lower due to the removal of the primer Perna used before applying the coating. However, both the Perna study and this experiment show that the coating basis weight of the coating with Nisaplin® is higher than the coating basis weight without Nisaplin®. It was expected that the coating with Nisaplin® would have a relatively higher basis weight than the coating without Nisaplin® due to the added ingredient of Nisaplin®.

The purpose of a tape test is to visually rate the adhesion of the coating to the surface of the film. It can be concluded that the adhesion of both the Treatment (N) and Control (C) coatings have very good adhesion properties to the surface of the LLDPE sealant side of the PET/LLDPE sealant.

The color (ΔE) of both the coating with Nisaplin® and the coating without

Nisaplin® were both less than or equal to 1.0, meaning that the color that could possibly be perceived by a consumer is not observable to the trained human eye. However, the ΔE of the coating with Nisaplin® had a ΔE much closer to the 1.0 than the coating without Nisaplin® by a ΔE difference of 0.823. The slight increase in coloration in coating with Nisaplin® is due to the presence of Nisaplin®, which has a slight brown tint. The results show that the ΔE of coating with Nisaplin® is low enough that the color it gives off can not be perceived by the average consumer. However, if the amount of Nisaplin® in the coating was increased in future formulations it could raise the ΔE above 1.0, making the coating visible to the human eye. This could draw attention to the coating on the package or change the appearance of the ready-to-eat deli meat inside of the package, causing consumers to question the quality of the product. As a result, the increase in Nisaplin® could lead to problems with consumer acceptance.

As a result of the heat seal test, all three treatments were considered sealable at a temperature of 300˚F or more, according to ASTM standards, stating a seal must have a minimum force greater or equal to 200 g/in. Both coated films had a lower overall seal strength compared to the uncoated film. At the end of the test (400˚F) the average seal strength of N was 2943 g/in, where coating without Nisaplin® was 1424 g/in, compared to uncoated film, which was 4315 g/in. As expected, this suggests that both the coating and the corona discharge treatment had an effect on the seal strength of the package but not enough to where a seal was not achievable. The results of coating with Nisaplin® heat seal profile test showed that the average seal strength of the film increased as temperature increased by a total average of by 2614 g/in. However, the overall average

seal strength of the coating without Nisaplin® only varied by 571 g/in from 300˚F to 400˚F and uncoated only varied by 60 in/g from start to finish. This suggests that the Nisaplin® within the coating could have an effect on the seal properties of the system. Further research would need to be conducted to better explain this result. Overall, the results of the heat seal test concluded that a seal is achievable for all three of the treatment films, including the Nisaplin® coated film.

Coliform bacteria are typically gram negative. As stated in the introduction, Nisaplin® is proven to be mostly effective towards the inhibition of gram positive bacterium, such as Lactobacillus. The graphs for Experiment 1 and Experiment 2 (Appendix B), show little to no inhibition in coliform microbial growth with the exception of Experiment 1, Day 6. Experiment 1, Day 6 showed statistical difference between the coating with Nisaplin® vs. the uncoated film and vs. the coating without Nisaplin®. There was no statistical difference between any of the treatments on any day for during Experiment 2. During Experiment 1, Day 6 the colony count (cfu/mL) for the coating with Nisaplin® was “too few to count”. Based off the results of Experiment 1, it could be concluded that on Day 6, the Nisaplin® was effective inhibiting coliform bacteria growth. However, when compared to the results of Experiment 2, Day 5 (which had no statistical difference between treatments) it is believed that experimental error occurred on Experiment 1, Day 6. Thus, it can be suggested that the Nisaplin® coating was ineffective against inhibiting growth of coliform bacteria.

The first experiment (Experiment 1) of the inhibitions test was to establish a maximum effective storage time that the coating with Nisaplin® could effectively inhibit

the growth of aerobic and coliform bacteria, as well as staph. The second experiment (Experiment 2) was to determine if the coating with Nisaplin® is effective in inhibiting growth of aerobic bacteria, coliform bacteria, and staph, 45 days after the coating was applied to the coating. Also, Experiment 2 was conducted to determine at which point the coating with Nisaplin® was no longer effective in inhibiting the growth of aerobic bacteria, coliform bacteria, and staph. As stated previously in the Results section, the colony counts (cfu/mL) of the Staph plates were "too few to count" for both Experiment 1 and Experiment 2. Additionally, during Experiment 2, there was contamination and growth of unidentified colonies. Therefore, the results of Staph were not further processed or discussed. Also, as discussed previously, the data from the results of the coliform plates in both Experiment 1 and Experiment 2 suggest that the Nisaplin® coating is not effective in inhibiting the growth of coliform bacteria. As a result, the majority of the study was focused on the Nisaplin® coating's ability to inhibit growth of aerobic bacteria.

Aerobic bacteria can categorize both gram-positive and gram-negative. The aerobic count Petrifilm plates used in both Experiment 1 and Experiment 2 included aerobic bacteria that was both gram positive and gram negative. Therefore, this could lead to less dramatic log reductions and statistical differences between the two control groups (the uncoated film and the coating without Nisaplin®) and the coating with Nisaplin®.

The statistical results for the aerobic count of the t-test for Experiment 1, Day 6 showed that there was a statistical difference between all three treatment groups.

However, there was a 2-log reduction between the control groups (uncoated film and the coating without Nisaplin®) vs. the coating with Nisaplin®. There was only a 0.5 log reduction between the two control groups and the colony count for both of the control groups were at the 6^{th} and 7^{th} log, which is considered potentially health hazardous and inedible. These data suggest the during Experiment 1, the Nisaplin® coating was effective in inhibiting the growth of aerobic bacteria. The t-test results for Experiment 1, Day 10 determined that there was no statistical difference between the two control groups and the coating with Nisaplin®, however there was a statistical difference between the two control groups. On Day 10, the aerobic colony counts of the coating with Nisaplin® was statistically the same as the aerobic colony counts of both the uncoated film and the coating without Nisaplin®. Also on Day 10, the aerobic colony counts for all three treatments were at the 7^{th} log. Thus, the data suggest that at Day 10, the Nisaplin® coating is no longer effective in inhibiting the growth of aerobic bacteria and that somewhere between 6 and 10 days is the maximum effective storage time for the coating with Nisaplin®.

Experiment 2 was conducted to determine at what point between Day 6 and Day 10 was the coating with Nisaplin® no longer effective in inhibiting aerobic microbial growth. It was also conducted to determine if the Nisaplin® coated film maintains antimicrobial efficacy 45 days after being applied to the film. ANOVA tests conducted on days 3, 5, 9, and 11 suggested that there was a statistical difference between the three treatments on Day 5 and Day 9 but no statistical difference between the three treatments on Day 3 or Day 11. On Day 5, the t-tests suggested that there was a statistical difference

between both of the control groups (uncoated film and coating without Nisaplin®) vs the coating with Nisaplin®. On Day 5, there was a 2-log reduction between both of the control groups vs the coating with Nisaplin®. There was no statistical difference between the two control groups on Day 5. These data suggest that on Day 5, the coating with Nisaplin® was effective in inhibiting aerobic bacteria growth. The experimental design called for experimental sampling to occur on Day 7, however, due to a power outage on campus, testing was unable to occur on Day 7. The refrigeration temperatures did not reach above required conditions, therefore, the study was continued on Day 9. The t-test on Day 9 also suggested that there was a statistical statistical difference between both of the control groups (uncoated film and coating without Nisaplin®) vs the coating with Nisaplin®, with no statistical difference between the two control groups. However, unlike Day 5, on Day 9 there was only a 1-log reduction between the control groups and the coating with Nisaplin®. Also, on Day 9 the colony count of the coating with Nisaplin® was at the 6^{th} log, meaning the turkey deli meat is no longer edible. However, the data suggest Nisaplin® is still effective in inhibiting aerobic microbial growth on Day 9. On Day 11, there was no statistical difference and the colony counts of all three treatments were at the 7^{th} log. This suggests that between Day 9 and Day 11 (Day 10), the coating with Nisaplin® was no longer effective in inhibiting aerobic microbial growth.

In conclusion, the results of Experiment 2, suggest that the Nisaplin® coating is effective in inhibiting growth of aerobic bacteria up to 9 days, 45 days after the coating has been applied to the substrate. This results of this study reflect the Perna (2016) study, where Perna found the coating without Nisaplin® had inhibition zones of 0.0±0 and the

coating with Nisaplin® had inhibition zones of 3.60±1.36 (n=14) in a film on lawn study inoculated with *L. monocytogenes*. Further research would need to be conducted to determine which aerobic bacterium the Nisaplin® coating is effective in inhibiting growth in a food challenge with ready-to-eat sliced deli turkey meat.

With the exception of a few outliers, the was relatively no significant difference between the the three sides of the film (O,C,D) and the inhibition against both coliform and aerobic microbial growth. The coating basis weights suggested that there was very little difference in coating weights between the three sides (O,C,D). Thus, it was expected to see that there would be little variation in the efficacy of the coating containing Nisaplin® among the three sides of the film.

The Perna study (2016) suggested that the addition of a primer could have an effect on the sealability of the Nisaplin® coated package. For this reason, the PEI primer was removed from the coating process. Seals were achieved for all three treatments, concluding that the Nisaplin® coated film can be commercially converted into a package. Also, the results of the inhibition test prove that the package was effective inhibiting some forms of aerobic microbial growth without the addition of the primer. Also, the tape test suggests that the coating with Nisaplin® adhered to the substrate without the application of the primer prior to coating.

CONCLUSIONS AND RECOMMENDATIONS

The purpose of this study was to determine if an antimicrobial coating containing Nisaplin® could be commercially viable while maintaining antimicrobial efficacy. The coating containing Nisaplin® was previously formulated by Dr. Michele Perna (2016) in a small batch quantity. During this study, the coating formulation was adapted from small batch to a large batch quantity, great enough to be printed on a flexographic printing press. The Nisaplin® coating maintained a good pH balance of 7.26 and a viscosity of 19.53 seconds when tested with a #3 Zahn cup. The coating was applied using a OMET VaryFlex530 flexographic printing press. During the press run both the coating containing Nisaplin® and the containing without Nisaplin® were applied to the LLDPE sealant side of a PET/LLDPE laminate web film that was corona discharge treated at 1660 watts prior to applying the coating. The press was run at 32 ft/min with a tunnel dry temperature of 200˚F and coated using a 15.2 BCM anilox roller and a rubber roller. The basis weight of the coatings suggested that the coatings were both evenly applied across the surface of each of the films. As expected, the total basis weight of the coating containing Nisaplin® (0.575±0.04 #/ream) had a slightly greater basis weight than the coating without Nisaplin® (0.478±0.16 #/ream). A tape test was conducted to visually rate the adhesion of each of the coatings to the substrate. Both the coating containing Nisaplin® and the coating without Nisaplin® had very good observable adhesion, with little to no removal of the coating off of the substrate surface on to the tape. The color

test, conducted with a colorimeter, resulted in a $\Delta E <= 1.0$ for both coatings tested. Therefore, the presence of both the coating with Nisaplin® and without Nisaplin® are undetectable to the average human eye. A heat seal profile test was conducted on the Treatment (N) film (film with coating containing Nisaplin®), Coating without Nisaplin® (film with coating containing no Nisaplin®) and the Raw (P) material (PET/LLDPE laminate). The results of the heat seal tests concluded that all three films are able to achieve a peal seal, above 200 g/in, at a temperature greater than or equal to 300˚F, with a dwell time of 2 seconds and an applied pressure of 35 psi.

The antimicrobial efficacy of the coating containing Nisaplin® was tested during an inhibition test. The inhibition test tested each side (Operator, Center, Drive) of each film treatment for its inhibition against aerobic bacteria, coliform bacteria, and staph colony growth. Two trials were conducted. After Experiment 1, it was suggested that the maximum effective storage time that Nisaplin® effectively inhibit aerobic bacteria growth was 10 days. Experiment 2 was conducted to determine if the Nisaplin® coating is effective in inhibiting microbial growth 45 days after the coating was applied and at what point the Nisaplin® coating was no longer effective in inhibiting certain microbial growth. During Experiment 2, Day 5 and Day 9 there was statistical difference between the coating containing Nisaplin® and the two control groups (uncoated film and coating without Nisaplin®) with a 2-log reduction on Day 5 and a 1-log reduction on Day 9 between the coating with Nisaplin® vs the coating without Nisaplin® and the coating with Nisaplin vs. the uncoated film. Experiment 1 and Experiment 2 suggested that there was little to no statistical difference between the coating with Nisaplin® and the control

groups (coating without Nisaplin® and the uncoated film). The results of the inhibition testing for staph was discarded for both Experiment 1 and Experiment 2 due to unidentified colony formation during, as well as “too few to count” colony counts growth for all three treatments. Overall, the results of the inhibition test in a food challenge with ready-to-eat turkey deli meat suggested that the coating with Nisaplin® is effective in inhibiting the growth of some aerobic bacteria, even after the coating has been applied for 45 days. The results also suggest that the Nisaplin® coating is not effective in inhibiting the growth of staph of coliform bacteria. Further research would need to be conducted to determine which aerobic bacteria the Nisaplin® coating is most effective in inhibiting growth.

Further research on this topic should be conducted to gain further knowledge on the commercialization and efficacy of an antimicrobial coating containing Nisaplin®. Further research should be conducted to determine the optimal press conditions for applying the coating with a flexographic printing press. For this experiment, the flexographic press was run at 32ft/min, this is the slowest speed the press could run. Commercial flexographic presses can run up to 2000ft/min, determining the optimal conditions and the maximum run speed could be more beneficial in making the coating commercially viable. At the end of this experiment, it was suggested that the antimicrobial coating with Nisaplin® was effective in inhibiting aerobic bacteria growth. Further research should be conducted to determine which exact aerobic bacteria colonies were inhibited. Also, research should be conducted to determine if the commercially applied coating containing Nisaplin® is effective in inhibiting the growth of

Listeria monocytogenes and anaerobic bacteria. Further more, an HPLC test should be conducted to determine how much of the Nisaplin® transferred from the coating to the turkey deli meat and how much remained in the coating that is applied to the surface of the substrate. This would provide further information on if the amount of Nisaplin® that is transferred to the food remains under the FDA set a maximum limit of 10,000 IU/g. Further research should be conducted to determine the overall efficacy of the antimicrobial containing Nisaplin® when applied to a stack of deli meat as opposed to a single slice. Also, further research should be conducted to determine if a Nisaplin® coated parchment paper in the form of a leaflet placed in-between the slices of the deli meat or other ready-to-eat foods would be more effective than placing a stack of sliced deli-meat in a film pouch. Both studies would be useful to determine if the Nisaplin® coating is effective in inhibiting bacteria growth in a true commercial food environment.

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APPENDICES

APPENDIX A: ANOVA AND T-TEST STATISTICS FOR INHIBITION OF AEROBIC MICROBIAL GROWTH

Aerobic Inhibition Experiment 1.

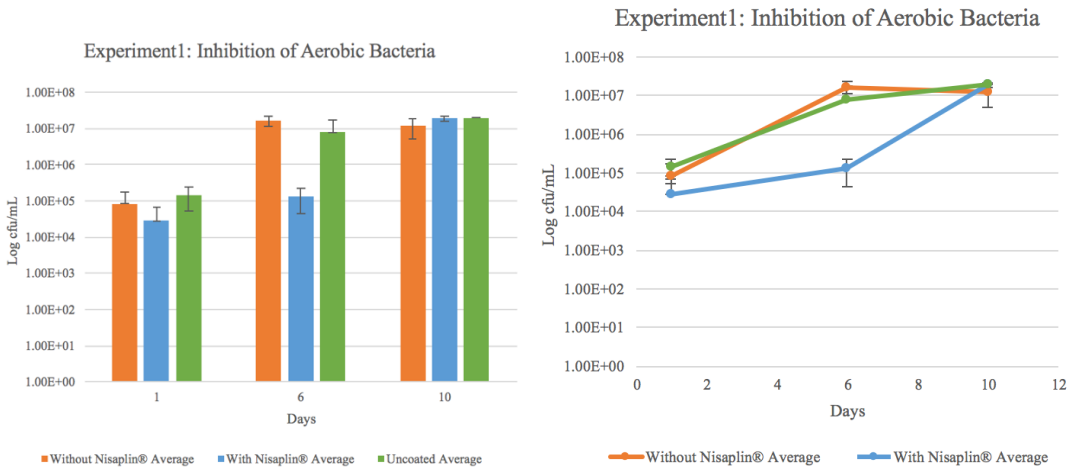

Figure A.1 Treatment effect on the inhibition of aerobic microbial growth: Experiment 1

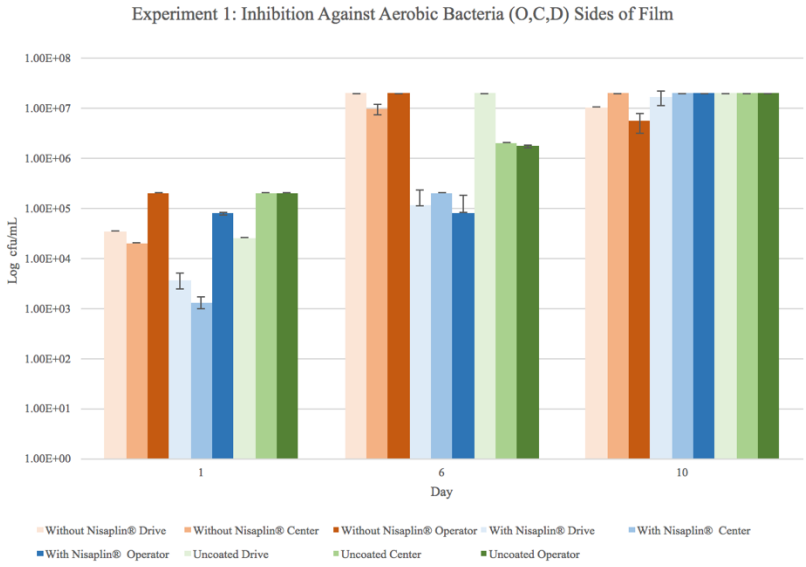

Figure A.2 Aerobic inhibition: Experiment 1 (breakdown per side of film (O, C, D))

Aerobic Inhibition Experiment 1: ANOVA Tests

Table A.1 ANOVA test of inhibition of aerobic microbial growth: Experiment 1, Day 6

ANOVA: Single Factor
Aerobic Count Experiment 1, Day 6
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	43.156428	7.192738	0.03028052
V2 With Nisaplin®	6	29.6877073	4.94795121	0.26094482
V3 Uncoated	6	39.6742363	6.61237272	0.28558321

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16.2923811	2	8.14619053	42.368602	6.7493E-07	3.68232034
Within Groups	2.88404271	15	0.19226951			
Total	19.1764238	17				

Reject The Null

Table A.2 ANOVA test of inhibition of aerobic microbial growth: Experiment 1, Day 10

ANOVA: Single Factor
Aerobic Count Experiment 1, Day 10
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	42.0414634	7.00691056	0.07908365
V2 With Nisaplin®	6	43.6157397	7.26928995	0.00604458
V3 Uncoated	6	43.80618	7.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.3127132	2	0.15635661	5.5101558	0.01606352	3.68232034
Within Groups	0.4256412	15	0.02837608			
Total	0.7383544	17				

Reject The Null

Aerobic Inhibition Experiment 1: T-Tests

Table A.3 t-Test for inhibition of aerobic microbial growth: Experiment 1, Day 6

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V3 Uncoated	*V2 With Nisaplin®*
Mean	6.612372723	4.947951213
Variance	0.285583208	0.260944819
Observations	6	6
Pooled Variance	0.273264013	
Hypothesized Mean Difference	0	
df	10	
t Stat	5.514837574	
P(T<=t) one-tail	0.0001282	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.0002564	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V3 Uncoated	*V1 Without Nisaplin®*
Mean	6.612372723	7.192737999
Variance	0.285583208	0.030280516
Observations	6	6
Pooled Variance	0.157931862	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.529456237	
P(T<=t) one-tail	0.014949379	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.029898758	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 6

	V1 Without Nisaplin®	*V2 With Nisaplin®*
Mean	7.192737999	4.947951213
Variance	0.030280516	0.260944819
Observations	6	6
Pooled Variance	0.145612667	
Hypothesized Mean Difference	0	
df	10	
t Stat	10.18910753	
P(T<=t) one-tail	6.69202E-07	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	1.34E-06	
t Critical two-tail	2.228138852	

Reject the Null

Table A.4 t-Test for inhibition of aerobic microbial growth: Experiment 1, Day 1

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V3 Uncoated	*V2 With Nisaplin®*
Mean	7.301029996	7.269289948
Variance	0	0.006044584
Observations	6	6
Pooled Variance	0.003022292	
Hypothesized Mean Difference	0	
df	10	
t Stat	1	
P(T<=t) one-tail	0.170446566	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.340893132	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V1 Without Nisaplin®	*V2 With Nisaplin®*
Mean	7.006910564	7.269289948
Variance	0.079083651	0.006044584
Observations	6	6
Pooled Variance	0.042564117	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.202766789	
P(T<=t) one-tail	0.026098022	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.052196043	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count Experiment 1: Day 10

	V3 Uncoated	*V1 Without Nisaplin®*
Mean	7.301029996	7.006910564
Variance	0	0.079083651
Observations	6	6
Pooled Variance	0.039541826	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.561863512	
P(T<=t) one-tail	0.014141316	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.028282633	
t Critical two-tail	2.228138852	

Reject the Null

Aerobic Inhibition Experiment 2:

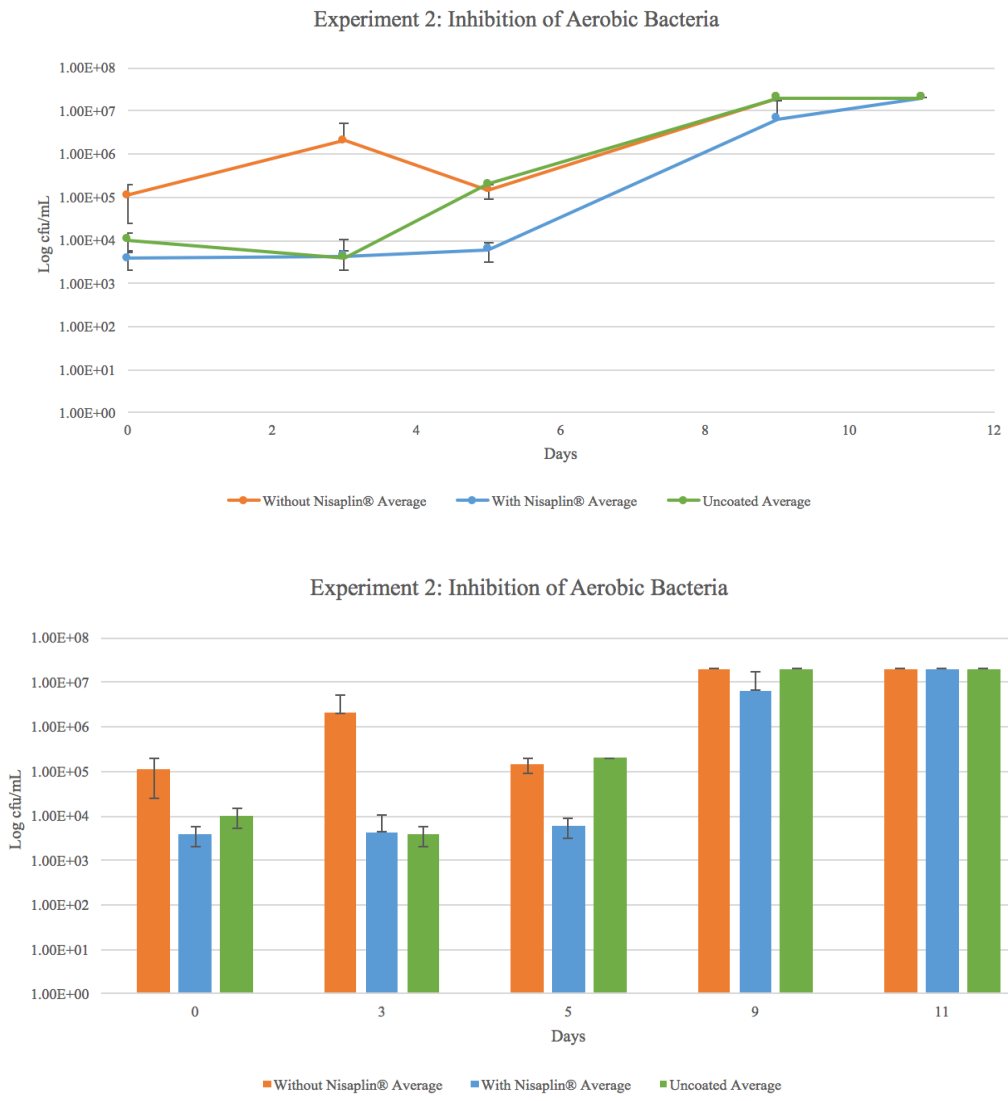

Figure A.3 Treatment effect on the inhibition of aerobic microbial growth: Experiment 2

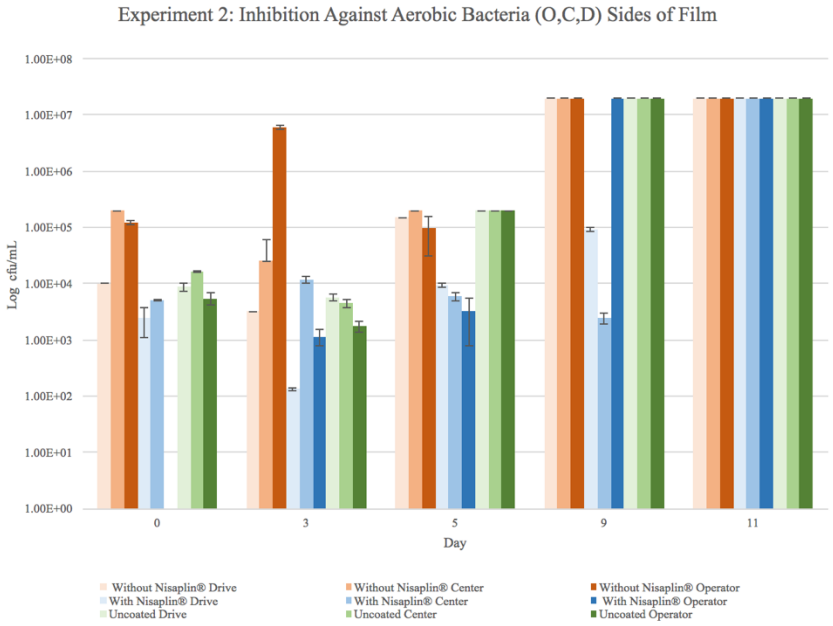

Figure A.4 Aerobic inhibition: Experiment 2 (breakdown per side of film (O, C, D))

Aerobic Inhibition Experiment 2: ANOVA Tests

Table A.5 ANOVA test of inhibition of aerobic microbial growth: Experiment 2, Day 3

ANOVA: Single Factor
Aerobic Count: Experiment 2: Day 3
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Wihtout Nisaplin®	6	28.286911	4.71448516	2.88919139
V2 With Nisaplin®	6	18.4893256	3.08155427	0.75842846
V3 Uncoated	6	21.2671177	3.54451962	0.06181481

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.49923912	2	4.24961956	3.43687376	0.05905552	3.68232034
Within Groups	18.5471733	15	1.23647822			
Total	27.0464124	17				

Accept the Null

Table A.6 ANOVA test of inhibition of aerobic microbial growth: Experiment 2, Day 5

ANOVA: Single Factor
Aerobic Count: Experiment 2: Day 5
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Wihtout Nisaplin®	6	30.7963967	5.13273278	0.04970489
V2 With Nisaplin®	6	22.3429958	3.72383264	0.08637535
V3 Uncoated	6	31.80618	5.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.00175017	2	4.50087508	99.2254716	2.2436E-09	3.68232034
Within Groups	0.68040116	15	0.04536008			
Total	9.68215133	17				

Reject the Null

Table A.7 ANOVA test of inhibition of aerobic microbial growth: Experiment 2, Day 9

ANOVA: Single Factor
Aerobic Count: Experiment 2: Day 9
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Wihtout Nisaplin®	6	43.80618	7.30103	0
V2 With Nisaplin®	6	31.322386	5.22039767	3.09607089
V3 Uncoated	6	43.80618	7.30103	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.3161235	2	8.65806173	8.38940261	0.00358635	3.68232034
Within Groups	15.4803544	15	1.03202363			
Total	32.7964779	17				

Reject the Null

Aerobic Inhibition Experiment 2: T-Tests

Table A.8 t-Test for inhibition of aerobic microbial growth: Experiment 2, Day 5

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V3 Uncoated	*V1 Wihtout Nisaplin®*
Mean	5.301029996	5.132732782
Variance	0	0.049704886
Observations	6	6
Pooled Variance	0.024852443	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.849068545	
P(T<=t) one-tail	0.047094413	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.094188826	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V3 Uncoated	*V2 With Nisaplin®*
Mean	5.301029996	3.723832636
Variance	0	0.086375347
Observations	6	6
Pooled Variance	0.043187673	
Hypothesized Mean Difference	0	
df	10	
t Stat	13.14518686	
P(T<=t) one-tail	6.17135E-08	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	1.23427E-07	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 5

	V1 Wihtout Nisaplin®	*V2 With Nisaplin®*
Mean	5.132732782	3.723832636
Variance	0.049704886	0.086375347
Observations	6	6
Pooled Variance	0.068040116	
Hypothesized Mean Difference	0	
df	10	
t Stat	9.355312908	
P(T<=t) one-tail	1.4588E-06	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	2.91761E-06	
t Critical two-tail	2.228138852	

Reject the Null

Table A.9 t-Test for inhibition of aerobic microbial growth: Experiment 2, Day 9

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V3 Uncoated	*V1 Wihtout Nisaplin®*
Mean	7.301029996	7.301029996
Variance	0	0
Observations	6	6
Pooled Variance	0	
Hypothesized Mean Difference	0	
df	10	
t Stat	65535	
P(T<=t) one-tail	0.00E+00	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.00E+00	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V3 Uncoated	*V2 With Nisaplin®*
Mean	7.301029996	5.220397672
Variance	0	3.096070889
Observations	6	6
Pooled Variance	1.548035445	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.896446549	
P(T<=t) one-tail	0.007965135	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.01593027	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Aerobic Count: Experiment 2: Day 9

	V1 Wihtout Nisaplin®	*V2 With Nisaplin®*
Mean	7.301029996	5.220397672
Variance	0	3.096070889
Observations	6	6
Pooled Variance	1.548035445	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.896446549	
P(T<=t) one-tail	0.007965135	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.01593027	
t Critical two-tail	2.228138852	

Reject the Null

APPENDIX B: ANOVA AND T-TEST STATISTICS FOR INHIBITION OF COLIFORM MICROBIAL GROWTH.

Coliform Inhibition Experiment 1.

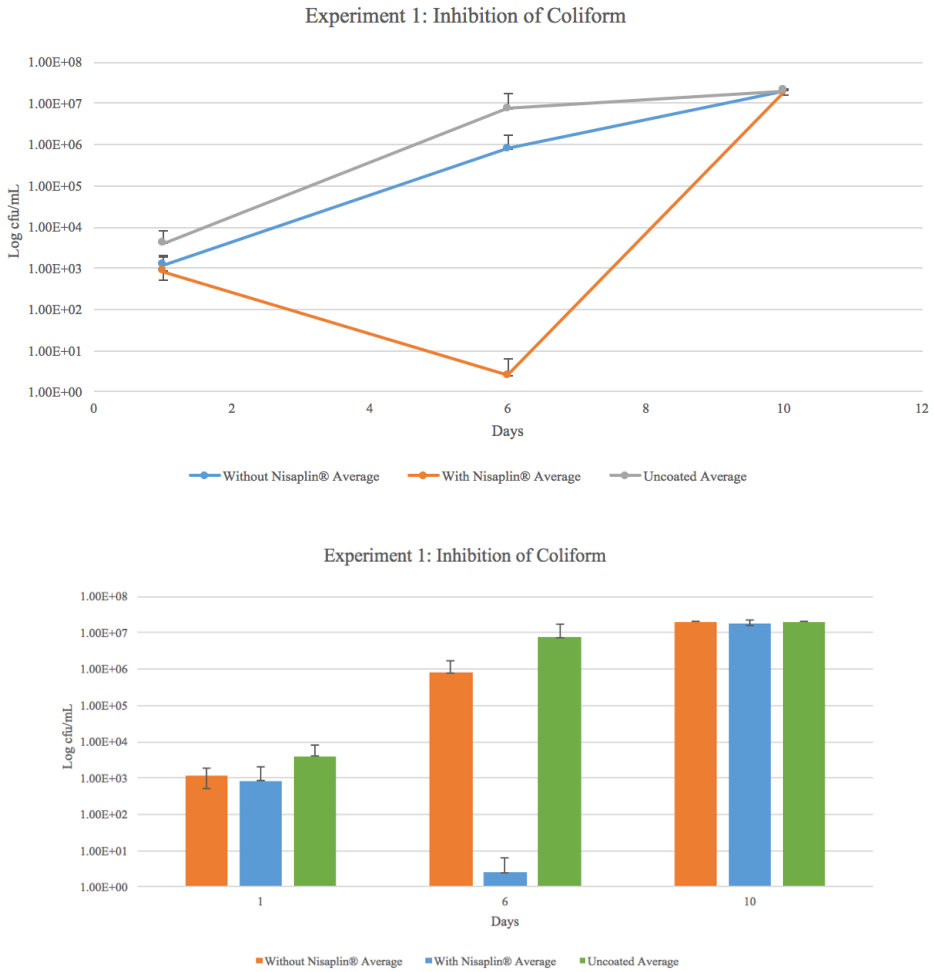

Figure B.1 Treatment effect on the inhibition of coliform microbial growth: Experiment 1

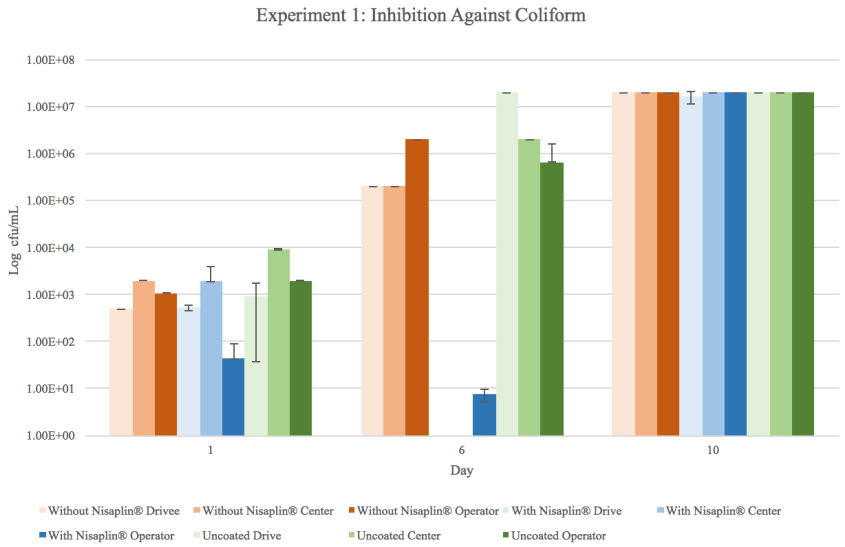

Figure B.2 Coliform inhibition: Experiment 1, (breakdown per side of film (O, C, D))

Coliform Inhibition Experiment 1: ANOVA Tests

Table B.1 ANOVA test of inhibition coliform microbial growth: Experiment 1, Day 6

ANOVA: Single Factor
Experiment 1: Day 6
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Coating without Nisaplin®	6	33.80617997	5.634363329	0.266666667
V2 Coating with Nisaplin®	6	1.73239376	0.288732293	0.203180022
V3 Uncoated	6	33.61909333	5.603182222	7.022610464

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	113.6402429	2	56.82012147	22.75092949	2.86694E-05	3.682320344
Within Groups	37.46228576	15	2.497485718			
Total	151.1025287	17				

Reject the Null

Table B.2 ANOVA test of inhibition coliform microbial growth: Experiment 1, Day 10

ANOVA: Single Factor
Experiment 1: Day 10
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Coating without Nisaplin®	6	43.80617997	7.301029996	0
V2 Coating with Nisaplin®	6	43.61573969	7.269289948	0.006044584
V3 Uncoated	6	43.80617997	7.301029996	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.004029722	2	0.002014861	1	0.391126762	3.682320344
Within Groups	0.030222919	15	0.002014861			
Total	0.034252641	17				

Accept the Null

Coliform Inhibition Experiment 1: T-Tests

Table B.3 t-Test for inhibition of coliform microbial growth: Experiment 1, Day 6

t-Test: Two-Sample Assuming Equal Variances
Experiment 1: Day 6

	V3 Uncoated	*V1 Without Nisaplin®*
Mean	5.603182222	5.634363329
Variance	7.022610464	0.266666667
Observations	6	6
Pooled Variance	3.644638565	
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.028289478	
P(T<=t) one-tail	0.488993942	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.977987884	
t Critical two-tail	2.228138852	

Accept the Null

t-Test: Two-Sample Assuming Equal Variances
Experiment 1: Day 6

	V3 Uncoated	*V2 with Nisaplin®*
Mean	5.603182222	0.288732293
Variance	7.022610464	0.203180022
Observations	6	6
Pooled Variance	3.612895243	
Hypothesized Mean Difference	0	
df	10	
t Stat	4.842741219	
P(T<=t) one-tail	0.000339353	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.000678705	
t Critical two-tail	2.228138852	

Reject the Null

t-Test: Two-Sample Assuming Equal Variances
Experiment 1: Day 6

	V1 without Nisaplin®	*V2 with Nisaplin®*
Mean	5.634363329	0.288732293
Variance	0.266666667	0.203180022
Observations	6	6
Pooled Variance	0.234923345	
Hypothesized Mean Difference	0	
df	10	
t Stat	19.1027776	
P(T<=t) one-tail	1.6799E-09	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	3.3598E-09	
t Critical two-tail	2.228138852	

Reject the Null

Coliform Inhibition Experiment 2:

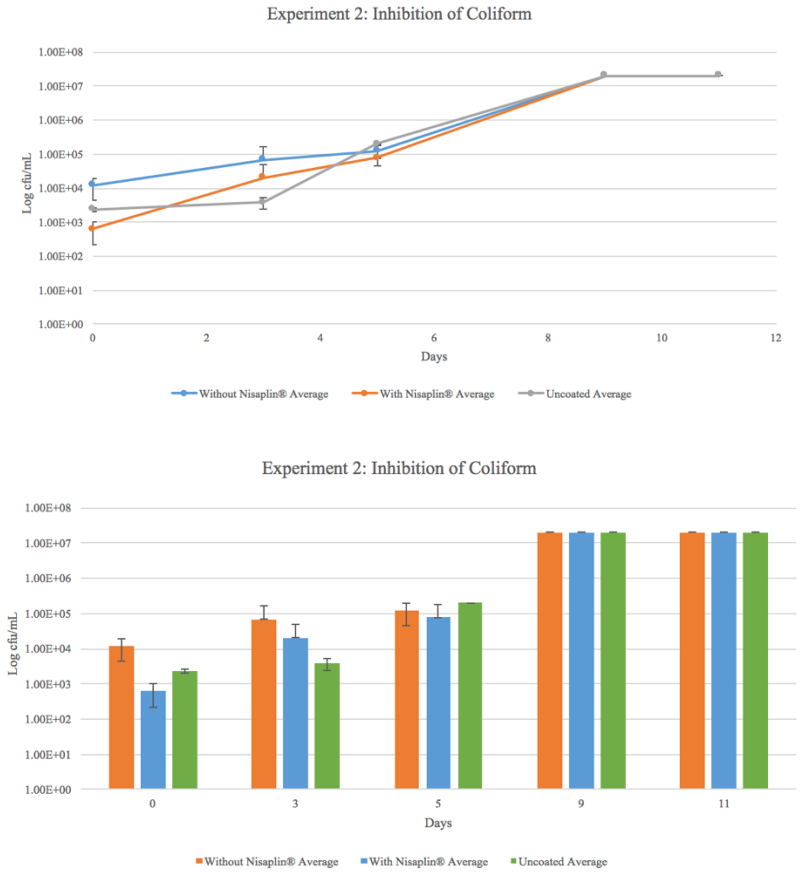

Figure B.3 Treatment effect on the inhibition of coliform microbial growth: Experiment 2

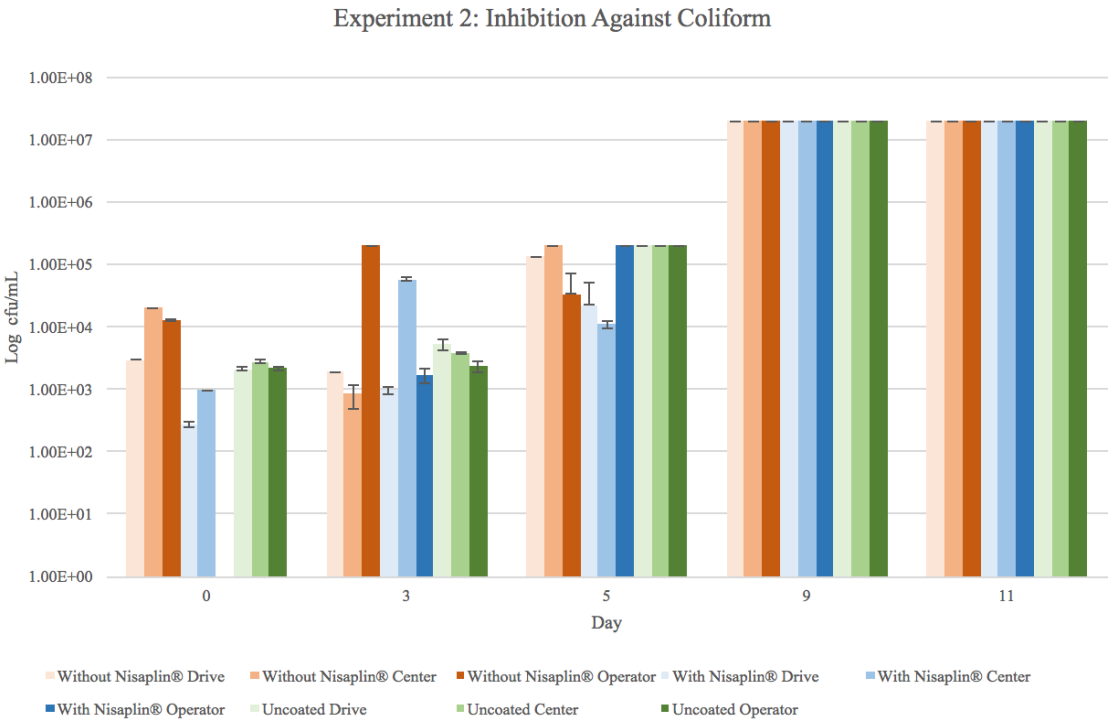

Figure B.4 Coliform inhibition: Experiment 2 (breakdown per side of film (O, C, D))

Coliform Inhibition Experiment 2: ANOVA Tests

Table B.4 ANOVA test of inhibition of coliform microbial growth: Experiment 2, Day 3

ANOVA: Single Factor
Experiment 2: Day 3
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	22.97790643	3.829651071	1.333253203
V2 With Nisaplin®	6	21.94970095	3.658283491	0.75144047
V3 Uncoated	6	21.30887952	3.55147992	0.028742533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.23630608	2	0.11815304	0.167716971	0.847157033	3.682320344
Within Groups	10.56718103	15	0.704478735			
Total	10.80348711	17				

Accept the Null

Table B.5 ANOVA test of inhibition of coliform microbial growth: Experiment 2, Day 5

ANOVA: Single Factor
Experiment 2: Day 5
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
V1 Without Nisaplin®	6	29.47480115	4.912466858	0.313518438
V2 With Nisaplin®	6	26.78161178	4.463601964	0.552781789
V3 Uncoated	6	31.80617997	5.301029996	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.107493428	2	1.053746714	3.649127683	0.051127294	3.682320344
Within Groups	4.331501137	15	0.288766742			
Total	6.438994565	17				

Accept the Null

APPENDIX C: CONVERSION FACTOR FOR NISAPLIN® COATING

The following is the conversion used to convert the small batch coating formulated in the Perna (2016) study to the amount (1 gallon per coating) used to perform a press run during this experiment.

Conversions:

1 batch of coating ~ 1750 mL

2.16 batches = 1 gallon

1 gallon = 3785.41 mL

1 pound = 453.6 grams