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# Evaluation and Development of Nisin-Containing Packaging for Ready-to-Eat Meats, Utilizing Methods Feasible for Future Commercialization

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EVALUATION AND DEVELOPMENT OF NISIN-CONTAINING PACKAGING FOR
READY-TO-EAT MEATS, UTILIZING METHODS FEASIBLE FOR FUTURE
COMMERCIALIZATION

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ABSTRACT

Antimicrobial food packaging may extend shelf life, reduce spoilage, maintain food quality and eliminate foodborne pathogens in ready-to-eat (RTE) deli meat. Nisin is a polypeptide with natural antimicrobial activity against gram-positive microorganisms. This food additive is generally recognized as safe (GRAS) in the United States. In order to combat *Listeria monocytogenes*, continued good manufacturing practices, continued proper handling by food workers, and additional secondary safety measures such as antimicrobial packaging are necessary. However, current research for antimicrobial packaging is in preliminary stages and is primarily based on theoretical lab scale testing. Antimicrobial coatings containing nisin were developed and studied. Diffusion was successfully measured by agar well diffusion method and high performance liquid chromotography. Microscopy was examined as a new method for tracking nisin diffusion in the food and films and found to be useful. A food challenge study on turkey bologna demonstrated that the coatings were able to inhibit a *L. monocytogenes* cocktail compared to the control coating. In addition, antimicrobial extruded films containing nisin and bovine albumin were developed and tested for antimicrobial activity. The results demonstrated that there was significant increased inhibition of *M. luteus* when the bovine albumin was used in combination with the nisin Z. Also, there was a significant difference between the type of polymer and the amount of inhibition of *M. luteus*. This research is directed toward the development of an antimicrobial vacuum skin package for RTE meat. It provides new and necessary information for future commercialization of antimicrobial packaging.

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CHAPTER I

INTRODUCTION

*Listeria monocytogenes* is an ongoing global problem. Outbreaks, recalls, illness

and death often occur, costing billions of dollars. Current United States regulations state

that there is a zero tolerance of *L. monocytogenes* in ready-to-eat foods (such as

delicatessen meats). This means that all of the food supply would need to be tested in

order to truly achieve this regulation, leaving no supply for consumption. Therefore,

other non-destructive measures are required to reduce the presences of *L. monocytogenes*.

Approaches to aid in controlling the menace of *L. monocytogenes* include: good

manufacturing methods, continued proper handling by food workers, and additional

secondary safety measures. An antimicrobial packaging (a coating or extruded film)

would be examples of an additional secondary safety measure to reduce the population of

*L. monocytogenes* on the surface of the ready-to-eat food. However, current research for

antimicrobial packaging is in preliminary stages and is primarily based on theoretical lab

scale testing.

Nisin is an antimicrobial that targets gram-positive pathogens such as *L.*

*monocytogenes.* It is food safe, colorless and is currently generally recognized as safe by

the United States government. For these reasons nisin is an ideal antimicrobial to be

incorporated into the coating/extrudate for direct ready-to-eat food contact. However, the

antimicrobial cannot be a stand-alone coating/film due mainly to its cost (approximately

twenty times more expensive compared to the control, according to the experiment found

in Section 3.1). Therefore, a carrier is required, such as pectin to develop an antimicrobial

coating. Pectin is a food safe carrier that is commonly used in drug delivery coatings, which allow for a slow release.

To date, the research conducted to help commercialize antimicrobial packaging has gaps. There is a need for both food challenge studies and diffusion studies for antimicrobial packaging (measuring the entire shelf life of the food product). New detection methods may be needed to track the antimicrobial's diffusion. It is necessary to understand the safety and potential success for antimicrobial packaging on RTE-foods instead of liquid media. Slow release of the antimicrobial packaging such as in common methods used in drug release could be studied. It is also important to focus on substrates and methods of coating/extrusion that would be common in industry. Targeting foods that commonly use packaging in direct contact and do not require sealing would be beneficial.

In addition, cost and consumer perception (color, flavor etc.) are important factors to consider when commercializing a coating/extrudate. As consumers drive demand, a product cost that is prohibitive (based on its packaging) would diminish sales. Color or haze in packaging can scare consumers, who may feel that the product is unsafe or cause them to buy a competing product. If production is feasible, not considering these factors will limit the product's economic potential. The researchers goal is to aid in bridging the gap between current literature and the demand needed for commercialization.

CHAPTER II

LITERATURE REVIEW

Foodborne illness is a major concern in the United States as it affects
approximately 1 in 6 people, according to the Centers for Disease Control  (Centers for
Disease Control and Prevention (CDC) 2013 C). These estimated 48 million cases occur
each year in the United States and include 128,000 hospitalizations and 3,000 related
deaths (Centers for Disease Control and Prevention (CDC) 2013 C). Of these, it is
estimated that the 31 most pathogenic strains found in foods consumed in the United
States each year, caused nearly 9.5 million illnesses, nearly 56,000 hospitalizations, and
1,351 deaths, as seen in Table 1 (Batz et al. 2011). Furthermore, the top seven strains
accounted for 90% of all illnesses  (Batz et al. 2011). Of those 1,351 deaths, nearly 30%
were caused by *Listeria monocytogenes*, which indicates the pathogen's lethality (Batz et
al. 2011, Chen 2012), as the pathogen causes about 2,500 cases a year, according to the
FDA.

**Table 2.1. Top 10 Pathogen-Food Combinations in Terms of Annual Disease Burden**

| Pathogen Food Combinations | Cost of Illness (Billions) | Number of Illnesses | Hospitalizations | Deaths |
|---|---|---|---|---|
| Campylobacter – Poultry | $1.257 | 608,231 | 6,091 | 55 |
| Toxoplasma – Pork and Beef | $1.908 | 55,623 | 2,041 | 210 |
| *Listeria – Deli Meats and Dairy* | *$1.810* | *1,085* | *992* | *174* |
| Salmonella – Poultry, Produce, Eggs, Other | $2.260 | 70,1967 | 13,209 | 258 |
| Norovirus – Other Foods | $0.914 | 2,494,222 | 6,696 | 68 |
| **Totals** | **$8.151** | **386,1128** | **29,830** | **765** |

Adapted from (Batz et al. 2011).

*L. monocytogenes* is the leading cause of death associated with deli meats (Batz et al. 2011); in fact 83% of all listeriosis cases in the United States are attributable to deli meats  (Food Safety and Inspection Service (FSIS) 2013) and listeriosis is over 15 times more likely to be found in deli meats than any other source (Oliver 2013).  Of further concern, *Listeria* has a very high combined public health burden with an economic cost of almost two billion dollars, despite the relative lack of comparative occurrences (Batz et al. 2011). Oliver (2013), presented that despite massive efforts in the prevention of listeriosis, there are still too many confirmed cases since 2004, when a *Listeria* initiative addressed to outline concerns and establish surveillance techniques (Cartwright et al. 2013). The trend on listeriosis is "flatlining" as opposed to decreasing despite more time and effort being put into its prevention (Oliver 2013, Cartwright et al. 2013).

**Listeria**

Listeria species are non-spore forming, catalase-positive, gram-positive rods, and facultative anaerobes that are motile through flagella with the ability to produce lactic acid from fermentable sugars, including glucose (Jay, Loessner & Golden 2005, Hitchins, Jinnerman 2013, Swaminathan, Gerner-Smidt 2007, Posfay, Wald 2009). *Listeria monocytogenes* became known in 1926, when British scientists discovered what was described as "a non-spore forming, Gram-positive rod that infected blood monocytes," of which resulted in rabbit death; "bacterium monocytogenes" was the initial nomenclature for the pathogen (Murray, Webb & Swann 1926). Around the same time, (Pirie 1927) whilst investigating gerbil deaths in South Africa, discovered what he referred to as "*Listerella hepatolytica."* The two researchers sent their results to the National Type Collection at the Lister Institute in London, which noticed the strong similarity between the two new microorganisms and suggested that Murray and Pirie contact each other: after agreeing that the organisms were the same, the tag *Listerella monocytogenes* was bestowed (Rocourt, Buchriser 2007). The generic name "*Listerella"* was rejected in 1939 (due to a previous designation for a mycetozoa), so the current designation and genus, *Listeria monocytogenes* was given to the organism) based on its catalase-positive, and gram-positive rods (Rocourt, Buchriser 2007, Pirie 1940, Hof 2003). The pathogen's first outbreak in the USA occurred in 1979, when 23 patients from the Boston area were infected with the pathogen, with raw vegetables being implicated (Ho et al. 1986). Two years later, a listeriosis outbreak occurred in Canada, with coleslaw being the implicated food, along with a fatality rate of 27% (Schlech III et al. 1983).

This family of bacteria is closely related to *Bacillus, Lactobacillus* and *Streptococcus* species. The genus *Listeria* contains seven identified species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. martihii*, *L. ivanovii*, and *L. rocourtiae* (Graves et al. 2010, Leclercq et al. 2010, Jay, Loessner & Golden 2005). In addition, these different species are distinguished through four different phylogentic lineages, yielding 17 different serotypes (Jay, Loessner & Golden 2005, Chatterjee et al. 2006, Nadon et al. 2001, Roberts et al. 2006, Wagner, McLauchlin 2008, Ward et al. 2004). The primary species is the pathogen *L. monocytogenes* that has 13 different serotypes that of which, serotypes 1/2a, 1/2b, and 4b have been associated with the vast majority of foodborne infections (Chen 2012, Wagner, McLauchlin 2008, Jay, Loessner & Golden 2005, Latorre et al. 2007, Swaminathan, Gerner-Smidt 2007, Meloni et al. 2009).

In order to grow, *Listeria* species require four B vitamins (biotin, riboflavin, thamine and alpha-lipoic acid) and five essential amino acids (cysteine, glutamine, isoleucine, leucine and valine) (Jay, Loessner & Golden 2005). *Listeria* species' ideal growth happens with pH range of 6-8; however studies have shown growth in a much wider range: as low as pH 4.1 and as high as 9.6 (Jay, Loessner & Golden 2005, Camejo et al. 2009, Posfay, Wald 2009). Like most pathogens, *listeria* can grow at body temperature (37ºC). However, *Listeria* has shown the ability to grow at refrigeration temperatures and below, as *L. monocytogenes* is unique due to the bacterium being extremely hardy: it is both salt-tolerant and able to survive and even grow in temperatures below 1°C, unlike most pathogens (Jay, Loessner & Golden 2005, Posfay, Wald 2009,

Camejo et al. 2009). Also *L. monocytogenes* is second only to *staphylococci* species as a foodborne pathogen being able to grow at $A_w$ values $< 0.93$. *L. monocytogenes* can be found in many different environments and species, including numerous mammalian species, birds, some fish species, soil, farms, decaying vegetation, silage, animal feces, sewage, water and various food sources (such as raw vegetables, cheeses, milk and deli meats) (Centers for Disease Control and Prevention (CDC) 2013 C, Fenlon 1986, Lyautey et al. 2007, Posfay, Wald 2009, Chen 2012).

The unique pathogenesis of *L. monocytogenes* is what makes the bacterium dangerous, as the organism is able to spread directly from cell to cell in the host (Chen 2012). When *L. monocytogenes* enters the host's "monocytes, macrophages, or polymorphonuclear leukocytes", it quickly reproduces and becomes blood-borne; groups of proteins on the *L. monocytogenes* cell surface allow for survival in phagocytic cells, increasing its capability to spread from cell to cell (Chen 2012).

This bacterium is also the cause of listeriosis, a potentially fatal infection. The infective dose is believed to vary with the strain and susceptibility of the host; the food matrix involved can also affect the dose-response relationship (Chen 2012). Major outbreaks of listeriosis have been caused by a variety of food categories, including soft cheeses, fish, poultry, deli meats, and vegetable products (Schlech, Acheson 2000, Posfay, Wald 2009). In cases associated with raw or inadequately pasteurized milk, for example, it is likely that fewer than 1,000 cells may cause disease in susceptible individuals (Chen 2012). Pregnant women, who are disproportionately infected with *L. monocytogenes*, may experience mild, flu-like symptoms; however, their offspring do not

fare as well – they may abort or be stillborn, and those born alive may have bacteremia and meningitis. One-third of confirmed cases of maternal-fetal *L. monocytogenes* infections lead to abortion or stillbirth. The severe form of the infection has a case-fatality rate ranging from 15% to 30%, overall. When listerial meningitis occurs, the overall case-fatality rate may be as high as 70%; this compares to septicemia with 50% case fatality rate, and to perinatal/neonatal infections, which has 80% case fatality (Chen 2012). *L. monocytogenes* is an opportunistic pathogen (Mascola et al. 1988) infecting neonatal, elderly, or immunocompromised people. Most reported cases of listeriosis are life threatening and present one of three clinical syndromes: neonatal listeriosis, blood stream infection, or meningoencephalitis (Schlech, Acheson 2000, Swaminathan, Gerner-Smidt 2007). *Listeriosis* affects humans in two different ways:  a non-invasive gastrointestinal illness, which has a relatively short incubation period, which generally does not affect otherwise healthy people, and, a much more serious form of the illness, which is known to cause either septicemia or meningitis. This invasive version can have a long incubation period, with estimates from 3 days to 3 months (Chen 2012).

Because of the pathogenicity of *L. monocytogenes,* and its increasing incidence in the 1980's*,* the Food Safety and Inspection Service (FSIS), a division of the United States Department of Agriculture (USDA), worked to improve safety procedures of processing plants to emphasize the zero-level of tolerance (meaning "no detectable level permitted") in RTE products (Shank et al. 1996, Food Safety and Inspection Service (FSIS) 2000). *L. monocytogenes* is considered "detectable" when two separate 25 g samples of food are tested and found to have the pathogen, as defined by the Federal Food Drug and

Cosmetic Act, 21 U.S.C. 342(a)(1) (Shank et al. 1996). Furthermore, the USDA, through

the Federal Meat Inspection Act and the Poultry Inspection Act, 21 U.S.C. 601(m), states

that if meat or poultry products are contaminated with *L. monocytogenes*, the products are

considered adulterated, and as such, "unfit for human food" (Food Safety and Inspection

Service (FSIS) 2003).

Not only is the loss of life a concern with *L. monocytogenes* outbreaks, but also

the huge economic losses that can arise when a company needs to recall a product. The

USDA has three different recall classifications: I. This is a health hazard situation where

there is a reasonable probability that the use of the product will cause serious, adverse

health consequences or death; II. This is a health hazard situation where there is a remote

probability of adverse health consequences from the use of the product; III. This is a

situation where the use of the product will not cause adverse health consequences

(Bagley 2013). In all reported instances, *L. monocytogenes* is considered a class I recall,

as the pathogen can be dangerous, as noted previously. In the USA alone, recalls resulted

in economic costs of nearly two billion dollars; a single, small product recall could lead

to millions of dollars in loss (Ivanek et al. 2005, Batz et al. 2011).

For instance, in 2011, there was a recall of nearly 16,000 pounds of deli meats

(Food Safety and Inspection Service (FSIS) 2011) and in 2010, a recall of nearly

380,000lbs of deli meats, because of possible *L. monocytogenes* contamination (Food

Safety and Inspection Service (FSIS) 2010). There was no illness attributed to either case.

(Cochran 2013) reports a July 2012 recall of 324,000 lbs of frozen, RTE and poultry

products from a New Jersey company due to potential *L. monocytogenes* contamination.

Two weeks later, this same company had an additional 72,000 lbs recalled for the same

reason (Bagley 2013). Table 2 demonstrates other high-volume *L. monocytogenes*

incidents.

**Table 2.2. Notable recent recalls for *L. monocytogenes*.**

| Type of Food | Amount Recalled | Date and Company Location | Source |
|---|---|---|---|
| RTE Meats & Poultry | 15,880 lbs | August 2012 MN | (Bagley 2013 B) |
| Meat & Poultry Salad Products | 13,600 lbs | August, 2012 WI | (Bagley 2013 C) |
| Fully Cooked Meat and Poultry products | 33,500 lbs | January 2013 ID | (Khan 2013) |
| Chicken Sausage Products | 6,120 lbs | March 2012 AR | (Lindenberger 2013) |

*RTE: Ready-to-eat

The 2002 poultry incident in Table 3 had a recall of 27 million lbs of product

(Burros 2002). Sales reductions of RTE foods due to *L. monocytogenes* recalls are

estimated to be approximately 25% for months following the aftermath of the recall

(Thomsen, Shiptsova & Hamm 2006). Furthermore, all of these incidents have the

potential for legal action, which could cause exponential economic loss.

**Table 2.3. Notable incidences of *L. monocytogenes* outbreaks.**

| Cause | Year | Sickened | Deaths | Source |
|---|---|---|---|---|
| Farmstead Cheese | 2013 | 6 | 1 (1 miscarriage) | (Centers for Disease Control and Prevention (CDC). 2013) |
| Ricotta Cheese | 2012 | 22 | 4 (1 miscarriage) | (Centers for Disease Control and Prevention (CDC). 2012) |
| Cantaloupe | 2011 | 147 | 33 (1 miscarriage) | (Centers for Disease Control and Prevention (CDC). 2012 B) |
| Celery | 2010 | 10 | 5 | (Gaul et al. 2013) |
| RTE meat | 2008 | 22 | | (Gilmour et al. 2010) |
| Milk | 2007 | 4 | 2 | (Mcdonald 2007) |
| Cheese | 2003 | 12 | | (Swaminathan, Gerner-Smidt 2007) |
| Cheese | 2002 | 17 | | (Swaminathan, Gerner-Smidt 2007) |
| Cheese | 2002 | 47 | | (Pagotto et al. 2006) |
| Cheese | 2002 | 86 | | (Pagotto et al. 2006) |
| Poultry | 2002 | 46 | 10 (3 miscarriages) | (Burros 2002) |
| RTE Meats | 1998 | 101 | 21 (6 miscarriages) | (Food Safety and Inspection Service (FSIS) 2000) |
| Chocolate milk | 1994 | 44 | | (Dalton et al. 1997) |
| Soft-style Cheeses | 1985 | 142 | 47 (29 infant deaths) | (Centers for Disease Control and Prevention (CDC) 1998) |
| Milk | 1983 | 49 | | (Flemming 2002) |
| Coleslaw | 1981 | 41 | | (Schlech III et al. 1983) |
| Vegetables | 1979 | 23 | | (Ho et al. 1986) |

*RTE: Ready-to-eat

*Microbial Contamination of Food Products*

The food supply can be subject to different types of contamination, caused by bacteria, yeasts, viruses and fungi; these microbial reactions deteriorate the flavor, color and sensory properties of foods (Appendini, P., Hotchkiss, J.H. 2002, Vermeiren, L., Devlieghere, F., van Beest, M, de Kruijf, N., Debevere,J. 1999a, Han 2005). Those microorganisms are concerning because they can also cause foodborne illness, (Padgett, Han & Dawson 1998, de Oliveira et al. 2007, Davidson, Sofos & Branen 2005, Cha, Chinnan 2004). In foods, a variety of intrinsic factors such as pH, Aw, nutrient content, natural antimicrobial compounds, energy of activation, biological structure, enzymes and natural microbial flora affect microbial growth (Jay, Loessner & Golden 2005).

The control of moisture content in foods is one of the oldest preservation strategies (United States Food and Drug Administration (FDA) 2013). Water activity is defined as the ratio of water vapor pressure of a food to the vapor pressure of pure water at the same temperature (Jay, Loessner & Golden 2005). Increasing the acidity of foods, either through fermentation or the addition of weak acids, has been used as a preservation method since ancient times (United States Food and Drug Administration (FDA) 2013). Another important characteristic of a food to consider when using acidity as a control mechanism is its buffering capacity; the buffering capacity of a food is its ability to resist changes in pH; therefore foods with a low buffering capacity will change pH quickly in response to acidic or alkaline compounds produced by microorganisms as they grow (United States Food and Drug Administration (FDA) 2013).

Microorganisms require basic nutrients for growth and maintenance of metabolic functions of which the amount and type of nutrients required range dependent on the microorganism. These nutrients include water, a source of energy, protein, vitamins, and minerals (Jay, Loessner & Golden 2005, United States Food and Drug Administration (FDA) 2013, Mossel et al. 1995). Foodborne microorganisms can derive energy from carbohydrates, alcohols, and amino acids although most microorganisms will metabolize simple sugars such as glucose; there are certain few that can metabolize more complex carbohydrates, such as starch or cellulose found in plant foods, or glycogen found in muscle foods (United States Food and Drug Administration (FDA) 2013).

Jay, Loessner & Golden (2005), indicates that the extrinsic factors associated with growth are temperature, relative humidity, gas concentrations, and presence of other microorganisms. Other extrinsic treatments such as heating/pressure (canning, pasteurization etc.), salting, acidification, fermenting, drying, oxygen removal and carbon dioxide have been used traditionally to control the microbial growth (United States Food and Drug Administration (FDA) 2013) Traditional food preservation techniques have used combinations of temperature, pH, $a_w$, atmosphere, numerous preservatives, and other inhibitory factors. Food microbiologists have often referred to this phenomenon as the "hurdle effect" (United States Food and Drug Administration (FDA) 2013). For example, certain processed meat products and pickles may use the salt-to-moisture ratio (brine ratio) to control pathogens.

Many scientific studies have demonstrated the antimicrobial activity of gases at ambient and sub-ambient pressures on microorganisms important in foods (Loss,

Hotchkiss 2002, United States Food and Drug Administration (FDA) 2013). Gases inhibit

microorganisms by two mechanisms. A first mechanism was that gases could have a

direct toxic effect that can inhibit growth and proliferation. Carbon dioxide ($CO_2$), ozone

($O_3$), and oxygen ($O_2$) are gases that are directly toxic to certain microorganisms (United

States Food and Drug Administration (FDA) 2013). This inhibitory mechanism is

dependent upon the chemical and physical properties of the gas and its interaction with

the aqueous and lipid phases of the food. Oxidizing radicals generated by $O_3$ and $O_2$ are

highly toxic to anaerobic bacteria and can have an inhibitory effect on aerobes depending

on their concentration. Carbon dioxide is effective against obligate aerobes and at high

levels can deter other microorganisms. A second inhibitory mechanism is achieved by

modifying the gas composition, which has indirect inhibitory effects by altering the

ecology of the microbial environment. When the atmosphere is altered, the competitive

environment is also altered. Atmospheres that have a negative effect on the growth of one

particular microorganism may promote the growth of another. This effect may have

positive or negative consequences depending upon the native pathogenic microflora and

their substrate. Carbon dioxide replacement of oxygen is an example of this indirect

antimicrobial activity (Loss, Hotchkiss 2002).

All microorganisms have a defined temperature range in which they grow, with a

minimum, maximum, and optimum. An understanding of the interplay between time,

temperature, and other intrinsic and extrinsic factors is crucial to selecting the proper

storage conditions for a food product. Temperature has a dramatic impact on both the

generation time of an organism and its lag period. Over a defined temperature range, the

growth rate of an organism is classically defined as an Arrhenius relationship (Mossel et al. 1995).

When considering growth rate of microbial pathogens, time and temperature are integral and must be considered together: increases in storage and/or display temperature will decrease the shelf life of refrigerated foods since the higher the temperature, the more permissive conditions are for microbial growth (Branen 1983).

*Active Packaging*

Over its history, the human population has found ways to preserve freshly harvested foods for later use, which utilized some form of heat, cold, drying or fermenting treatments  (Branen 1983). Active packaging, which has been in existence for decades, has been defined as "a type of packaging that changes the condition of the packaging environment after sensing internal or external environmental change and responds by changing its own properties or attributes; this can extend shelf-life or improve safety or sensory properties while maintaining the quality of the food (Vermeiren, L., Devlieghere, F., van Beest, M, de Kruijf, N., Debevere,J. 1999a, Brody, Strupinsky & Kline 2001). Active packaging was introduced as a response to the demands of consumers for high quality, safety and extended chilled shelf-life of food products while accommodating the changes in retail and distribution practices, (for instance, online commerce and "retail superstores"), which have pressed logistic limits, as distribution distances have increased and longer storage times are required (Vermeiren, L., Devlieghere, F., van Beest, M, de Kruijf, N., Debevere,J. 1999a, Quinatavalla, Vicini 2002). Active packaging applications, including the incorporation of antimicrobials (and

subsequent release into the product) has received considerable attention as a means of extending the bacterial lag phase, leading to slower growth of microorganisms (Han 2000, Guerra et al. 2005).

The direct addition of antimicrobials (organic acids, spice extracts, chelating agents, metals, enzymes or bacteriocins, such as nisin) into the food product or its packaging have also shown a decrease in antimicrobial growth because of leaching into the food matrix, and cross-reaction with other food components such as lipids or proteins (Han, Floros 1997, Davies et al. 1999, Hoffman, Han & Dawson 2001). Given that there are numerous legal approved antimicrobials, their utilization in food is dependent on a variety of factors, including the properties of the food in question, the type of preservation system being used, the characteristic of the microorganism that is being contested (including type, number of, etc.) and the cost effectiveness of the antimicrobial (Branen 1983). An estimated 20% of the world's food supply is lost due to microbial spoilage; antimicrobials that could be applied are needed to provide the appropriate food supply levels in the future (Branen 1983, Fulton 1981). Even an additional two or three days of microbial prevention (in some instances, if it increases the shelf life of a product) could significantly help offset the costs of using an antimicrobial (Davidson, Branden 1981).

Antimicrobial packaging is produced to control undesirable microorganisms by means of incorporation of an antimicrobial compound into the packaging by various methods (Cha, Chinnan 2004). These methods include coating on packaging, extrusion where an antimicrobial is included in the packaging or volatizing the compound into the

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products headspace within the packaging (Lagarón, Ocio & López-Rubio 2011). The blending of antimicrobial agents directly to polymeric packaging is a continuingly increasing development, including sorbic acid, plant extracts, silver-substituted zeolite, lysozyme and chlorine dioxide, successfully incorporated in packaging materials to confer antimicrobial activity in food packaging (Vermeiren, L., Devlieghere, F., van Beest, M, de Kruijf, N., Debevere,J. 1999b, Appendini, P., Hotchkiss,J.H. 2002, Quinatavalla, Vicini 2002). Packaging films containing antimicrobial agents have shown improved efficacy by a controlled migration of the compound into the food, allowing for initial inhibition of undesirable microorganisms, and subsequent residual activity over the course of the distribution stage of the food cycle (Quinatavalla, Vicini 2002). Table 4 demonstrates that bacteriocins and other biologically derived antimicrobials, including nisin, have also been increasingly used in packaging materials (Siragusa, G.R., Cutter, C.N., Willett, J.L. 1999a).

**Table 2.4. Examples of Antimicrobial Packaging.**

| Examples of Antimicrobial Packaging | | |
|---|---|---|
| Author(s) and Year of Publication | Type | Polymer / Food Incorporated Into |
| | **Antimicrobials in Coatings** | |
| (Kim, Y.M., An, D.S., Park, H.J., Park, J.M., Lee,D.S. 2002) | Nisin | Acrylic and VAE |
| (Matthews et al. 2010) | Rosemary | Cellulose |
| (Mangalasary, Cooksey 2009) | Chitosan | Methyl-cellulose |
| (Brown, Wang & Oh 2008) | Lactoferrin | Edible Film |
| (Castellan et al. 1993) | Hydroquinone | HPC |
| (Wong et al. 1992) | Fatty Acid Esters | Chitosan Film |
| | **Direct Contact – Antimicrobial is blended into extrudate or coating** | |
| (Oral et al. 2009) | Oregano Essential Oil | Absorbent Pads |
| (Taptim, Sombatsompop 2011) | Silver Zeolite | Metallic Compounds |
| (Camilloto et al. 2010) | Triclosan | PE / Cellulose |
| (Cutter, C.N., Willett, J.L., Siragusa,G.R. 2001a) | Nisin | PEO Resin |
| (Pelissari, Yamashita & Grossmann 2011) | Essential Oregano Oil | Extrusion Parameters |
| (Taylor, T.M., Bruce, B.D., Weiss, J., Davidson,P.M. 2008) | Encapsulation | Polymer |
| | **Indirect Antimicrobial Packaging – Gases in Headspace** | |
| (Daifas et al. 2000) | Ethanol Vapor Generator | Crumpets |
| (Popa et al. 2007) | Chloring Dioxide Gas | Blueberries |
| (Gabler et al. 2010) | Sulfur Dioxide Gas Generator | Grapes |
| (Espitia et al. 2012) | Oregano, Lemongrass, Cinnamon Essential Oils | Papaya |
| (Chounou et al. 2013) | Oxygen Absorber | Ground Meat |
| (Scussel et al. 2011) | Carbon Dioxide Gas Generator | Brazillian Tree Nuts |

**Nisin**

Nisin is a polypeptide antibacterial substance produced from the fermentation of a modified milk medium by strains of the lactic acid bacterium, Lactococcus *lactis* (Delves-Broughton 1990, Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009a). A polypeptide is a chain of amino acids that are the basis of proteins (Kimball 2011).

Nisin is made up of about 34 amino acid residues; the molecule possesses amino and carboxyl end groups, and five thio-ether bonds form internal rings (Delves-Broughton 1990). Figure 1 demonstrates the structure of nisin A.



ABU =Amino butyric acid
DHA = Dehydroalanine
ALA-S-ALA = Lanthionine
DHB = Dehydrobutyrine (β - Methyldehydroalanine)
ABU-S-ALA = β Methyl lanthionine
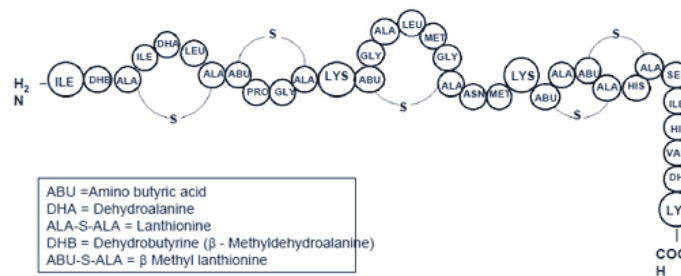
**Figure 2.1. The amino acid residue structure of nisin A. From: (Delves-Broughton 1990).**

*History*

Nisin was discovered in England in 1928, when inhibitory streptococci were considered to be a problem in cheesemaking (Jeevaratnam, L., Jamuna, M., Bawa, A.S. 2005). Streptoccoci are generally gram-positive cocci that grow in chains (Todar 2012b). It was first noticed when in certain batches of milk starter development was slow, resulting in faulty cheese (Jeevaratnam, L., Jamuna, M., Bawa, A.S. 2005). Initial research concerning the properties of nisin was more focused on the potential for either veterinary or clinical uses (Delves-Broughton 1990). However, given the comparatively

narrow antibacterial spectrum, a low solubility in body liquids and physiological pH instability, nisin was deemed unsuitable for those purposes (Hurst 1981).

Nisin's potential in food preservation was first suggested by Hirsch et al. (1951)*,* who found that nisin-producing starter cultures could prevent clostridial gas (from the bacteria *Clostridium tyrobutyricum*) formation in cheese. Afterwards, research studies showed adverse characteristics such as interference by nisin with starter culture performance during cheese formation (Delves-Broughton 1990). The literature indicates that food preservation then became the main research focus of nisin (Delves-Broughton 1990, Jeevaratnam, L., Jamuna, M., Bawa, A.S. 2005). Nisin was first used in processed cheese, and then expanded to various dairy products, milk, canned foods and alcoholic beverages (Delves-Broughton 1990). As the knowledge and biochemistry of nisin increased, a commercially available format was released as Nisaplin®, which possessed "a high and consistent antimicrobial activity" (Delves-Broughton 1990).

*Production*

Nisin can be produced in a sterilized medium of non-fat milk solids or of a non-milk-based fermentation source, such as yeast extract and carbohydrate solids (Anonymous 2007). Also, nisin can be recovered from fermentation by various methods, "such as injecting sterile, compressed air, membrane filtration, acidification, salting out; and spray-drying" (Anonymous 2007). Standard nisin preparation consists of nisin and sodium chloride with an activity of not less than 900 units per milligram; the activity can be adjusted by the concentration of sodium chloride in the solution (Anonymous 2007). Other non-fat milk solids or solids from other fermentation sources can be present in the

preparation, which is stable at ambient temperatures and upon heating under acid conditions; maximum stability occurs at pH 3 range (Anonymous 2007).

Commercially sold nisin can be produced in a few different ways. As mentioned previously, nisin comes from strains of *Lactococcus lactis*, which is found in abundance in dairy products. *Nisaplin®,* is a brand name for a nisin product made by Danisco, a subsidiary of DuPont. *Nisaplin®* contains approximately 2.5% nisin, the balance consisting of milk and milk solids derived from the fermentation of a modified milk medium by nisin producing strains of *L. lactis* (Delves-Broughton 2005). Handary Company, which is based in Belgium, produces nisin slightly differently: they obtain fermented *Lactococcus lactis* from sauerkraut and produce both the A and Z variants of nisin (Handary 2013). Other Suppliers include the Chinese-based Zheijang Silver Elephant Bio-Engineering Co., Ltd, who specializes in Nisin A and Sigma-Aldrich Company, a merged corporation with both US and Germany Roots, who has their own formulation for 2.5% concentrated nisin.

*Safety of Nisin*

Nisin is produced from subspecies of *L. lactis*, which is a gram-positive lactic acid bacterium (LAB) that is used extensively in the production of various dairy products, including various cheeses, butter and sour cream (Todar 2012). As this LAB is naturally occurring in milk, which has been consumed by both humans and animals for millennia, it is indicative of non-toxic nature of nisin (Delves-Broughton 1990). In 1969, nisin was approved for use as an antimicrobial in food by the Joint FAO/WHO Expert Committee on Food Additives (which is run by the United Nations); nisin has since been given the

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food additive number 234 and has been approved for use in over 50 countries (Delves-Broughton 2005). Nisin is the most abundantly used of all bacteriocins (Marth 1998), as it is an effective antimicrobial (Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009a). However, while nisin is GRAS (generally recognized as safe) by the FDA since 1988 (Marth 1998), there is a concentration limit of 10,000 IU (international units) in food. Nisin is used in a variety of dairy products (primarily cheeses) though its application as an active packaging material is still being researched. Nisin has shown to be inactivated by enzymes; when consuming a nisin-contained liquid, it cannot be detected in human saliva after 10 minutes (Delves-Broughton 1990, Jay, Loessner & Golden 2005). Higher international concentrations of nisin probably stem from the fact that nisin is easily digested by the upper GI tract of the human digestive system (Bower, C.K., McGuire, J., Daeschel, M.A. 1995a, Deshpande 2002).

Since its discovery, nisin has proven to be an effective inhibitor of gram-positive bacteria (Bower, C.K., McGuire, J., Daeschel, M.A. 1995a)and is now approved for use in 57 countries around the world and has been affirmed as generally recognized as safe (GRAS) in the United States (Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009a). Nisin is considered nontoxic to humans because it is inactivated by proteolytic enzymes in the digestive tract, and assumed to be safe for use as a food preservative (Bower, C.K., McGuire, J., Daeschel, M.A. 1995a). Barrett, Woessner & Rawlings (2004), describes proteolytic enzymes as any group of enzymes that break down protein chains into amino acids.

Because nisin has non-toxic qualities, is heat stable, and will not create off-flavors in food products, it is used in a variety of commercially produced foods, including dairy products, meats, fish, and eggs  (Le Blay, G., Lacroix, C., Zihler, A., Fliss, I. 2007, Schillinger, U., Geisen, R., Holzapfel, W.H. 1996). While nisin is primarily active against gram positive bacteria according to  (Jin, T., and Zhang, H. 2008a), including *Clostridium, Bacillus, Staphylococcus,* and *Listeria* species, nisin can be effective against gram negative bacteria too, but only when used in combinations with other antimicrobials, including lysozyme (which is found in egg whites and human tears according to (Kimball 2001) and rosemary extract (Matthews et al. 2010).

*Class*

While classification system for lantibiotics has changed over time, the most current classification has three classes (I, II, III). This is based on the genetics, biochemistry of the compounds, and the pathway by which the peptide is modified and whether it demonstrates antimicrobial activity  (Jay, Loessner & Golden 2005, Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a, Willey, J.M., van der Donk.,W.A. 2007). Nisin is a Class I lantibiotic. Class I lantibiotics are different from other classes as they are made up in a more linear structure and by their ability to demonstrate antimicrobial activity; some examples include: nisin, subtilin, Epidermin, streptin and Pep5  (Willey, J.M., van der Donk.,W.A. 2007, Piper, C., Cotter, P.D., Ross, R.P., Hill,C. 2009a). Class II are typically small heat stable peptides that possess only a single large enzyme to carry out the dehydration and cyclization duties, including various lactacin strains, cinnamycin, and mersacidin  (Willey, J.M., van der Donk.,W.A. 2007, Piper, C., Cotter, P.D., Ross,

R.P., Hill,C. 2009a, Jay, Loessner & Golden 2005). Class III lantibiotics are heat stable, while containing lanthionine but lack any notable antimicrobial activity (Willey, J.M., van der Donk.,W.A. 2007, Piper, C., Cotter, P.D., Ross, R.P., Hill,C. 2009a, Jay, Loessner & Golden 2005).

*Synthesis of Nisin*

Lantibiotic synthesis is a unique process. At first the unmodified form of the structural peptide is ribosomally synthesized and then subjected to extensive post-translational modifications, which makes the peptide active (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a). The antimicrobial activity of a lantibiotic is based on the "depolarization of energized bacterial cell/plasma membranes, which are originated by the establishment of aqueous transmembrane pores" (Bactibase 2013). This post-translational process uses 2,3-didehydroalanine (Dha), 2,3-didhydrobutyrine (Dhb) to form lanthionine or methyl-lanthionine, which explains the name "lantibiotic (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a, Willey, J.M., van der Donk.,W.A. 2007). Dha and Dhb are atypical amino acids generally not found in nature and are formed by the dehydration of serine (L-Ser) and threonine (L-Thr) residues then followed by the interaction of the cysteine (Rihakova et al. 20009, Willey, J.M., van der Donk.,W.A. 2007). This is followed by membrane translocation (when the transport of proteins in and out of the endoplasmic reticulum occurs, according to (Kimball 2011b). Specifically, the double bond in Dha or Dhb interacts with the thiol group in the cysteine group. As explained by Figure 2 (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a), when L-Ser is dehydrated and interacts with Dha (where the double bond interacts with thiol on

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cysteine) Lanthionine is formed (Ala-S-Ala). When L-Thr is dehydrated and interacts

with Dhb (where the double bond interacts with thiol on cysteine) Methyl-lanthionine is

formed (Abu-S-Ala). Both of these processes can be seen in Figure 2 after this paragraph,

which was originally created by (Piper, C., Cotter, P.D., Ross, R.P., Hill,C. 2009a). Both

lanthionine and methyl-lanthionine are referred to as the "lanthionine bridges",

"intramolecular bridges" or simply "rings," due to the polycyclic structure that is formed

(Bactibase 2013). These cyclic "rings" increases the stiffness of the peptide and provides

increased resistance to proteolytic degradation and thermal stress; it has been

hypothesized that the change in chirality of one of the two alpha carbons involved

contributes to the activity of the compound (Piper, C., Cotter, P.D., Ross, R.P., Hill, C.

2009a, Willey, J.M., van der Donk.,W.A. 2007, Suda et al. 2010).
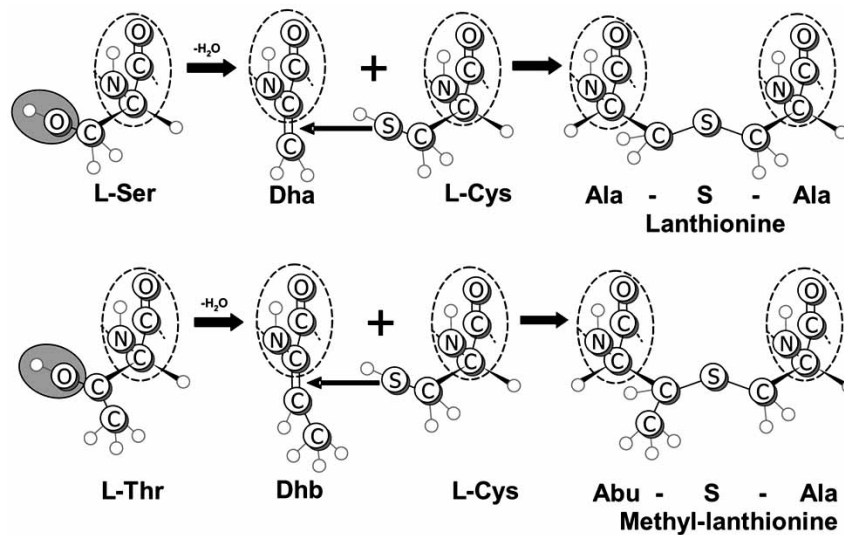


**Figure 2.2. From (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009b) showing Lantibiotic Synthesis**

*Type*

L. lactis strains produce at least three structural variants of nisin: Nisin A is considered the prototype, nisin Z differs by one amino acid, and nisin Q differs at four positions: all are 34 residues in length (Willey, J.M., van der Donk.,W.A. 2007). The two most studied variations of nisin are nisin A and nisin Z. The different amino acid is at position 27; for nisin A, it is histidine and in nisin Z, it is asparagine (De Vos et al. 1993). See figure 3 for more detail. This difference between the two strains is shown when comparing the solubility between the two: nisin A strains exhibits higher solubility at lower pH values, whereas nisin Z is comparatively decreased; At neutral and higher pH values, the solubility of both "strains" was comparable (Rollema, H.S., Kuipers, O.P., Both, P., de Vos, W.M., Siezen, R.J. 1995). The antimicrobial activity for both was found to be comparable during all studies (Rollema, H.S., Kuipers, O.P., Both, P., de Vos, W.M., Siezen, R.J. 1995). There are other less studied variants such as: Q, N, U, K etc. (Immonen, N., Karp, M. 2007). Streptococcus uberis is known to produce nisin U, which has 78% sequence identity to nisin, but lacks the C-terminal three residues, according to (Willey, J.M., van der Donk.,W.A. 2007). With relation to packaging, nisin A is more ideal for coating to utilize the hurdle concept (as it has greater inhibition at lower pH), whereas nisin Z is better for extrusion (has better inhibition at neutral pH) where corrosion from low pH is a concern.
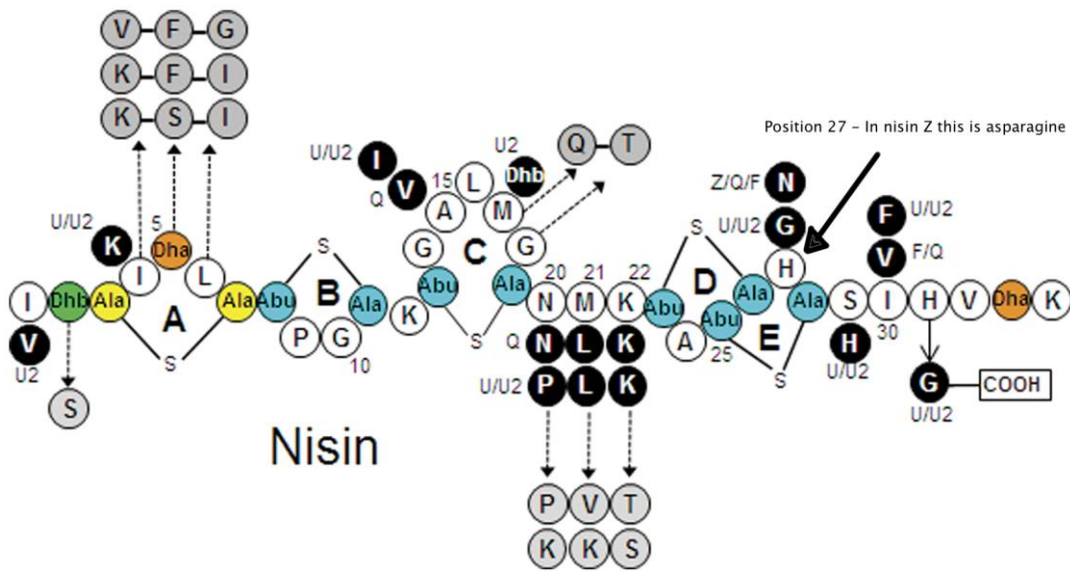
**Figure 2.3. Adapted from (Field et al. 2012) which shows how various nisin strains differ from one another, with emphasis on the differences of nisin A and nisin Z.**

## 2.2.7 Mode of Action of Nisin

Davidson & Branden (1981) postulated that the mode of action of an antimicrobial falls into three categories: 1. Reaction with the cell membrane, which causes permeability and loss of cellular constituents; 2. Inactivation of essential enzymes; 3. Destruction or functional inactivation of genetic material. While many modes of action have been proposed for nisin, (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl, H-G. 2001a, Piper, C., Cotter, P.D., Ross, R.P., Hill,C. 2009c), it was originally proposed that permeabilization of the bacterial cell membrane was mode of action of antibactieral peptides (Jenssen, Hamill & Hancock 2006). A combination of theories will be discussed, however. There are many elements that determine how nisin causes microbial inhibition. The stage of growth of the bacteria is one element that will be discussed. It is known that nisin will react differently depending

on the concentration of nisin present and availability of the lipid II molecule (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a), however, it does not always cause inhibition via the same mode of action (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl,H-G. 2001b). Figure 4 shows the Nisin-lipid II interaction.
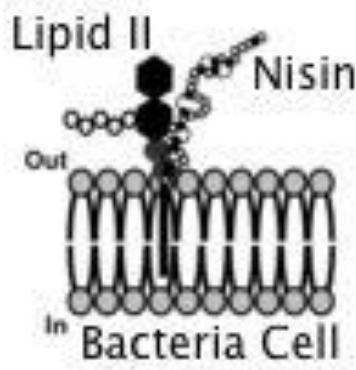


**Figure 2.4. Adapted from (Zendo, Yoneyama & Sonomoto 2010) and shows the nisin and lipid II interaction in the cell wall.**

Willey, & van der Donk (2007), stated that nisin is considered a "relatively flexible molecule that has two amphiphilic domains consisting of three N-terminal rings (labeled A, B ,C) and the C-terminal D and E rings, which are joined by a flexible hinge region" The lipid II molecule consists of bactoprenol-carrier lipid and a monomeric disaccharide-pentapeptide peptidoglycan subunit (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl, H-G. 2001b).

Nisin binds to the lipid II molecule through pore-forming activity; in this instance, lipid II is a "docking molecule" (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl, H-G. 2001b). Nisin binds the pyrophosphate division of lipid II with one of three N-terminal rings (Willey, J.M., van der Donk.,W.A.

2007, Piper, C., Cotter, P.D., Ross, R.P., Hill,C. 2009a), which involves five intramolecular hydrogen bonds. It has been demonstrated by (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl, H-G. 2001b) that nisin binds with high affinity to the lipid II molecule; when present; nisin is more effective at causing inhibition against gram-positive microorganisms. This is because the act of binding allows nisin to be transported to a developing cell and as a result inhibit the cell wall from forming (meaning it inhibits peptidoglycan synthesis). If the bacteria cell wall is already formed then the lipid II molecule will aid in the pore formation process.

For nisin molecules to form a pore complex. Recall the N-terminus of the nisin is interacting with the lipid II molecule, seen in Figure 4. Then the hinge region (ring clusters) of the nisin is important in pore formation. This allows the nisin to bend in half, therefore having the C-terminus end contact the cell wall. The C-terminus end of nisin is important for translocation across the cell membrane. This will disrupt the barrier function of the bilayer and form pores. The pores are typically 2nm in size. This permeabilization of the membrane causes dissipation of vital ions and small metabolites and ultimately results in the dissipation of the proton motive force (PMF) leading to the cessation of all metabolic and biosynthetic processes leading to cell death (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009c). This pore formation process with the presence of lipid II molecule can happen at very low concentration of nisin. (Wiedemann, I., Bruekink, E., van Kraaj, C., Kulpers, O.P., Bierbaum, G., de Krujiff, B., Sahl, H-G. 2001b) hypothesizes nM concentrations will allow for pore formation when lipid II is present.

If lipid II molecule is not present, nisin must be present in high concentrations in order to form pores. This is done in a targeted-independent fashion. The membrane of the cell wall should have 50-60% negatively charged phospholipids in order to allow the C-terminus end to bind to the cell wall. Pores formed under this mode of action are anion-selective and depend on the membrane potential to support pore formation. This is one mechanism in which the nisin may still be able to cause inhibition, when the hinge region is mutated. Since the N-terminus does not bind to the lipid II molecule, the nisin is not required to bend for the C-terminus to contact the bacteria cell wall.

The last mode of action that will be discussed is the disruption of cell division. Nisin has been shown to cause cell wall degradation between dividing cells (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009c). Nisin releases two cell wall hydrolyzing enzymes that are cationic and can bind by electrostatic interactions with negatively charged acids in the bacteria cell wall (Suda et al. 2010). Bacteria cell walls contain negatively charged acids such as teichoic and teichuronic acids (Mamo 1989). The binding alone will not cause cell death, when the displacement of these enzymes occurs it will result in cell lysis, however, (Suda et al. 2010) during cell division, large amounts of peptidoglycan synthase is organized in helical threads along the longitudinal axis of the cell (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a). If lipid II cannot co-localize with peptidoglycan synthesis then cell wall formation is inhibited and bacteria are killed (Piper, C., Cotter, P.D., Ross, R.P., Hill, C. 2009a). It is thought that the lipid II molecule has a functional location in gram positive bacteria and nisin has the ability to displace this molecule (Willey, J.M., van der Donk.,W.A. 2007). Knowledge of the mode of action of

antimicrobials, and the ability of the organism to overcome this mode of action can be helpful in determining the efficiency and usefulness of an antimicrobial.

*Methods of Testing Inhibition*

The spot-on-lawn assay is one of the most direct (and widely used) methods for preliminary screening of large numbers of strains, as it is a relatively simple process (Tagg, J.R., Dajani, A.S., Wannamaker, L.W. 1976). In this method a specific quantity of the antimicrobial solution is pipetted onto the surface of an agar plate and the resulting zones of inhibition are measured after 24-48 hours of incubation.

A popular variation of this method involves wells cut into agar plates freshly seeded with the test organism. A small quantity of the antimicrobial solution is pipetted into the well and inhibition zones are measured after 24-48 hours of incubation. A study using the spot-on-lawn or drop assay measured the efficacy of nisin release from a coated polyethylene film against *Listeria monocytogenes* (Grower, J.L., Cooksey, K., Getty, K. 2004b). Matthews et al. (2010), used spot on lawn assay to test the efficacy of nisin in barrier film with cellulose coating in inhibiting *L. monocytogenes.* A study conducted by (An, D.S., Kim, Y.M., Lee, S.B., Paik, H.D., Lee, D.S. 2000a) measured the efficacy of bacteriocins against several bacteria, yeasts and molds using the spot on lawn assay; after observing positive initial results, the researchers applied the bacteriocins to low density polyethylene (LDPE) films, which was then measured for antimicrobial activity by the agar diffusion test.

The agar diffusion method has probably been the most widely used method for determination of antimicrobial activity throughout recent history (Davidson, Sofos &

Branen 2005). In this test, an antimicrobial compound is added to an agar plate on a paper disk; the compound diffuses through the agar, resulting in a concentration gradient that is inversely proportional to the distance from the disk  (Davidson, Sofos & Branen 2005). The degree of the molecule's movement can be related to the concentration of the molecule (Tolman 2013). The smallest concentration of an antibiotic (or antimicrobial) that inhibits growth of a bacterium after a specified incubation period is called the Minimal Inhibitory Concentration, or more commonly known as MIC  (Davidson, Sofos & Branen 2005, Wiegand, Hilpert & Hancock 2008).

MIC, indicated by a zone of no growth around well, is dependent on the rate of diffusion of the compound and cell growth (Barry 1986). An evaluated antimicrobial should not be highly hydrophobic because the compound will not diffuse properly and little or no inhibition will be detected and a test microorganism must also grow rapidly and uniformly (Davidson, Sofos & Branen 2005). The results of the agar diffusion test will not give an indication of microbial reduction in terms of cell numbers.  Variations of the agar diffusion method exist, including the agar well diffusion method, which provides an accurate, rapid, simple and sensitive tool to detect and quantify the antimicrobial activity of *L. monocytogenes* strains without equipment requirements  (Ruiz, Silva & Laciar 2009).

There have been numerous studies where the researchers used the agar diffusion as a method of testing the ability of nisin.  Sebti, I., Ham-Pichavant, F., Coma,V. (2002a), added nisin to HPMC based film against *L. monocytogenes, Staphyloccocus aureus and Micrococcus luteus* and tested the efficiency using agar diffusion method.  (Mauriello,

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G., De Luca, E., La Storia, A., Villani, F., Ercollini, D. 2005) found that nisin-coated films were effective in inhibiting *M. luteus* by using agar diffusion assay. Le Blay, G., Lacroix, C., Zihler, A., Fliss, I. (2007), tried to compare *pediocin PA-1* versus nisin strains inhibitory ability against various common intestinal bacteria (mostly Gram positive), and found that nisin was successful in inhibiting the microorganisms, including *Enterococcus faecium.* Neetoo, H., Ye, M., Chen, H., Joerger, R.D., HIcks, D.T., Hoover, D.G. (2008)*,* tested the resistance of numerous strains of *L. monocytogenes* against nisin using agar well diffusion to determine the most resistant strain of *L. monocytogenes*. The study then used nisin-coated films on vacuum packaged cold-smoked salmon against these nisin resistant strains and found that nisin still had inhibitory success, dependent on the concentration of the nisin, the time exposed, and the temperature. In a study to determine nisin diffusion in protein films,  Teerakarn. A., Hirt, D.E., Acton, J.C., Rieck, J.R., Dawson, P.L. (2002), used agar diffusion for the quantification of nisin activity by measuring the response of the nisin against *Lactobacillus plantarum* 1752.

**Table 2.5. Summary of Selected Antimicrobial Screening Methods**

| Method | Author | Level of Nisin | Organism Tested | Reduction |
|---|---|---|---|---|
| Spot-on-Lawn | (Grower, J.L., Cooksey, K., Getty,K. 2004b) | 100000 IU/cm$^2$ | *Listeria monocytogenes* ATCC15313 | 7 and 6.75mm |
| | (Matthews et al. 2010) | 5.49 mg/ml | *Listeria monocytogenes* ATCC15313 | Inhibition observed – compared to control (no antimicrobial). |
| | (An, D.S., Kim, Y.M., Lee, S.B., Paik, H.D., Lee,D.S. 2000b) | 10% bacteriocin solution | Various nonpathogenic, pathogenic bacteria, yeast and molds | + or - |
| Agar Diffusion | (Sebti, I., Ham-Pichavant, F., Coma,V. 2002a) | 2800IU (used 40IU nisin and 70ul of nisin solution) | *M. luteus IP270 S. aureus IP 58156 L. monocytogenes ATCC 15313* | Not reported |
| | (Mauriello, G., De Luca, E., La Storia, A., Villani, F., Ercollini,D. 2005) | 1g 2.5% nisin in 5 ml | *Micrococcus luteus* ATCC 10240 | Tested for activity of nisin 51200AU-1 |
| | (Teerakarn. A., Hirt, D.E., Acton, J.C., Rieck, J.R., Dawson,P.L. 2002) | 0.00028 to 0.0125mg purified nisin/mL | *L. plantarum* 1752 | Produced a standard curve |
| | (Neetoo, H., Ye, M., Chen, H., Joerger, R.D., HIcks, D.T., Hoover,D.G. 2008) | 0 (not reported), 625, 1250, 2500, 5000, 10000 IU/ mL | *Listeria monocytogenes* PSU1, PSU2, PSU 21 | PSU1: 8.00+/-0.41, 9.58+/-1.05, 10.73+/-0.98, 12.52+/-0.56, 14.10+/-0.23 PSU2: 7.94+/-0.85, 9.88+/-1.55, 11.42+/-1.50, 12.64+/-1.67, 13.88+/-1.76 |

| | | | | PSU21:6.60+/-1.05, 9.14+/-1.09, 11.51+/-1.06, 13.80+/-1.09, 15.35+/-0.83 |
|---|---|---|---|---|
| | (Le Blay, G., Lacroix, C., Zihler, A., Fliss,I. 2007) | Nisin A and Nisin Z 40ul | *Bacteroides sp. Fusobacterium nucleatum subsp. Polymorphum* ATCC 10953, *E. coli* DSM 5698, *Bifidobacterium sp., Lactoballus sp. Clostridium sp., Clostridium sp. Eubacterium biforme* DSM 3989, *Enterococcus faecium* DSM 20477, *Ruminococcus productus* DSM 2950, *Streptococcus salivarius* DSM 20560 | Wide variety of inhibition |

Table 5 attempts to summarize some of the current literature on screening

methods used to determine the effectiveness of nisin for eventual incorporation into food

packaging applications.  Collectively, the research shows that nisin is an effective

antimicrobial but the methods of measurement, levels of nisin tested, indicator

organism(s) and method of reporting effectiveness are so varied that it becomes very

difficult to compare between studies.  For example, of the agar diffusion assays shown in

table 5, each study reports their data as a measurement of a zone of inhibition, percentage

of the packaging material or "wide variety of inhibition".  If progress is to be made with regard to nisin for food packaging, it is important to try and find a way to standardize some of the methods and reporting units so that comparisons between studies can be made.

**Coating**

Coating refers to the ability to deposit liquid (sometimes air) uniformly onto a solid surface or, substrate, and is recognized as a barrier against gases, moisture and microorganisms  (Chawengkijwanich, Kopermsub 2012). Coating is commonly used in the manufacturing of ink/paint, tapes (adhesives), photographic films, paper plastic, glass and metal.  The two key properties that need to be understood when developing coatings are rheology and surface chemistry, specifically surface tension.

Rheology, in a broad sense, is the study of the physical behavior of all materials, specifically liquids or pastes, when placed under stress (Gilleo 2006). Essentially, it is the "science of flow and deformation of matter" (Oesterle, Palmer 1969) or the study of viscosity over a wide range of conditions (Oil and Colour Chemists Association, Australia (OCCA) 1984).

Surface tension is the measure of attractive or repulsive forces of molecules (Gilleo 2006) and can be defined as the excess force per unit length at the surface (Chan, Venkatraman 2006); in this case, this measures the liquid-solid interface of the coating. The porous network structure of the coating can allow for additional active characteristics to be realized as the incorporated active agent can subsequently be released through the

pores onto the food surface in a controllable manner  (Chawengkijwanich, Kopermsub 2012).

All molecules have forces either attractive or repulsive; these forces are measured in force per unit length and noted as dynes per centimeter. This measurement, surface tension, is important with respect to coating: when a surface is ideally wetted, there are no droplets formed on the substrate. A drop is formed when uneven distribution of forces occurs (and typically occurs with liquid with higher surface tensions, such as water); molecules are pulled in every direction. There are a few attributes that affect surface tension: liquids are not only attracted to other liquid (intramolecular) but when placed on a solid, intermolecular attraction can also occur.

One of the more important rheology characteristics is the resistance to flow, or viscosity (Oesterle, Palmer 1969). Mathematically, this is the ratio of shear stress to shear rate. Shear stress is the force per unit area (measured in dynes per square centimeter) applied to the coating. Coatings can behave differently depending on viscosity (Glass 1978a, Soules et al. 1998). Newtonian flow is when the coating is unchanged when shear is applied, whereas non-Newtonian changes occur when shear is applied (Oesterle, Palmer 1969). Two common behaviors are shear thickening (dilatant) or shear thinning (pseudoplastic) (Oil and Colour Chemists Association, Australia (OCCA) 1984). In addition, the coating can be viscoelastic and develop tensile and compressive elastic stresses when sheared or extended. High viscosity coatings need a lot of force to change shape and take longer to flow out. The yield point is the minimum amount of shear applied to initiate flow (Oil and Colour Chemists Association, Australia (OCCA) 1984).

There are many principles that should be considered when developing a coating, according to (Harrington 2006, Oil and Colour Chemists Association, Australia (OCCA) 1984):

1. What type of substrate is ideal for coating?

2. Does the coated substrate seal? The substrate will be used on what types of products? Is it then food safe? Will it seal through grease or lipid if in contact with that type of food product?

3. Once the substrate is chosen. Will the substrate need to be pretreated?

    a. Or will the coating stick to the substrate without substrate treatment?

    b. If substrate pretreatment is needed, what type of treatment is best?

*Properties of Coating*

Properties of the coating should be considered during development of coatings. Physical properties such as viscosity are needed. The coating should be able to flow without extreme shear stress or elevated temperature (Oil and Colour Chemists Association, Australia (OCCA) 1984). Viscosity of coating can be measured in many ways. Brookfield viscometer is a typical lab method of measuring centipoise and torque. Zahn cup and Bostwick Consistency Meter are two other commercial ways of measuring viscosity (Rolin, de Vries 1990). Time, temperature and volume all affect the viscosity and should not be reported without this important information (Oil and Colour Chemists Association, Australia (OCCA) 1984).

Other physical properties of the coating include: corrosion, color and flexibility. pH should be measured when developing coatings because a low pH coating may cause

corrosion of equipment color should be measured. A colorimeter can be used if the coating has color. However, if the coating is transparent, ASTM D1003 can be used to measure clarity of haze (ASTM 2013). Depending on the use of the coating, color may be unattractive to the consumer. Also percent solids should be measured. A typical commercial coating has percent solids between 15-40%. The percent solids should be in this range to run on current commercial equipment (Gilleo 2006). Flexibility is important for storage shipping and use. Coatings should not break off the substrate when the final material is handled. Useful knowledge includes, drying time, curing (such as oven and UV treatment) and equipment desired for use in scale up of the coating formula. Finally, cost and toxicity are also important for the coating to be commercialized and legally approved (Oil and Colour Chemists Association, Australia (OCCA) 1984).

*Mayer rods*

Wire-wound metering rods such as mayer rods have been used for more than 75 years to apply coating to flexible substrates (Macleod 2006). This coating method is cost effective, versatile (with respect to both types of coating and changing of production type), and allows for easy cleanup, making this viable for commercial production (Hull 1991).  Mayer rods are typically used for production of tape, labels and flexible packaging. Coating with a wire wound rod is one of the least complex methods for applying coatings or adhesives (Hull 1991). The rods are made today of stainless steel and are tightly wound to coat exact amounts of liquid/coating onto the substrate. Mayer Rods come in many different sizes (which are differentiated by number), and the lay

down of the coating is correlated with the number of the Mayer Rod; the higher the number of the Mayer Rod, the thicker the lay down of the coating.

The coating thickness can be controlled within an accuracy of 0.0001 in (0.1 mil) and is controlled by the cross-sectional area between the wire coils  (Hull 1991, Macleod 2006). Additionally, the coating is in strips from the grooves between the wires in a mayor rod. The coating is rapidly pulled together from the surface tension creating an unevenly distributed coating. When viscosities are high, the coating can adhere to the surface of the mayor rod, which can negatively affect the process. There are other factors that commercially affect coating thickness such as web speed and web tension. Mayer rods that are shorter are called "lab rods" and can be used in initial development process. These "lab rods" can be placed on a flat surface with the substrate attached near the top and drawdowns can be completed manually. This flat surface can be called a drawdown table. Some drawdown tables have a magnetic strip, which holds the substrate in place. See Figure 5 for an example of a drawdown table.
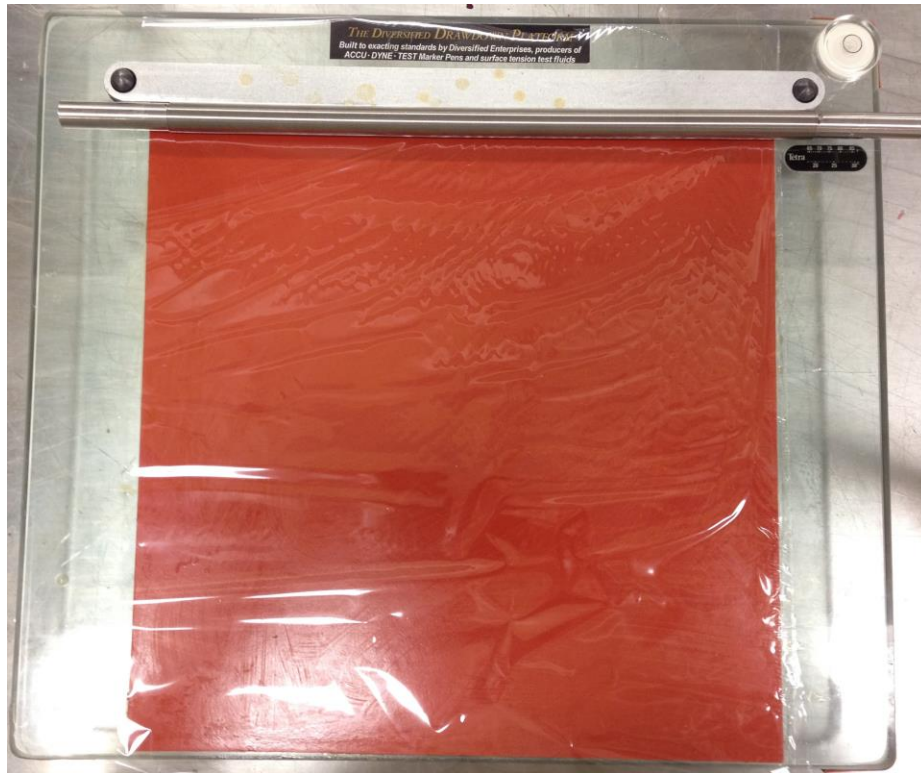
**Figure 2.5. Drawdown Table utilized in research**

*Defects*

Defects that can occur with surface coatings include: Aeration, coagulation, gassing, settling, skinning, viscosity increase, viscosity decrease, coverage, sticky application or streaking. Aeration is when air bubbles form during mixing and become trapped in the coating and are unable to escape. It forms foam that leaves uneven coating. Coagulation is when the emulsion breaks from too much shear. Gassing is when an ingredient in the coating formulation produces gas. This can be dangerous especially during storage. Settling is when an ingredient does not stay in solution and due to density falls to the bottom of the solution. Skinning is when the coating develops a thick "skin" on the surface that can no longer be mixed into the rest of the coating solution. Viscosity

decrease is when the coating is shear thinning. The more the coating is exposed to shear the thinner the coating. This will result in a different coverage on the substrate. Viscosity increase occurs when the coating is shear thickening. The more shear applied to the coating the thicker the coating becomes. This can sometimes be prevented by changing solvents. Coverage is the rate of spreading across the substrate. Poor coverage happens when the viscosity is too high or when the coating becomes sticky. Sticky application can be controlled by changing the viscosity or avoiding high temperatures. Finally streaking is when the coverage is uneven. This can happen with the coating itself or with a pigment in the coating. This can be prevented with proper mixing and viscosity (Oil and Colour Chemists Association, Australia (OCCA) 1984).

Other issues in coating are more wire rod coating specific: this method of coating has a limited viscosity range (the efficacy of metering decreases as viscosity increases) and the coating mechanism has issues with change in the dimensional inconsistencies in the web (Hull 1991). Defects that appear from the act of coating include streaks in the coating (generally in the direction of the machine), which are caused by particulate matter trapped between the rod and the web (Hull 1991). "Rod streaks" are another common issue; which are caused by the wire pattern on the rod (Hull 1991).

*Carrier: Pectin*

There are numerous carriers used in antimicrobial coating development research, such as methylcellulose, alginate, soy, corn zein, and cellulose. Pectin as a carrier is important as it can allow for the slow release of an antimicrobial. Pectin is a naturally occurring polymer mainly obtained from the non-woody cell walls of land growing plants

(Rolin, de Vries 1990, United States Food and Drug Administration (FDA) 2013b).

Pectin is a mixture of polysaccharide structures that help those plants grow as shown in

Figure 6. The chemical structure of pectin is different between plants.
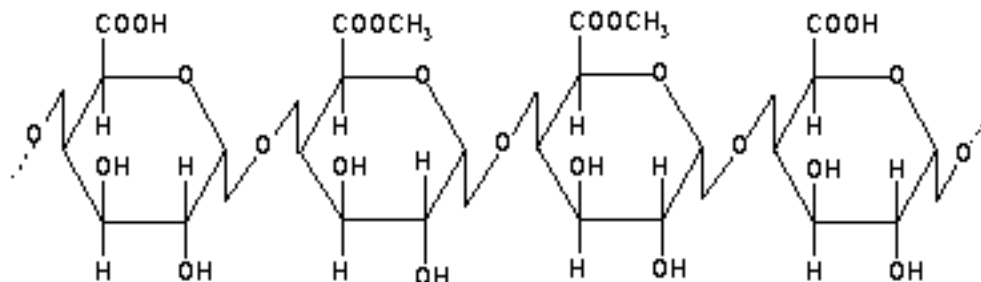


**Figure 2.6. Chemical Structure of Pectin Adapted from (Anonymous 2012)**

Pectin is a soluble dietary fiber that binds cholesterol and slows glucose

absorption during digestion (Brown et al. 1999). It is safe for human consumption as it is

currently part of daily diets and is GRAS approved (United States Food and Drug

Administration (FDA) 2013b). It has been used successfully for many years in the food

and beverage industry as a thickening agent, a gelling agent and a colloidal stabilizer

(Allwyn et al. 2012). In coatings pectin is the carrier agent.

Commercial pectin production is mainly obtained from citrus fruits (lemons,

limes, oranges and grapefruits), apple pomace, and various vegetables; the pectin is then

produced into a dry white or brown powder (Rolin, de Vries 1990, Sriamornsak 2003).

CP Kelco is the world's leading producer of pectin, and their pectin is derived from both

citrus peels and beet pulp (CPKelco 2013). There are many types of commercial pectins.

However, most pectins are high in galacturonic acid. Therefore the main component in

the majority of commercial pectin is methanol-esterified 1-4 linked alpha-D-galacturonic

acid (See Figure 3).

In nature about 80% of the carboxyl groups are esterified with methanol. However, commercial pectins are produced in a large range from 80% methylesterification and trending lower. This percentage varies by production method and it imparts changes in the functionality of the pectin. Solutions with more than 1% pectin are pseudoplastic. Commercial pectins are divided into two groups based on the degree of methyl esterification (DE): high-methylester pectins (HM) and low-methylester (LM) pectins. The DE influences the functional properties of both HM and LM, but in an inverse manner.

The process of manufacturing pectin is confidential for most commercial manufactures. However, a common method starts with extraction. Acidified water (pH 2) is used to extract pectin from fruits or vegetables at 70ºC. During this process de-esterification can take place so considerations must be taken depending on manufacturing of which type of pectin. Then a vacuum filter removes peel and the peel is sent off as animal feed.

Pectin can be dissolved in warm water with the presence of shear. Pectin is not soluble in ethanol. Pectin is a candidate for pharmaceutical use, due to its capability in controlled drug delivery / controlled releases. Sriamornsak, Nunthanid (1998), modified drug release patterns by utilizing a pectin/calcium delivery system; there was a delayed release of the drug in the colon. According to Marathe (2008), pectin shows better dissolving capability at low pH (being 2.5) or below the pka of pectin (3.95), which allowed for the better release of nisin. The study concluded that if pH was above the pka, then there was not release of nisin. Viscosity can be reduced under low pH conditions.

Viscosity increases with increasing pectin solution. Pectin solutions are viscous, but pectin is not particularly efficient as a thickener compound compared to other water soluble –polymers. The rheological properties of pectin solutions are very dependent on the presence of salts, calcium or similar non alkali metals, and on the pH. Other important facts are the chemical properties of pectin, including the degree of ester, and the average molecular weight.

*Pectin and Calcium*

High-methylester pectins (DE above 50) form gels in the presence of low pH combined with low water activity (such as the addition of sugar). Gelation for low-ester pectin relies on calcium being added to the mixture.  Grant et al. (1973), was the first to coin the term "egg-box model" in order to describe the gelation of pectin and other alginates.  The mechanism involves junction zones created by the ordered, side-by-side associations of galacturonans, whereby specific sequences of GalA monomer in parallel or adjacent chains are linked intermolecularly through electrostatic and ionic bonding of carboxyl groups. The gel structure is a net-like formation of cross-linked pectin molecules. The cross-linkages formed by ionic bonds between the carboxyls are strong and produce a rather brittle, less elastic than those formed by hydrogen bonding as in regular pectin. See Figure 7 for the diagram of the egg-box. With pectins of lower DE, there is an increasing probability for the formation of cross-links with a given amount of calcium. As the number of reactive carboxyl groups that can form a salt bridge increases, the greater the chances are that the bridge will be formed. Because de-esterified

molecules are straighter than the esterified ones, they will be more likely to form calcium

linkages (Thibault, Rinaudo 1985, Sriamornsak 2003).
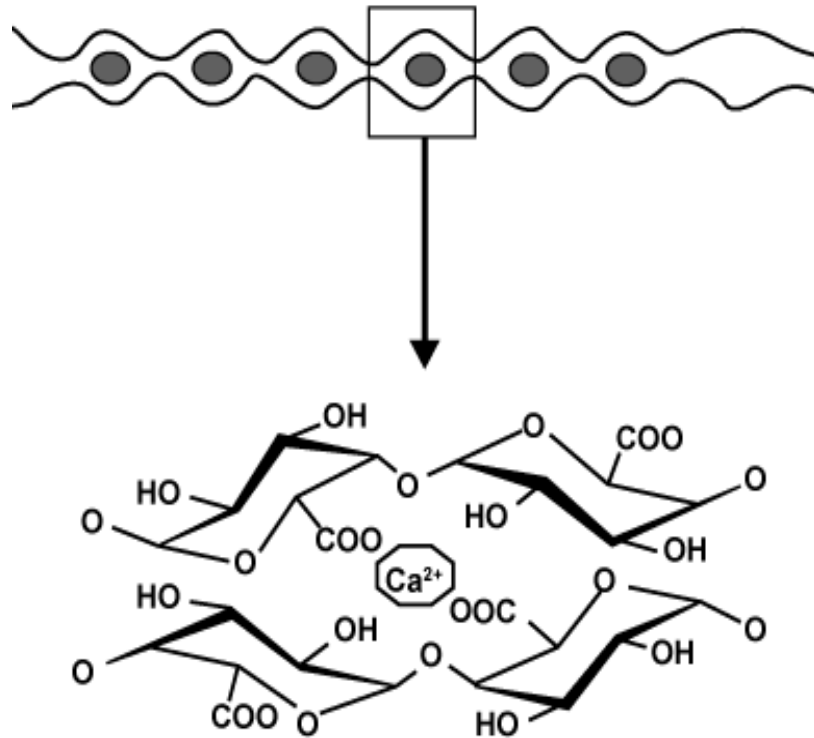


**Figure 2.7. Representation of calcium binding to polygalactoronate sequences: egg box dimer and egg box model (Allwyn et al. 2012, Axelos, Thibault 1991).**

*Glycerol*

Glycerol has previously been utilized in food coatings (Marathe 2008,

Bangyekan, Aht-Ong & Srikulkit 2006) in order to assist in the coating flow of a

formulation. Glyceryol is a trihydroxy sugar alcohol  (PubChem 2013) and is a plasticizer

and is colorless syrupy liquid that is miscible in water. Its structure can be seen in Figure

8 below. A plasticizer is a substance that can be added to a rigid plastic in order to

increase flexibility or workability or extensibility. Glycerol, when added to a coating,

ensures that the coating maintains the properties of the film.
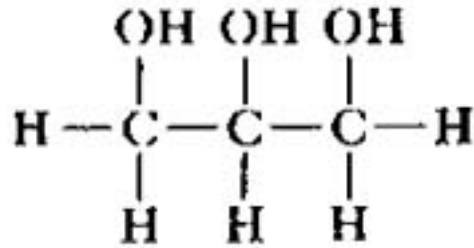


**Figure 2.8. Chemical structure of glycerol adapted from (Kousen 2013).**

### Nisin in Packaging

While nisin has been around for years as an antimicrobial food preservative, there

has been no commercial implementation into packaging (at least not on a large scale).

There are a variety of methods used to incorporate antimicrobial agents into food

packaging materials.  Nisin is a bacteriocin of choice because it is relatively heat stable

(Le Blay, G., Lacroix, C., Zihler, A., Fliss, I. 2007) and because of its efficacy against

gram-positive bacteria (Jin, T., and Zhang, H. 2008a). However, as the research indicates,

progress is being made towards the commercialization of nisin based films, but there is

still a gap between what can be done in academia (or research laboratories) and what is

able and willing to be done by current packaging converting firms. The main reason is

because the research has not yet proven all of the variables needed to get it approved for

packaging. These variables include the extreme heat used in commercial packaging

(which could potentially inactive the nisin), and the diffusion of nisin over time (so legal

concentration levels are not exceeded).

*Extrusion*

Extrusion of nisin into films is a commercial way of producing antimicrobial films. Jin, T., Liu, L., Zhang., H., Hicks.,K. (2009b), extruded nisin into bio-based thermoplastic polylactic acid (PLA), and found that their preparations showed excellent antimicrobial activity against *L. monocytogenes.* Another study, conducted by Cutter, C.N., Willett, J.L., Siragusa, G.R. (2001b), used extrusion of nisin with polyethylene or polyethylene oxide resin to produce a film. Other authors have encapsulated nisin, in order to study if it had a positive effect on nisin natural properties. There have been several studies evaluating various properties of nisin-containing films. Nisin, when combined into a film, has had varying effects on the films to which it was added. The tensile strength of a composite pectin/polyactic acid (PLA) film that was extruded with nisin was presented (Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009a). While the study found a resulting decrease in tensile strength (19% decrease and a 40% decrease for fracture energy; it was not attributable to the addition of nisin. Rather, it was believed that the cause was the reduction of the PLA phase. Padgett, Han & Dawson (1998), also measured the efficacy of nisin based films created through heat press and casting and found that both formed excellent films with strong inhibitory capabilities against *Lactobacillus plantarum.* Taylor, T.M., Davidson, P.M., Bruce, B.D., Weiss, J. (2005), used liposomal-encapsulated nisin, to determine the antimicrobial activity of the encapsulated nisin. The study showed that encapsulation helped increase the thermotropic solubility and release of nisin, although this study did not go through with an actual

extrusion. Taylor, T.M., Bruce, B.D., Weiss, J., Davidson, P.M. (2008), found that liposome encapsulated nisin produced inhibition against *L. monocytogenes.*

*Coating*

The use of nisin-containing containing coatings on the surface of polymeric films has the potential for delivery and transfer of nisin to the surface of foods.

A study incorporated nisin into a food grade packaging material involved a methylcellulose blend that was cast coated onto low-density polyethylene (Grower, J.L., Cooksey, K., Getty, K. 2004b). This allowed nisin to be blended in an acidic solution prior to blending in hydroxyl propyl methylcellulose, which helped activate the nisin. The purpose of coating was to act as a carrier of nisin and dissolve slowly when in contact with a semi-wet media (such as agar), which would dissolve and release nisin. This method proved to be effective for nisin release but inhibition was not consistent over time (Grower, J.L., Cooksey, K., Getty, K. 2004a). Another study, by Sebti, I., Ham-Pichavant, F., Coma,V. (2002b), used a combination of stearic acid and nisin in a hydroxyl propyl methyl cellulose film (HPMC) to develop packaging film that not only acts as a moisture barrier and as an antimicrobial, but also biodegrades. Because stearic acid is a fatty acid, the combination with nisin caused a high decrease in tensile strength of the HPMC. Among those are coating onto a substrate material such as low-density polyethylene (LDPE), heat press, absorb the antimicrobial into a substrate material and extrusion. Of these methods mentioned, only coating and extrusion can be considered among the more commercially viable methods (Cooksey 2005). These examples, in addition to a study by Cutter, C.N., Willett, J.L., Siragusa, G.R. (2001c), that utilized a

nisin-coated PE/PE oxide film on beef, suggest that nisin-coated films potentially also have considerable efficacy in environments other than liquid. The reason for the preponderance of nisin as antimicrobial in films is perhaps its relatively settled regulatory status as a food additive, but the urgent goal of inhibiting *L. monocytogenes* on foods is presumably also a contributing factor. This bacterium is of concern to food safety for a number of reasons and, as a Gram-positive bacterium, is sensitive to nisin.

Adsorption of nisin into films is another way of increasing their antimicrobial activity. A study by (Dawson et al. 2005) looked at the adsorption of nisin in silica and starch powders, and found nisin was efficient at both adsorption in the powders and the release of antimicrobial activity. Bower, C.K., McGuire, J., Daeschel, M.A. (1995b), looked at how a protein, such as nisin, can adsorb to a food contact surface, giving it the potential to prevent against pathogenic growth, including *L. monocytogenes;* the study found that the use of nisin is feasible in this regard. While using nisin solely as an antimicrobial agent that was incorporated into both corn zein and gelatin films, (Ku, K., Song, K.B. 2007) found that nisin both increased tensile strength and decreased film permeability for both film types. Corn zein films were affected much more then the gelatin type film. The study also reported increased antimicrobial activity with increased nisin concentration.

*Food Challenge Studies*

In order to fully understand nisin's potential as a reliable antimicrobial, it is imperative that a food challenge study be completed, as it can replicate real world situations. Fang, Lin (1994), completed a food challenge study that utilized nisin as an

antimicrobial. This study was done in conjunction with modified atmosphere packaging in cooked pork and tested against *L. monocytogenes* and *Psudomonas fragi*. The results of this study was that the combination of MAP with nisin was effective at inhibiting both organisms, although the study noted that nisin efficacy was increased as nisin concentration increased. Harris et al. (1989), conducted a study to find out if lactic acid bacteria were effective L. *monocytogenes* antimicrobials. The study found that lactic acid bacteria, including some nisin strains were effective in inhibiting L. *monocytogenes* and could be a way to help prevent its abundance in the food system. (Mangalassary, S., Han, I., Rieck, J., Acton, J., Dawson, P.L. 2008) conducted a study that utilized a combination of nisin and lysosome in RTE turkey bologna for a 12 week period; the study found that the combination of the two antimicrobials were both factors in reducing log counts of L. *monocytogenes*, which was a "hurdle effect" with the in package pasteurization process. (Shefet, S.M., Sheldon, B.W., Klaenhammer, T.R. 1995) utilized a nisin-based treatment (with EDTA) against *Salmonella* Typhimurium to extend the shelf life of broiler carcasses. The study found that nisin treated chicken had a slightly better prevention than against the control.

Scannell, A.G.M., Hill, C., Ross., R.P., Marx., S., Hartmeier, W., Arendt,E.K. (2000), measured adsorption of Nisaplin® and lacticin 3147 as a bioactive food packaging material against *Lactococcus lactis, Listeria innocua, and Staphylococcus aureus*. Only the Nisaplin® film was able to maintain activity over a three month period under refrigeration against sliced cheese and ham store in modified atmosphere packaging.

*Diffusion Studies*

Aly, S., Floury, J., Famelart, M-H., Madec, M-N., Dupont, D., Le Gouar, S., Lortal, S., Jeanson,S. (2011), conducted a nisin quantification study to find the diffusion coefficient in cheeses. This study chose nisin Z as the relevant model solute in order to investigate mass transfer properties of peptides during ripening within the cheese matrix. In order to find the diffusion that this study utilized an enzyme-linked immunosorbent assay (ELISA) that model diffusion coefficients for nisin, according to Fick's law. Although successful, modeling would need to be investigated for different nisin variants and other food products.

Hanusova, K., Stastna, M., Votavova, L., Klaudisova, K., Dobias, J., Voldrich, M., Marek,M. (2010), measured migration from a dried natamycin solution on a coated LDPE film into water. In order to determine diffusion, the study utilized agar well diffusion method. Natamycin was released in amounts that inhibited microrganisms, however, the study indicated that the natmycin broke down after eight days of exposure to light.

Kim, Y.M., An, D.S., Park, H.J., Park, J.M., Lee, D.S. (2002), incorporated nisin into acrylic polymer and vinyl acetate ethylene co-polymer and coated onto paper. The study measured (via agar well diffusion) the release of nisin into different contact solutions, which included water, 2% sucrose solution, 2% citric acid solution, and a 2% NaCl solution; these solutions represented sweet, acidic, and salty foods, respectively. The study also measured the inhibition with against *Micrococcus flavus* in a nutrition broth medium; the study showed that there was inhibition against the microorganism.

Jin, T., and Zhang, H. (2008b), in studies utilizing nisin as an antimicrobial in polylactic acid polymer, also measured the release of nisin. The standard curve was prepared by a seeded lawn overlay spot (also completed by (Siragusa, G.R., Cutter, C.N., Willett, J.L. 1999b) with some modifications to the procedure. The nisin was heated (100° C for 5 minutes) and then cooled for 144 hours to obtain maximum release. The release kinetics from a PLA nisin matrix needs further investigation, the study indicated, however their hypothesis follows a Fickian diffusion behavior. The diffusion or extraction of nisin from the film was used by (Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009a) to test the bacterial inhibitions where pectin films or PLA films were placed in various liquid mediums (including BHI Broth, preservative free orange juice and pasteurized liquid egg whites), and were innoculated with *L. monocytogenes.*

Bastarrachea, L., Dhawan, S., Sablani, S.S., Powers,J. (2010), studied the release kinetics of nisin from poly-butylene adipate-co-teraphthalate (PBAT) to distilled water. The reseachers utilized agar well diffusion and were able to model using Fick's second law of diffusion, partition coefficient and the Weibull model. The study concluded that nisin diffused better with temperature, and in PBAT film when compared to other films.

A study by (Cha, D.S., Cooksey, K., CHinnan, M.S., Park.,H.J. 2003) measured the release of nisin from both heat press and casting-method films. The heat pressed films were monolayer films that blended polyethylene powder and biopolymers containing nisin, and the casting method used PE film with a biopolymer containing nisin. Both methods showed that the films prepared with nisin, exhibited inhibition against the tested microbial, in this instance, *M. luteus*.

More studies need to be completed that utilize new diffusion techniques such as microscopy or HPLC. In addition, there diffusion studies need to be conducted on food (not food simulants) over a longer shelf life. Further research needs to be conducted on the breakdown of nisin and its variants during refrigeration over a longer period of time.

## Statement of the Problem

Current US regulations dictate zero tolerance policy for *L. monocytogenes* in Ready-to-eat foods (Food Safety and Inspection Service (FSIS) 2000). This zero tolerance policy is difficult to achieve since testing methods are destructive, training has limited success and post process-contamination is common (Oliver 2013). As mentioned previously, FSIS risk assessments found that 83% of all identified cases of *Listeriosis* are associated with deli meat sliced at retail delis (Food Safety and Inspection Service (FSIS) 2013).

Oliver (2013), demonstrated that training alone is not enough to eliminate food safety issues; an example is that it takes about eight hours to properly take apart and clean a deli case, which given modern retail food stores, is impractical. In her presented study, thirty retail delis were sampled for presence of *L. monocytogenes* before and after employee training. The intervention was not able to statistically decrease *L. monocytogenes* for both low and high prevalence of *L. monocytogenes* in retail delis. Pre-intervention food-contact surfaces (deli case, slicer blade etc.) sampled contained 4.5% prevalence of *L. monocytogenes*, while post intervention measured that 4.0% of food contact surfaces sampled contained *L. monocytogenes*. Out of the thirty tested stores, eleven demonstrated evidence of persisting *L. monocytogenes* strains (Oliver 2013).

*L. monocytogenes* is "ubiquitous" and can form biofilms: It is found everywhere including food contact sites, soil, consumers, drains, water (Fenlon 1986, Posfay, Wald 2009, Centers for Disease Control and Prevention (CDC) 2013 C, Valderrama, Cutter 2012). These studies have indicated that it is nearly impossible to prevent *L. monocytogenes* from entering the food system.

In addition, both culture and employee's attitudes are other challenges to overcome (Richard et al. 2013). There is a clear need for alternative control measures of *L. monocytogenes* in retail delis (Oliver 2013). In order to combat this ever-present menace, *L. monocytogenes* should be controlled by continuing good manufacturing methods, continued proper handling by food workers, and necessary additional secondary safety measure such as antimicrobial coating. However, current research for antimicrobial coatings is in preliminary stages and is primarily based on theoretical lab scale testing. There is a need for both food challenge studies and diffusion studies for antimicrobial coatings. It is necessary to understand the safety and potential success for antimicrobial coatings on RTE-foods instead of liquid media. There are many current gaps in the research including:

1. Research/food challenge studies with *L. monocytogenes* are needed instead of indicator organisms. The food challenge studies should last the entire shelf life of the food to ensure resistance is not occurring. This may require a possible slow release of antimicrobial over the course of the shelf life of the food.

2. It is important to select substrates that would typically be used at retail delis/industry and can easily be coated.

3. It is also important that the application is such that success can most likely be achieved. For example, this would include packaging that does not require sealing, or excessive heating. Also, it must be in direct contact with the food.

4. Research with food safe ingredients that are easily soluble and colorless.

5. Studies should be cost cognitive and percent loss of antimicrobial during processing or storage should be considered.

6. Coating should be designed to be scaled-up for commercial equipment. Coatings should be thin to resemble real world use.

7. There is a need for effective (measurable) detection methods for diffusion in the food. Also methods should be compared since methods are not consistent in the literature.

**Research Objectives**

1.  To develop antimicrobial coating containing nisin that is opaque, food safe, thin, cost effective, and slow release.

2.  To coat two substrates (with intent for food contact without heat and sealing properties) with a thin coating using laboratory Mayer Rods to produce a uniform coating.

3.  To conduct food challenge studies using a ready-to-eat food (ie. Deli turkey meat) inoculated with a cocktail of *L. monocytogenes* in direct contact with the antimicrobial coating over the full chilled shelf life of the product.

4.  To compare agar diffusion, spot on lawn and challenge study results.

5. To measure diffusion (the amount leaving the film when in contact with the

   food) by high performance liquid chromatography.

6. To identify diffusion of nisin intensity in food using confocal microscopy.

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CHAPTER III

DEVELOPMENT OF NISIN PECTIN COATING, ANALYSIS OF PROPERTIES FOR
COMMERCIAL APPLICATIONS AND FOOD CHALLENGE STUDY ON READY-
TO-EAT TURKEY BOLOGNA INOCULATED WITH *LISTERIA MONOCYTOGENES*
COCKTAIL

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**Abstract**

*L. monocytogenes* is the leading cause of death associated with deli meats (Batz et
al. 2011); in fact, 83% of all listeriosis cases in the United States are attributable to deli
meats (Food Safety and Inspection Service (FSIS) 2013) and listeriosis is over 15 times
more likely to be found in deli meats than any other source (Oliver 2013). Antimicrobial
packaging is produced to control undesirable microorganisms by means of incorporation
of an antimicrobial compound, such as nisin, into the packaging by various methods
(Cha, Chinnan 2004). While research indicates that progress is being made towards
commercialization of nisin-based films, there is still a gap between what can be done in
academia (or research laboratories) and what current packaging firms are capable of or
willing to do. Pectin is an excellent carrier of nisin in a coating and may allow for a slow
release of the antimicrobial. Findings from our current research suggest that the

developed coatings demonstrated antimicrobial activity against *L. monocytogenes*. The coatings were found to be food safe, colorless and within the commercial range for viscosity/percent solids. Both coating treatments (with and without Calcium Chloride) could inhibit *L. monocytogenes* for the entire shelf life of the turkey bologna and were significantly different compared to controls.

## Introduction

Food-borne illness is a major concern in the United States, as it affects approximately 1 in 6 people per year, according to the Centers for Disease Control (Centers for Disease Control and Prevention (CDC) 2013 C). An estimated 48 million cases occur each year in the United States, and include 128,000 hospitalizations and 3,000 related deaths (Centers for Disease Control and Prevention (CDC) 2013 C).

*L. monocytogenes* is the leading cause of death associated with deli meat-derived food-borne illness (Batz et al. 2011); in fact, 83% of all listeriosis cases in the United States are attributable to deli meats (Food Safety and Inspection Service (FSIS) 2013) while listeriosis is over 15 times more likely to be found in deli meats than any other source (Oliver 2013). Of further concern, *Listeria* has a very high combined public health burden with an economic cost of almost two billion dollars, despite the relative lack of comparative occurrences (Batz et al. 2011). Oliver (2013) presented that despite massive efforts in the prevention of listeriosis, there are still been a significant number of confirmed cases since 2004, when a *Listeria* initiative addressed to outline concerns and establish surveillance techniques (Cartwright et al. 2013) was implemented; the trend on listeriosis is "flat-lining" as opposed to decreasing, despite more time and effort being put

into its prevention (Oliver 2013, Cartwright et al. 2013).

Current US regulations dictate a zero-tolerance policy for *L. monocytogenes* in Ready-to-eat foods (Food Safety and Inspection Service (FSIS) 2000). This zero-tolerance policy is difficult to achieve since testing methods are destructive, training has limited success and post process-contamination is common (Oliver 2013). As mentioned previously, (Food Safety and Inspection Service (FSIS) 2013) risk assessments found that 83% of all cases of *Listeriosis is* associated with deli meat sliced at retail delis.

Oliver (2013) demonstrated that training alone is not enough to eliminate food safety issues. For example, it takes about eight hours to properly take apart and clean a deli case, which in modern retail food stores, is not impractical. In the study, thirty retail delis were sampled for presence of *L. monocytogenes* before and after training. The intervention was not able to statistically decrease *L. monocytogenes* for either low or high prevalence of *L. monocytogenes* in retail delis. Pre-intervention food-contact surfaces (deli case, slicer blade, etc.) contained 4.5% prevalence of *L. monocytogenes*, while post-intervention food-contact surfaces measured that 4.0% prevalence. Of the thirty stores tested, eleven demonstrated evidence of persisting *L. monocytogenes* strains (Oliver 2013).

*L. monocytogenes* is "ubiquitous" and can form biofilms. As such, it is found everywhere, including food contact sites, soil, drains, water, and on consumers themselves (Fenlon 1986, Posfay, Wald 2009, Centers for Disease Control and Prevention (CDC) 2013 C, Valderrama, Cutter 2012). These studies indicate that it is nearly impossible to prevent *Listeria* from entering the food system.

Culture and employee's attitudes are other challenges to overcome (Richard et al. 2013) in the prevention of *Listeria* outbreaks. There is a clear need for alternative control measures against *L. monocytogenes* in retail delis (Oliver 2013). In order to combat this ever-present menace, *L. monocytogenes* should be controlled by continued good manufacturing methods, proper handling by food workers, and necessary additional secondary safety measures, such as antimicrobial packaging coatings. However, current research for antimicrobial coatings is preliminary and primarily based on theoretical lab scale testing. There is a need for both food challenge studies and diffusion studies for antimicrobial coatings. It is necessary to understand the safety and potential success of antimicrobial coatings on RTE-foods instead of liquid media.

Active packaging was first introduced as a response to the demands of consumers for high quality, safety and extended shelf life of food products; it has also suited the changes in retail and distribution practices, (for instance, online commerce and "retail superstores"), which have pressed logistic tensions. For example, as distribution distances have increased, the need for longer storage times has also increased(Vermeiren, L., Devlieghere, F., van Beest, M, de Kruijf, N., Debevere,J. 1999, Quinatavalla, Vicini 2002). Active packaging applications, including the incorporation of antimicrobials (and subsequent release into the product), have received considerable attention as a means of extending the bacterial lag phase, leading to slower growth of microorganisms (Han 2000, Guerra et al. 2005). Antimicrobial packaging is produced to control undesirable microorganisms by means of incorporation of an antimicrobial compound into the packaging by various methods (Cha, Chinnan 2004). These methods include coating onto

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packaging, inside surface extrusion where an antimicrobial is blended into the packaging or volatizing the compound into the product headspace within the packaging (Lagarón, Ocio & López-Rubio 2011).

The direct addition of antimicrobials (organic acids, spice extracts, chelating agents, metals, enzymes or bacteriocins, such as nisin) has also shown a decrease in antimicrobial growth due to leaching into the food matrix, and cross-reaction with other food components such as lipids or proteins (Han, Floros 1997, Davies et al. 1999, Hoffman, Han & Dawson 2001).  Packaging films containing antimicrobial agents have shown improved efficacy through a controlled migration of the agents into the food, allowing for initial inhibition of undesirable microorganisms, and subsequent residual activity over the course of the distribution stage of the food cycle (Quinatavalla, Vicini 2002).

Nisin is a polypeptide antibacterial substance produced from the fermentation of a modified milk medium by strains of the lactic acid bacterium, *Lactococcus lactis* (Delves-Broughton 1990, Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009). Nisin is made up of about 34 amino acid residues. The molecule possesses amino and carboxyl end groups, and five thio-ether bonds which form internal rings (Delves-Broughton 1990).

Nisin's potential in food preservation was first suggested in 1951 (Hirsch, A., Grinsted, E., Chapman, H.R., Mattick, A.T)*,* which found that nisin-producing starter cultures could prevent clostridial gas (from the bacteria, *Clostridium tyrobutyricum*) formation in cheese (Jung, D.S., Bodyfelt, F.W., Daeschel, M.A. 1991). As the knowledge regarding the biochemistry of nisin increased, a commercially available

format was released as Nisaplin®, which possessed "a high and consistent antimicrobial activity" (Delves-Broughton 1990).

The use of nisin-containing coatings on the surface of polymeric films has the potential for delivery and transfer of nisin to the surface of foods. A study incorporating nisin into a food grade packaging material involved a methylcellulose blend which was cast coated onto low density polyethylene (Grower, J.L., Cooksey, K., Getty,K. 2004b). This allowed nisin to be blended in an acidic solution prior to blending in hydroxyl-propyl-methylcellulose, which helped activate the nisin. The purpose of coating was to act as a carrier of nisin, which would dissolve slowly when in contact with a semi-wet media (such as agar), and subsequently releases the nisin. This method proved to be effective for nisin release, but inhibition was not consistent over time (Grower, J.L., Cooksey, K., Getty,K. 2004a).

Pectin is an excellent carrier of the antimicrobial, nisin, in a coating and may allow for slow-release of the antimicrobial. Pectin is a naturally occurring polymer mainly obtained from the non-woody cell walls of land growing plants (Rolin, de Vries 1990). Commercial pectin production is mainly obtained from citrus fruits (lemons, limes, oranges and grapefruits), apple pomace, and various vegetables; the pectin is distributed as a dry white or brown powder (Rolin & de Vries 1990, Sriamornsak 2003) Commercial pectins are divided into two groups, high methyl ester pectins (HM) and low methyl ester (LM) pectins, based on their degree of methyl esterification (DE) (Rolin & de Vries, 1990). The DE influences the functional properties of both HM and LM pectins, but in an inverse manner. High methyl ester pectins (DE above 50) form gels in the presence of

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low pH and low water activity (such as the addition of sugar), whereas, the gelation for low-ester pectin relies on calcium being added to the mixture (Endress, Christensen 2009). For these studies, LM pectin was chosen for its high reactivity to calcium in order to get optimum slow-release properties.

(Grant et al. 1973) were the first to coin the term "egg-box model" in order to describe the structure of the gelation of pectin and other alginates. This gel structure is a net-like formation of cross-linked pectin molecules. Cross-linkages are formed by ionic bonds between the carboxyl groups and are less elastic than those formed by hydrogen bonding as in regular pectin (Sriamornsak 2003). Because de-esterified molecules are straighter than the esterified ones, they are more likely to form calcium linkages (Thibault, Rinaudo 1985, Sriamornsak 2003).

Coating with a wire wound rod is one of the least complex methods for applying coatings or adhesives (Hull 1991). Wire-wound metering rods such as Mayer rods have been used for more than 75 years to apply coating to flexible substrates (Macleod 2006). This coating method is cost effective, versatile (with respect to both types of coating and changing of production type), and allows for easy cleanup, making this viable for commercial production (Hull 1991). New generation stainless steel rods are tightly wound to coat exact amounts of liquid/coating onto the substrate. The coating thickness can be controlled within an accuracy of 0.0001 inches (0.1 mil) and is controlled by the cross-sectional area between wire coils (Hull 1991, Macleod 2006).

The objectives of this study were (1) to develop antimicrobial coating formulations that were food safe with the intent for future commercialization; (2) to

determine the properties and characteristics of the coating formulations; (3) to identify an effective antimicrobial coating formulation for the inhibition of *L. monocytogenes* over the shelf-life of ready-to-eat turkey bologna; and (4) to determine whether calcium chloride could enhance the efficacy of the antimicrobial coating by a slow-release of nisin.

Packaging films containing antimicrobial agents have shown improved efficacy through a controlled migration of the agents into the food, allowing for initial inhibition of undesirable microorganisms, and subsequent residual activity over the course of the distribution stage of the food cycle (Quinatavalla, Vicini 2002).

## Materials and Methods

*Preparation of Coating*

Three different coating formulations were used: A pectin-based control coating (C), nisin added (A), and nisin & calcium chloride added (B). All coatings originated as pectin slurry. Preparation occurred by mixing sterile water (percentage varied with treatment to equal end volume of 100%), 2.79% sterile glycerol (Fisher Scientific, USA), 12% sterile acidified water and 4.62% pectin GENU® low methyl ester (LM)-12CG degree of methyl esterification (DE) 35 (kind gift from CPKelco a Huber Company). The coatings were stirred for 15 minutes at 75ºC, removed from the heat and allowed to cool to 50ºC. The antimicrobial, ultra-pure nisin A (>95%) (Handary, Belgium) was dissolved in sterilized acidified water (20% Acetic Acid). The final concentration of nisin was 10,000IU/g (for properties analysis) or 20,000IU/g (for food challenge study) in wet weight (~30% solids) based on Richard et al. 2014 (REF) diffusion studies (note: nisin %

not part of formulation). For coating C, no nisin was added; it contained only 12% sterilized acid (20% Acetic Acid). For coating B, 18% calcium chloride (0.7% calcium chloride solution) (Fisher Scientific, USA) was added. The coatings were stirred for 15 minutes at 50ºC. Substrates were coated using a sterile 16'' "lab Mayer rod" (See Figure 1). The coated substrates were allowed to dry for 24 h (See Figure 1) before being cut into 4 x 4 in$^2$ (16 square inches).

*Substrates Coated*

Table 1 summarizes the two substrates used, plastic (P) (gift from Sealed Air Corporation) and wax paper (W), with three types of coatings (C, A, B), for a total of six treatments (PC, WC, PA, WA, PB, WB). The only difference between treatment A and treatment B was the addition of calcium chloride to treatment B. The plastic substrate was a laminate coextruded forming web with a polypropylene skin. A plastomer sealant and ethylene vinyl alcohol barrier was used for the plastic treatments (PA, PB, PC). The plastic substrate is a low-heat-sealing material (105-180 ºC) with enhanced sealability through brines, marinates and sauces. Substrate (W) was a wax parchment paper purchased from Ingles Markets, SC, and was used for the wax paper treatments (WA, WB, WC). Substrates were treated with UV light for 15 minutes before being coated. Both wax paper and plastic substrates were evaluated, as both are industry standards for liners of ready-to-eat meats at the deli.

*Properties of coating solutions*

For the coating solutions, (A, B and C), the following properties were measured: pH, density, percent solids and viscosity. pH was measured using a pH meter (Model 63,

Jenco Electronics CTD China). Density was measured using a specific gravity pyrometer (VWR TG-15145-24). Percent Solids was measured by drying the samples in a Fisher Isotemp® oven 300 series model 338F (Fisher Scientific, USA) for 24 hr at 100ºC. Finally, viscosity was measured using three methods: Brookfield DV-E viscometer Model CVDVE115 (Middleboro, MA), Bostwick consistometer No 24925-000 (CSC Scientific company Inc., Fairfax, VA) and EZ® Zahn viscosity cup #2 (Garoco, USA). Viscosity was measured following ASTM D 4212 (ASTM, 2010). Parameters, (including temperature, volume, time) were kept constant between treatments and replications.

*Properties of coated film*

After the films were dried for 24 hours, additional properties could be measured. Basis weight was measured by using ASTM F 2217-13 (ASTM, 2013A). Transmittance, haze and clarity were measured using a BYK Gardner Haze-gard plus (Germany) at Printpack (Marshall, NC) following ASTM D 1003 (ATSM, 2013B). Thickness was measured using a Precision Micrometer Series 400 Tester (Amityville, New York). In addition, antimicrobial assessments were conducted (film on lawn, agar well diffusion and spot on lawn) as described below.

*Film on lawn*

"P" and "W" substrates each coated with one of the coating formulations, A, B, and C, were cut into circles (for a total of 6 circles) by using an X-acto precision instruments circle cutter (Elmer's products, OH, USA). The circles were placed on modified oxford agar plates (MOX) inoculated with sterile tweezers. The MOX plates were made using Oxford Medium Base (BD-Difco, Detroit, MI, USA) with the addition

of the modified oxford antimicrobial supplement (BD-Difco, Detroit, MI, USA). The plates were incubated for 48 h at 37ºC. Using a digital caliper (Control company, China) inhibition zones were measured in millimeters.

*Standard assay curve of nisin*

The standard assay curve of inhibition by nisin A solution was obtained by the method adapted by (Tramer, J., and Fowler, G.G. 1964). The effect of different nisin concentrations and the minimum inhibitory concentration (MIC) of nisin was determined using a stock nisin solution (concentration?) and serial dilutions ranging from 20,000IU/mL to 78 IU/mL. The stock solution was obtained by adding 0.05g of Nisin A to 100mL of acidified water (20% Acetic Acid). From the stock solutions, 10,000, 5,000, 2,500, 1,250, 625, 313, 156, and 78 IU/mL were made. Acidified water (20% Acetic Acid) was used as a negative control.

*Estimation of nisin in films*

After 24 h of drying, the coated films were cut into 4 x 4 square inch squares. The coated squares were placed in sterile polyethylene bags and 5mL of sterilized acidified water (0.02N Acetic Acid) were added. The bags were sealed with an impulse sealer Model FS-400 (Hualian, China) and placed on a Gyrotory® water bath shaker (Edison, NJ) and incubated at 4ºC. Samples were collected in test tubes at each sampling time and immediately stored at 4ºC until assay (to obtain nisin releasing from coating). Sample volume collected was 5mL (10-µl needed for spot on lawn testing and 50 µl needed for well diffusion testing). Time points were collected at 30 minutes, 5 h, 10 h, 24 h, 48 h, 72 h, and 7 days.

*Bacterial culture for spot on lawn and agar well release studies*

The *Listeria monocytogenes* strain (ATCC 43256) used in this study was obtained from the Food Microbiology Laboratory at Clemson University. The frozen stock culture was stored in brain heart infusion broth (BHI) (Difco, Detroit, MI, USA) with 20% glycerol at -80ºC. The working stock cultures were maintained by culturing on slants of BHI agar medium (Difco, Detroit, MI, USA) and incubating at 37ºC for 48 h. The prepared slants were stored at 4ºC until required for a maximum of 14 days. At the time of testing, the growth on the slant culture was streaked on to MOX and grown for 48 h. A single colony was isolated from the growth plate, suspended in 10mL of BHI broth and grown for 48 h (incubating at 37ºC, while shaking).

*Agar well diffusion assay*

The agar well diffusion method adapted by Barefoot, et al.(Barefoot, Klaenhammer 1983) was used to detect the activity of nisin against *L. monocytogenes* (ATCC 43256). MOX agar plates were overlaid with ~8mL of semisoft TSB agar (0.5% w/v agar) seeded with 1% broth culture of *L. monocytogenes* (ATCC 43256). The seed density was approximately $1x\ 10^6$ cfm ml$^{-1}$ of overlay. Plates were allowed to harden for 1 h before wells were made. 50-µl wells were made using a bore, and 50-µl of nisin-containing release samples were placed in each well. Plates were stored at 4ºC for 24 h and then incubated at 37ºC. Plates were evaluated for zones of inhibition in millimeters using a digital caliper (control company, China) in both horizontal and vertical directions and averaged. The experiment was repeated three times and reported as zones of inhibition (mm).

*Spot on lawn Assay*

The spot on lawn assay was also used to detect the activity of nisin against *L. monocytogenes (*ATCC 43256). A 10-µl drop of each nisin-containing release sample was placed onto the inoculated MOX agar plates. All plates were incubated at 37ºC for 48 h and zones of inhibition were measure in millimeters using a digital caliper (control company, China) in both horizontal and vertical directions and averaged. The experiment was replicated three times and reported as zones of inhibition (mm).

*Culture storage and preparation for challenge study*

*Listeria monocytogenes* strains: 15313, 43256, 7647, 13932, and Scott A were obtained from ATCC (Info here). These five strains were selected because they were considered to have real world application as they were obtained from food and/or human samples from actual outbreaks of listeriosis. The cultures were grown aerobically in Brain Heart Infusion (BHI) broth at 37°C for 24 h (while shaking). The cultures were stored at -80ºC in TSB and glycerol as stock cultures until needed for experiments. To prepare pathogen cultures for experiments, 100 uL volumes from frozen stock cultures were transferred to 10 mL of fresh BHI at 37°C for 24 h. The cultures were later transferred to Modified Oxford (MOX) plates (EMD Chemical Inc.), struck to isolation, and incubated at 37°C for 24 h. Finally, a single colony of each culture was taken from the MOX plate and transferred to a separate 500 mL bottle of BHI and allowed to grow statically for 24 h at 37°C to obtain a cell concentration of ~9 $\log_{10}$ CFU/mL. This transfer process was done in triplicate for each pathogen. The five pathogen cultures (10 mL each) were mixed in equal volumes to prepare a non-diluted cocktail for each

experiment. Stock cultures were struck on Trypticase soy agar (TSA) slants, stored at

4ºC, and properly maintained by re-streaking on fresh TSA every 10 days.

*Media and pathogen analyses for challenge study*

Modified Oxford medium was used as selective media for *L. monocytogenes*

enumeration and isolation following incubation for 48 h at 37°C. TSA (BD-Difco,

Detroit, MI) media was used as a non-selective media for background microflora

enumeration following incubation for 24-48 h at 30°C.

*Cocktail preparation for challenge study*

The cocktail preparation was made by using *Listeria monocytogenes* strains:

15313, 4698, 7647, 13932, and Scott A. After aerobic growth, 25mL of each *L.*

*monocytogenes* strains were centrifuged and the supernatants were discarded. The pellets

were re-suspended in 25mL Buffered Peptone Water (HiMedia Laboratories, India).

Then, 10mL of each of the *L. monocytogenes* strains were combined to yield a 50mL,

multi-strain cocktail.

*Challenge Study*

Turkey bologna (Oscar Mayer brand) was surface-inoculated with 100 μl of a $10^7$

CFU/ml suspension of *L. monocytogenes* cocktail. Three pieces of the meat were

randomly selected, aseptically placed in stomacher bags, weighed, diluted 10 times the

volume of the meat (25 g per slice) with Buffered Peptone Water (BPW) and stomached

for 2 min at 230 rpm (Stomacher 400; Seward, England). Samples were serially diluted in

BPW, and 0.1mL of each sample was spread plated in duplicate onto MOX plates. The

plates were incubated for 48 h at 37°C to determine initial inoculum level. The remaining

inoculated turkey bologna was placed, inoculated side of the meat in contact with coated substrates (4x4 in$^2$), inside a Low Density Polyethylene pouch. All pouches were vacuum packaged and stored at refrigeration (4-7°C) temperature. The study duration was 63 days with sampling taking place on days 0,1,2,7,14,21,28,35,42,49,56 and 63. Each sampling day, ten packages (two controls, two PA, two WA, two PB and two WB) were sampled in the same manner as described above. Three pieces of the un-inoculated turkey bologna were randomly selected, aseptically placed in stomacher bags, weighed, diluted 10 times the volume of the meat (25 g per slice) with Buffered Peptone Water (BPW) and stomached for 2 min at 230 rpm (Stomacher 400; Seward, England). Samples were serially diluted in BPW, and 0.1mL of each sample was spread plated in duplicate onto TSA plates to enumerate the background microflora naturally present on the meat.

*Statistical analyses.*

All coating solution parameters (pH, density, percent solids and viscosity methods) were conducted for each property in three replicates. Tables 2 and 3 demonstrate the averages of the results. The averages and standard deviations were calculated using Microsoft® Excel 2011 Version 14.1.2.  Statistical differences (P<0.05) were analyzed using 1-way analysis of variance (ANOVA), and Tukey's test (SAS, Cary, N.C., USA) was used to compare the three coating treatments (A, B, C). This test was completed separately for pH, density, percent solids and viscosity.

The properties that were measured on the dry coated substrates (basis weight, transmittance, haze, clarity and thickness) were conducted only on substrate "P". Ten samples were measured for each treatment.  Three measurements were taken per sample

and averaged (Table 4). The averages and standard deviations were calculated using Microsoft® Excel 2011 Version 14.1.2. Figure 2 shows photos of the inhibition achieved using the film on lawn method. Statistical differences ($P<0.05$) were analyzed using 1-way analysis of variance (ANOVA), and Tukey's test (SAS, Cary, N.C., USA) was used to compare the three coating treatments (A, B, C) on "P" for basis weight, transmittance, haze, clarity and thickness.

The log of nisin concentrations was plotted against the average diameter of inhibition zone to generate a nisin standard curve. A standard curve was generated for both spot on lawn and agar well diffusion methods. Using the standard curve's "equation of the line" unknown nisin concentrations were calculated for each assay. R-squared values were all greater than 0.97, where X is log units of nisin and Y is the diameter of inhibition. Statistical differences ($P<0.05$) were analyzed using true repeated measures analysis of variance (GLM) (SAS, Cary, N.C., USA) to compare the coating treatments (PA, PB, WA, WB, C). Table 5 compares methods (agar well diffusion vs. spot on lawn) for the evaluation of release of nisin from the films.

For challenge study statistical differences, ($P<0.05$) were analyzed using 1-way analysis of variance (ANOVA), and Tukey's test (SAS, Cary, N.C., USA) was used to compare the five coating treatments (PA, WA, PB, WB and C) over the 63 days shelf life.

*Results*

Tests were conducted on properties of coating solutions and dry coated substrates. Table 2 demonstrates the results averaged for the coating solution parameters (pH, density and percent solids. Table 3 compares the average viscosity results obtained for

the coating solution using three different methods (Brookfield viscometer, Zahn cup and

Bostwick consistency meter). Table 4 summarizes the averaged data measured on the dry

coated substrates (basis weight, transmittance, haze, clarity and thickness), which were

conducted only on substrate "P". Figure 2 consists of images of the inhibition achieved

by the film on lawn method on both substrates "P" and "W". Table 5 compares two

methods (agar well diffusion and spot on lawn) for the evaluation of the release of nisin

from the substrates. Challenge study results for reduction of *L. monocytogenes* over the

63 days testing period can be found in Figure 3.

*Discussion*

While nisin has been used for many years as an antimicrobial food preservative,

there has been no large-scale commercial implementation into packaging. Nisin is a

bacteriocin of choice because it is relatively heat stable (Le Blay, G., Lacroix, C., Zihler,

A., Fliss,I. 2007) and is highly efficacious against gram-positive bacteria (Jin, T., and

Zhang,H. 2008), which makes it a good specimen for potential commercialization.

However, as the literature indicates, while progress is being made towards the

commercialization of nisin based films, there is still a gap between what can be done in

academia (or research laboratories) and what is able and willing to be done by current

packaging firms. A coating would be a relatively easy way to establish a commercialized

nisin packaging.

When developing a coating, the properties of the coating should be considered

during its development. Physical properties, such as viscosity, need to be known. The

viscosity of a coating can be measured in several ways. Using a Brookfield viscometer is

a typical method of measuring centipoise and torque in laboratories, while a Zahn cup or Bostwick Consistency Meter are generally utilized in commercial settings (Rolin, de Vries 1990). The typical commercial range for viscosity of a coating is 35-200 centipoise (Argent et al. 1999). A Zahn cup reading that is accurate is in the range from 20-40 seconds (Argent et al. 1999). Most commercial coatings use Zahn cup #2 and #3. By measuring viscosity using all three methods (Brookfield viscometer, Zahn cup and Bostwick), the data collected will be useful for both research laboratories and industry. Future commercialization will require the formulation to be "scaled up" in order to be run on commercial equipment where viscosity testing measure such and Bostwick and Zahn cup will be the standard as opposed to the laboratory Brookfield viscometer method.

Time, temperature and volume all affect coating viscosity. As such, these values should be reported in addition to viscosity values (Oil and Colour Chemists Association, Australia (OCCA) 1984). Other important physical properties of the coating that should be noted include corrosion, color and flexibility. A coating with low pH can cause corrosion of costly coating equipment, and depending on the use of the coating, use of color may be unattractive to the consumer. Percent solids should be measured as well. A typical commercial coating has percent solids between 15-40% (Argent et al. 1999); therefore, percent solids of the coating should be in this range to run on current commercial equipment (Gilleo 2006). Finally, the flexibility is important for storage, shipping and use. Coatings should be flexible enough not to break off the substrate when handled.

All coating solution properties (density, percent solids, Zahn viscosity), for all treatments (A, B and C) were significantly different. Bostwick consistency meter, Brookfield viscometer and pH of coating A and coating C were not significantly different from one another, but were both significantly different from coating B. This was expected, as the addition of calcium chloride in coating B has an effect on the viscosity of the coating.  (Marathe, 2008) determined the addition of the nisin and the calcium could be added together or in any order without significant different behaviors for the coating. In the presence of calcium, pectin forms a gel or "egg box model" structure. Calcium chloride also adjusts the pH significantly, as it has a more alkaline pH. Both percent solids and viscosity of all the coatings fell in the acceptable commercial range, which would aid in future commercialization.

For dried coating properties: basis weight coating PA and coating PC were not significantly different from one another, but were both significantly different from coating PB. This may be explained by the differences in density and viscosity of the coatings. As expected, there were no significant differences in thickness between treatments (PA, PB, PC), indicating that the coatings were all evenly coated. There were no significant differences in transmittance amongst the three coatings; however, haze for treatments (PA, PB and PC) were all significantly different. For clarity coating B, coating A was significantly different from coating C; but coating B was not significantly different from coating A or coating C. It is important to note that to the naked eye all treatments were completely transparent. Film on lawn studies demonstrated that coatings A and B were antimicrobial compared to the control (Figure 2). It can also be observed that

coating A appears to have faster release than coating B, resulting in larger inhibition zones. It was difficult to see through the wax paper, "W" substrate, and so agar well diffusion studies were necessary.

Overall, the nisin released from the coated substrates containing 10,000IU/mL was effective in controlling the growth of *L. monocytogenes* (Table 5). As expected, control coatings (PC, WC), containing no nisin, did not produce zones of inhibition regardless of method or substrate coated. The nisin from the coatings (A and B) was released into 5mL of acidified water (20% Acetic Acid), which diluted the actual concentration of nisin in the coating by 5 fold. Table 5 demonstrates that nisin concentration multiplied by a factor of 5 to account for this dilution. Previous research demonstrates with low levels of nisin, the effectiveness can be reduced when diluted (Grower, J.L., Cooksey, K., Getty,K. 2004b).

. There is a significant difference in the nisin leaving the films when comparing diffusion methods (agar well diffusion vs. spot on lawn); however, the research determined that the agar well diffusion is the more reliable method, which is in agreement with the fact that it is the gold standard methodology most often reported in the literature. The agar well diffusion standard curve also had a higher R-squared value compared to standard curve of spot on lawn. Both methods (spot on lawn and agar well diffusion) showed significant differences between the various treatments PA, PB, WA, WB compared to the control over time, indicating a reduction of *L. monocytogenes* compared to the control coating for all coatings containing nisin. Either substrate (P or W) or coating treatment (A or B) is capable in causing reduction of *L. monocytogenes*.

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The newly developed coating did not show the expected slow release with the addition of the calcium chloride solution. Higher concentrations of $CaCl_2$ were not optimal for commercial coating parameters because viscosity was too high and the coating solution did not flow easily. The pH of the coating was designed to be less than the pka of the pectin (3.95) in order to achieve a higher percentage release of the nisin (Marathe 2008). The low methyl ester pectin used in the coatings was chosen for its high reactivity to calcium in order to optimize the slow release of nisin. Despite utilizing previous research findings, the percentage of release of nisin leaving the pectin coating remained very low.

The food challenge study was completed in triplicate. The average of the three replicate challenge studies are shown in Figure 3. As expected, all treatments (PA, WA, PB and WB) were statistically different from treatment C (control) in the reduction of *L. monocytogenes*. Statistical differences did not exist between treatments PA, WA, PB and WB (Figure 3). Therefore the addition of calcium chloride could be removed for cost reduction and ease of production. The cost of coating C is $0.005 cents, A is $0.0078 cents and the cost of coating B is $0.0114 cents. In addition, both substrates were successfully coated and achieved reduction of *L. monocytogenes*. Both substrates could be considered for future commercialization, with the lesser-cost substrate going into production.

The inoculated population of *L. monocytogenes* remained fairly constant over the time course of the study. *L. monocytogenes* is still able to grow at refrigeration temperature; therefore, a slight increase in population was expected. The background microflora was suppressed by the inoculation and did not increase over the time course of

the study (data not shown). The meat was not sterilized prior to the study in order to mimic a real world, worst-case scenario. Control meat was also sampled throughout the study in order to measure the impact of background microflora on inoculated organisms (Ceylan 2007).

## Conclusion

Findings from this research suggest that the developed coatings demonstrated antimicrobial activity against *L. monocytogenes*. The developed coatings were food safe, colorless and within the commercial range for viscosity and percent solids. Both coating treatments A and B were able to inhibit *L. monocytogenes* for the entire time course of the turkey bologna food challenge study, and were significantly different compared to controls. The addition of calcium chloride was not as beneficial as hypothesized, which was potentially due to the fact that slow release was not needed in the viscous pectin coating.

## Acknowledgements

**Table 3.1. Summary of Coding for treatments.**

| Codes (1st Letter) | Substrates | Codes (2nd Letter) | Coating |
|---|---|---|---|
| P | Sealed Air laminate coextruded forming web with a polypropylene skin, a plastomer sealant and EVOH barrier | C | Control: No Nisin or Calcium Chloride |
| W | A wax parchment paper from Ingles | A | Coating with nisin |
| | | B | Coating with nisin and Calcium Chloride |

**Table 3.2. Average pH, density and percent solids of coating solutions (A, B and C).**

| Method | Treatment | Average Result | Standard Deviation |
|---|---|---|---|
| Density (g/cm3) | A | 2.07[a] | 0.001 |
| | B | 2.06[b] | 0.0005 |
| | C | 2.06[c] | 0.001 |
| Percent Solids | A | 29.01[b] | 0.11 |
| | B | 29.78[a] | 0.15 |
| | C | 28.59[c] | 0.06 |
| pH | A | 2.50[b] | 0.01 |
| | B | 2.55[a] | 0.02 |
| | C | 2.50[b] | 0.01 |

[a]Treatment A: coating C + nisin added
[b]Treatment B: coating C + nisin and $CaCl_2$ added
[c]Treatment C: control coating
[d]a-c denote statistical differences (P<0.05) for between treatments for each property

**Table 3.3. Average viscosity results measured by Bostwick, Zahn cup and Brookfield viscometer for coating solutions (A, B and C).**

| Method | Treatment | Average Result | | Standard Deviation | |
|---|---|---|---|---|---|
| Bostwick time 30sec | A | 18.17 cm[a] | | 0.29 | |
| | B | 7.50 cm[b] | | 0.50 | |
| | C | 19.00 cm[a] | | 0.00 | |
| Zahn Cup #2 | A | 27.27 sec[a] | | 0.209 | |
| | B | N/M | | N/M | |
| | C | 28.43 sec[b] | | 0.081 | |
| Brookfield Time 90sec, Temp:45ºC, Volume: 80mL | A -Used Spindle #2 RMP: 60% | 95.85 CP (mPa*s)[a] | 30.43 torque | 0.15 | 0.08 |
| | B- Used Spindle #1 RPM 12% | 66.5 CP (mPa*s)[b] | 16.33 torque | 0.30 | 0.25 |
| | C-Used Spindle #2 RMP: 60% | 112.65 (mPa*s)[c] | 36.17 torque | 0.90 | 0.85 |

[a]Treatment A: coating C + nisin added
[b]Treatment B: coating C + nisin and CaCl$_2$ added
[c]Treatment C: control coating
[d]a-c denote statistical differences (P<0.05) between treatment for each viscosity measurement.

**Table 3.4. Dry coated film average properties (basis weight, thickness, transmittance, haze and clarity) for coating A, B and C.**

| Method | Treatment | Average Result | Standard Deviation |
|---|---|---|---|
| Basis Weight (g/m2) | A | 422.59[b] | 0.00 |
| | B | 578.94[a] | 0.01 |
| | C | 415.60[b] | 0.00 |
| Thickness (mm) | A | 2.61[a] | 0.10 |
| | B | 2.79[a] | 0.17 |
| | C | 2.76[a] | 0.17 |
| Transmittance | A | 90.99[a] | 0.10 |
| | B | 90.96[a] | 0.05 |
| | C | 91.08[a] | 0.48 |
| Haze | A | 8.63[b] | 2.32 |
| | B | 12.41[a] | 0.59 |
| | C | 4.62[c] | 3.11 |
| Clarity | A | 92.81[a] | 0.38 |
| | B | 91.90[ab] | 0.69 |
| | C | 90.76[b] | 1.68 |

[a]Treatment A: coating C + nisin added
[b]Treatment B: coating C + nisin and $CaCl_2$ added
[c]Treatment C: control coating
[d]a-c denote statistical differences (P<0.05) between treatment for each property measurement.

**Table 3.5. Average nisin leaving the films overtime measured by agar well diffusion and spot on lawn, accounting for the acetic acid dilution.**

| Nisin Leaving Films (IU/mL) over time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Method | Treatment | 30 minutes | 5 hours | 10 hours | 24 hours | 48 hours | 72 hours | 7 days |
| Agar Well Diffusion | PA | 0 | 0 | 850.73 | 1245.8 | 1252.2 | 1245.8 | 2617.83 |
| | WA | 0 | 0 | 0 | 1075 | 1365.3 | 1075 | 1075 |
| | PB | 0 | 1297.55 | 2153.5 | 2513.45 | 2591.34 | 2413.34 | 1699 |
| | WB | 0 | 0 | 0 | 0 | 1925.1 | 1451.2 | 1699 |
| | PC | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | WC | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Spot on Lawn | PA | 0 | 0 | 19.25.1 | 2100.1 | 2291 | 2291 | 2291 |
| | WA | 0 | 0 | 0 | 1559.6 | 1617.6 | 2291 | 2291 |
| | PB | 0 | 1246 | 1617.6 | 1674.9 | 1925.1 | 2100.1 | 2291 |
| | WB | 0 | 0 | 0 | 0 | 1925.1 | 2100.1 | 2291 |
| | PC | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | WC | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Treatment PA: coating C + nisin added on polymer
[b]Treatment PB: coating C + nisin and $CaCl_2$ added on polymer
[c]Treatment PC: control coating on polymer
[d]Treatment WA: coating C + nisin added on wax paper
[e]Treatment WB: coating C + nisin and $CaCl_2$ added on wax paper
[f]Treatment WC: control coating on wax paper
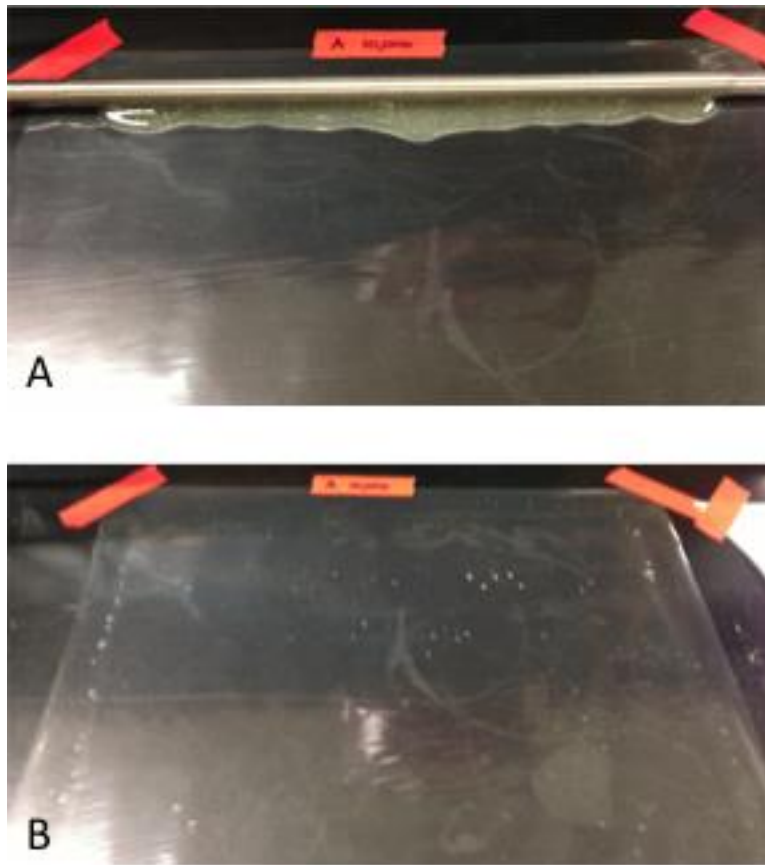
**Figure 3.1. A. Coating by Draw down method B. Coated Film Drying for 24 hours**
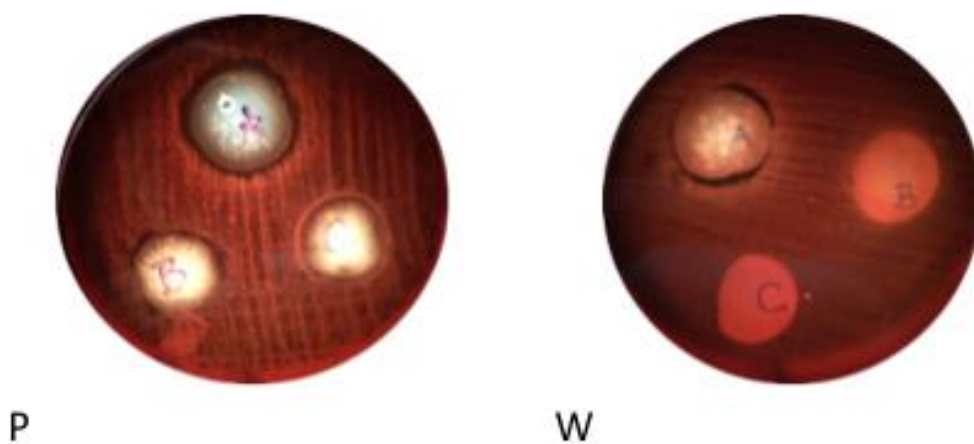
**Figure 3.2. Film on lawn of coatings A, B and C coated on substrate "P".**

[a] Treatment A: coating C + nisin added
[b] Treatment B: coating C + nisin and $CaCl_2$ added
[c] Treatment C: control coating
[d] Coated on Sealed Air Corporation Polymer: "P"
[e] Coated on Wax paper: "W"
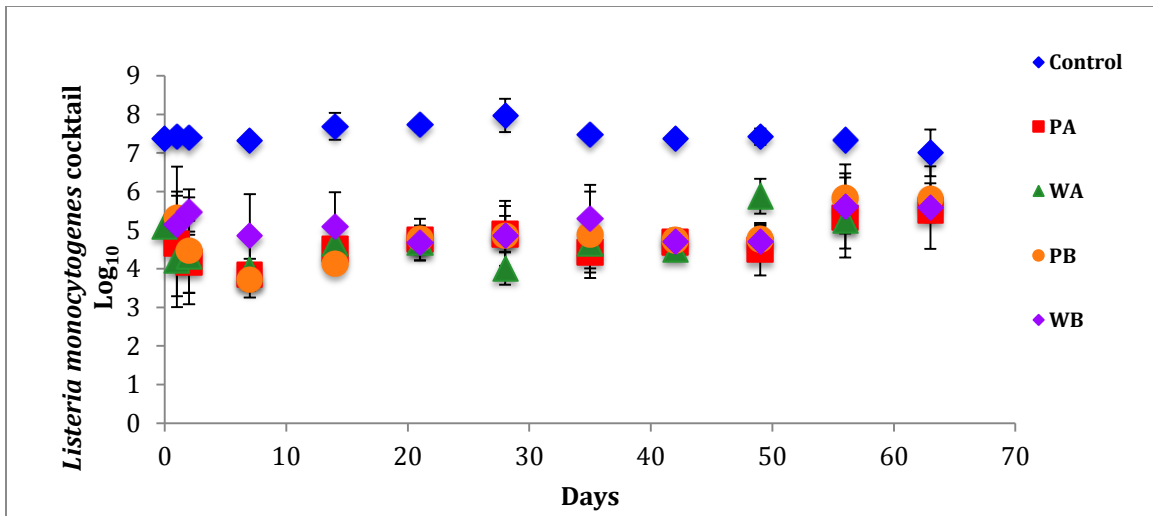
**Figure 3.3. Challenge Study on Ready-to-eat Turkey Bologna**

[a]Control : Average of Wax paper and Plastic controls
[b]PA: Plastic substrate, Treatment A
[c]WA: Waxpaper substrate, Treatment A
[d]PB: Plastic substrate, Treatment B
[e]WB: Waxpaper substrate, Treatment B
[f]Treatment A: coating C + nisin added
[g]Treatment B: coating C + nisin and $CaCl_2$ added
[h]Treatment C: control coating

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# CHAPTER IV

# MEASURING RELEASE OF NISIN FROM PECTIN COATING INTO READY-TO-EAT TURKEY DURING STORAGE

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**Abstract**

*Listeria monocytogenes* should be controlled by continuing good manufacturing methods, continued proper handling by food workers, and necessary additional secondary safety measure. An example of an additional secondary method is an antimicrobial coating. However, current research for antimicrobial coatings is in preliminary stages and is primarily based on theoretical lab scale testing. There is a need diffusion studies for antimicrobial coatings. It is necessary to understand the safety and potential success for antimicrobial coatings to consider being commercialized. An antimicrobial coating containing nisin was developed using common commercial methods and studied. Diffusion was successfully measured by agar well diffusion method. It was determined that approximately 50% of the nisin remained entrapped in the pectin coating. Microscopy was examined as a new method for tracking nisin diffusion in the food and films and found to be useful. Finally, HPLC results demonstrated that the nisin might

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have been breaking down when in contact with the turkey bologna. The research is necessary in order to fill the gap in the literature to produce a future commercial coating.

## Introduction

Since its discovery, nisin has proven to be an effective inhibitor of gram-positive bacteria, such as *Listeria monocytogenes* (Bower, C.K., McGuire, J., Daeschel,M.A. 1995) and is now approved for use in 57 countries around the world and has been affirmed as generally recognized as safe (GRAS) in the United States (Jin, T., Liu, L., Zhang., H., Hicks.,K. 2009). Nisin is considered nontoxic to humans because it is inactivated by proteolytic enzymes in the digestive tract, and assumed to be safe for use as a food preservative (Bower, C.K., McGuire, J., Daeschel,M.A. 1995). (Barrett, Woessner & Rawlings 2004) describes proteolytic enzymes as any group of enzymes that break down protein chains into amino acids.

Because of its non-toxic qualities, being heat stable, in that it does not create off-flavors, nisin is used in a variety of commercially produced foods, including dairy products, meats, fish, and eggs (Le Blay, G., Lacroix, C., Zihler, A., Fliss,I. 2007, Schillinger, U., Geisen, R., Holzapfel,W.H. 1996). Given the abundance of legal antimicrobials, utilization in food is dependent on a variety of factors, including the properties of the food, the type of preservation system being used, the characteristic of the microorganism that is being contested and the cost effectiveness of the antimicrobial (Branen 1983). An estimated 20% of the world's food supply is lost due to microbial spoilage and antimicrobials are needed to ensure proper food supply is maintained (Branen 1983, Fulton 1981). Packaging films containing antimicrobial agents have shown

improved efficacy by a controlled migration of the compound into the food, allowing for initial inhibition of undesirable microorganisms, and subsequent residual activity over the course of the distribution stage of the food cycle (Quinatavalla, Vicini 2002).

Current US regulations dictate zero tolerance policy for *L. monocytogenes* in Ready-to-eat foods (Food Safety and Inspection Service (FSIS) 2000). In order to combat this ever-present menace, *L. monocytogenes* should be controlled by continuing good manufacturing methods, continued proper handling by food workers, and necessary additional secondary safety measure such as antimicrobial coating. However, current research for antimicrobial coatings is in preliminary stages and is primarily based on theoretical lab scale testing. There is a need diffusion studies for antimicrobial coatings. It is necessary to understand the safety and potential success for antimicrobial coatings to consider being commercialized.

Pectin is an ideal carrier for the antimicrobial nisin in a food safe coating for ready-to-eat (RTE) meats. Pectin is a soluble dietary fiber that binds cholesterol and slows glucose absorption during digestion (Brown et al. 1999). It is safe for human consumption as it is currently part of daily diets and is GRAS approved (United States Food and Drug Administration (FDA) 2013b). It has been used for many years in the food and beverage industry as a thickening agent, a gelling agent and a colloidal stabilizer, respectively (Allwyn et al. 2012). In coatings, pectin is the carrier agent. Pectin is an interesting candidate pharmaceutical use, specifically for its utilization in controlled drug delivery / controlled release. (Sriamornsak, Nunthanid 1998) modified drug release

patterns by utilizing a pectin/calcium delivery system; there was a delayed release of the drug in the colon.

High methyl ester pectins (degree of methyl esterification above 50) form gels in the presence of low pH combined with low water activity (such as the addition of sugar). Gelation for low-ester pectin relies on calcium being added to the mixture. (Grant et al. 1973) was the first to coin the term "egg-box model" in order to describe the gelation of pectin and other alginates. LM pectin is ideal for slow release antimicrobial coatings when it is combined with calcium.

The objectives of this study were thus to (1) identify the concentration of nisin leaving the film by agar well diffusion method to (2) determine whether microscopy methods could enhance the understanding of diffusion of nisin and to (3) measure diffusion of nisin from coating using high performance liquid chromatography (HPLC).

**Materials and Methods**

*Coating Preparation*

Table 1 summarizes the two substrates used plastic (P) and wax paper (WP) with three types of coatings (C, A, B) for a total of six treatments (PC, WC, PA, WA, PB, WB). Sealed Air Corporation donated substrate (P). It is a Sealed Air laminate coextruded forming web with a polypropylene skin, a plastomer sealant and ethylene vinyl alcohol barrier was used for the plastic treatments (PA, PB, PC). This substrate is a low sealing material with enhanced sealability through brines, marinates and sauces. The sealing range is 105-180ºC. Substrate (W) is a wax parchment paper from Ingles Markets,

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SC. It was used for the wax paper treatments (WA, WB, WC). Substrates were ultraviolet light (UV) treated for 15minutes before being coated.

*Coating Preparation*

The coating was prepared semi-commercially according to section 3.1. The formulation is the same with various nisin concentrations ranging from 10,000-80,000 for initial agar well diffusion studies. Microscopy and high-pressure liquid chromatography (HPLC) release studies contained 60,000IU/g of nisin.

*Standard assay curve of nisin*

The standard assay curve of nisin A solution was obtained by the method adapted by  (Tramer, J., and Fowler,G.G. 1964). The effect of different nisin concentrations and the minimum inhibitory concentration (MIC) of nisin was determined with a stock solution and serial dilutions ranging from 80,000IU/mL to 78 IU/mL. A stock solution was obtained by adding 0.05g of Nisin A into 100mL of acidified water (0.02N Acetic Acid).  From the stock solutions 80,000, 40000, 20,000, 10,000, 5,000, 2,500, 1,250, 625, 313, 156, 78 IU/mL were made. Acidified water (20% Acetic Acid) was used as a negative control.

*Estimation of nisin in films*

Coated films containing 10,000IU/mL, 20,000IU/mL, 40,00IU/mL and 80,000IU/mL of nisin (wet concentration) each analyzed separately. After 24 h of drying were cut into 4 x 4 square inches. The squares were placed in sterile polyethylene bags and 5mL of sterilized acidified water (20% Acetic Acid) was added. The bags were sealed with an impulse sealer Model FS-400 (Hualian, China) and placed on a Gyrotory®

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water bath shaker (Edison, NJ) at 4ºC. Portions of 500ul of nisin- containing release samples were collected into test tubes after the stipulated release time and immediately stored at 4ºC until assay. The times of collection were 30 minutes, 5 h, 10 h, 24 h, 48 h, 72 h, and 7 days. Total removal of the coating was completed using 50% ethanol solution (See extraction of nisin from coating section, for full method).

*Agar well diffusion assay*

The agar well diffusion method adapted by (Barefoot, Klaenhammer 1983) was used to detect the activity of nisin against *L. monocytogenes* ATCC 4325. Modified Oxford (MOX) agar plates were overlaid with ~8mL of semisoft TSB agar (0.5% w/v agar) seeded with 1% of broth culture of *L. monocytogenes* ATCC 4325. The seed density was approximately $1x\ 10^6$ cfm ml$^{-1}$ of overlay. Plates were allowed to harden for 1 h before wells were made. 50-ul wells were made using a bore. Then 50-ul of nisin- containing release samples were placed in each well. Plates were stored at 4ºC for 24 h and then incubated at 37ºC.

*Microscopy*

Coated substrates "W" and "P" were coated cut and super-glued to fit a microscope slide. A total of 12 coating microscope slides were prepared by using the following treatments in duplicate: PA, WA, PB, WB, PC and WC (Figure 1). Ready-to-eat Turkey (Ingles, SC) was also cut and glued to a different set of 12 microscope slides (Figure 2). The super glue was necessary to hold the substrates and turkey meat in place over sampling time period of 63 days.  Each slide containing coating and turkey was imaged on day 0 and used as a control. Then each turkey slide was placed ontop of a

coating slide so the coating was in contact with the turkey and vacuum packaged. The slides were then separated and imaged on days 1, 2, 7, 14, 21, 28, 35, 42, 49, 56 and 63. After each imaging session the slides were placed by together, vacuum packaged and storage at refrigeration. Nisin A is naturally inherently fluorescent (Figure 2) and the objective was to track the nisin leaving the film and entering the turkey meat.  Figure 3 demonstrates the fluorescence of nisin A in the coated film. Images of the coated film and meat were obtained using a Nikon Eclipse Ti Microscope (Nikon Instruments, Melville, NY) in wide field fluorescence mode. A 10x dry objective (10X Plan Apo; IM=air; NA=0.45; WD=4mm), and a Nikon GFP filter cube (C-FL GFP HC HISN; Exciter= 450-490 nm; Dichroic= 495 LP; Emitter = 500-550), were used with a camera exposure time of 50 msec (Photometrics Cool-Snap HQ2, Photometrics, Tucson, AZ). NIS-Elements AR version 3.0 (Nikon Instruments, Melville, NY) was used to analyze the images.

*Extraction of Nisin from Coating*

The turkey bologna (Ingles, SC) was vacuum packaged in contact with the 4 x4 square coated films (PA, WA, PB, WB, PC, WC) and stored in the dark at refrigeration temperature. Each sampling day 0, 1, 2, 7, 14, 21, 28, 35, 42, 49, 56 and 63 three of treatment were sampled. The meat was separated from the coated film. The coating was removed from the substrate by using a razor blade and 50% ethanol. The removed coating was combined in a test tube containing three coatings for each treatment. Method of isolating the nisin was modified from (Xiao et al 2010). The solution was stirred, centrifuged and the supernatant was stored at 4ºC with foil to prevent light until analyzed

by high-performance liquid chromotography. In addition, the pellet was rehydrated and analyzed for remaining nisin after extraction (data not shown).

*High performance liquid chromatography*

High-performance liquid chromatography (HPLC) was carried out on a Water HPLC system with a UV/VIS detector and a Waters XBRIDGE column (inside diameter, 4.6 mm; length 250 mm; packed with 5-um beads) Method was modified from Liu and Hansen, 1990. A gradient elution with water- acetonitrile gradients (0.9 ml/min) containing 0.1% TFA were used. The gradient was from 50 to 100% acetonitrile over 40 minutes. Peaks were monitored at 254 nm, and quantitated by total area obtained by integration.

*Statistics*

The log of nisin concentration was plotted against the average diameter of inhibition zone to generate a nisin standard curve (See Figure 4). Unknown nisin concentrations were estimated using the equation generated for each assay. The R-squared value was 0.979. Where X is log units of nisin and Y is the diameter of inhibition.

The agar well diffusion experiments were replicated three times. Plates were evaluated for zones of inhibition in millimeters with a digital caliper (Control company, China) in both horizontal and vertical directions and averaged. From the standard curve (Figure 4) the equation of a line was used to determine the nisin concentration leaving the films (Table 2). The calculation were completed in Microsoft® Excel 2011 Version 14.1.2. Statistical differences (P<0.05) were analyzed using true repeated measures

analysis of variance (GLM)  (SAS, Cary, N.C., USA) was completed to compare the four

coating treatments (PA, WA, PB and WB) and difference of concentration of nisin A.

Microscopy images were taken in duplicate. The statistical method region of

interest (ROI) was used to analyze the same area on every image taken. This helped

removed sampling bias. Then the mean fluorescence intensity was calculated and

averaged for each treatment on each day. Finally the control fluorescence was subtracted

on each day for both coating and meat.  Statistical differences ($P<0.05$) were analyzed

using 1-way analysis of variance (Anova), Tukey's test (SAS, Cary, N.C., USA) was

completed to compare the four coating treatments (PA, WA, PB and WB) and difference

of fluorescence intensity by microscopy overtime. In addition, same test was completed

for the meat fluorescence intensity between the four treatments overtime.

### Results

The method agar well diffusion can be used to estimate the amount of nisin

leaving the coated substrates. Using the standard curve for agar well diffusion, which can

be found in Figure 4, the amount of nisin releasing from the coated substrates was

estimated. Table 2 summarizes the nisin concentration leaving the coated substrates (by

agar well diffusion method) with four different nisin concentrations (10,000, 20,000,

40,000 and 80,000).  Another method of measuring diffusion of nisin is HPLC. The

coated substrates were placed in contact with turkey meat to measure diffusion from

coated substrates to food. Table 3 summarizes the diffusion of nisin leaving the coated

substrates and entering the turkey (measured by HPLC) over the first 48 hours.

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Microscopy can be used to visualize the diffusion of nisin from the coated substrate to the food. Figure 3A shows nisin fluorescing in the coated substrates (10x magnification) day 0 (before in contact with the meat). Figure 3B then shows an image take after the coated substrate was in contact with the turkey for 63 days (10x magnification). Images were taken over the entire 63 days shelf life of the turkey meat with the coated substrates vacuum packaged to the turkey meat. The data is summarized in Figure 5 of the nisin leaving the film over 63 days measured by reduction in fluorescence intensity. Figure 6 then demonstrates the nisin entering the meat over 63 days measured by fluorescence intensity.

## Discussion

There is a demand for food safe commercial antimicrobial coatings. However, diffusion studies are necessary to determine the levels of antimicrobials that are effective and concentrations of the antimicrobial entering the food system before commercialization can occur. Carriers such as pectin can aid in the delivery of antimicrobials, by providing viscosity and percent solids in order for the solution to coat the packages surface.

The researchers first objective was to determine the concentration of the nisin leaving the pectin coatings by agar well diffusion method. A standard curve seen in Figure 4 allowed the researchers to estimate the nisin concentration leaving the coated substrates. The results demonstrated that despite high levels of nisin being added to the coating solution low levels of nisin was releasing from the substrates after 7 days. Meaning a higher concentration of nisin may be incorporated into the pectin coating

formulation and the diffusion of nisin from the substrates would still be within the legal limit. Approximately half of the nisin added to the coated substrates was measured leaving the substrates (Table 2). Therefore, if the nisin added in the coating formulation were double the legal limit (20,000IU/g) the release of nisin from the substrates into the food would be equal or less than legal maximum (10,000IU/g). The remaining nisin is hypothesized to be trapped in the pectin coating and unable to diffuse or breaking down and unable to be measured.

The second objective was to determine if microscopy could assist in the understanding of how the nisin diffused from the coated substrate into the food (turkey). The research was able to detect the nisin in the coated substrates and in the food by using fluorescence microscopy. Figure 2 demonstrates the auto-fluorescence of nisin A (95% purity). Although the meat and pectin displayed auto-fluorescent properties, the nisin A demonstrated the strongest auto-fluorescence. This allowed the background auto-fluorescence to be subtracted using controls and only evaluate the nisin. Fluorescence microscopy was useful in identifying the dispersion of the nisin through the coated substrates. In Figure 3, it is obvious that the nisin is present and dispersed throughout the coating.

Microscopy can be used to measure nisin diffusion as a non-quantitative measure. Figure 5 demonstrates the Nisin A leaving the coating by the reduction in fluorescence over time. Figure 6 demonstrates an increase in fluorescence intensity over time due to the diffusion on the Nisin A into the turkey. After 28 days, the diffusion was difficult to measure because the turkey began to attach to the coated film and was hard to sample.

Although statistical results may show statistical significance between treatments (PA, WA, PB and WB) and the fluorescence intensity over time, no practical significance was observed. There was not a significant difference between meat fluorescence intensity and coating treatment in contact with the meat.

Objective 3 was to measure diffusion of nisin from the coating using HPLC. This objective was attained but had limitations. The researchers were able to isolate nisin and develop a standard curve. However, the researcher's collected HPLC data over the entire 63-day shelf life but after analysis discovered the nisin had a conformational between day 2 and day 7. This resulted in the remaining sampling days 7,14, 21, 28, 35, 42, 49, 56 and 63 having values that did not correspond with the original nisin A standard curve. This data was discarded. The data from the first 48 hours was used.

The HPLC peak area data was averaged and, using the standard curve equation of a line, concentration was calculated (See Figure 7). Also, the dilution during the extraction process was accounted for and adjusted. The control substrates and blank (50% ethanol) did not show any peak when using the same HPLC method. The concentration calculated was the amount of nisin left in the substrate after storage (in contact with the meat). As the nisin left the substrates it was assumed to be diffusing into the meat see Table 3.

HPLC demonstrated similar results to agar well diffusion and microscopy images in the diffusion concentration of nisin A leaving the pectin coating. Approximately half the nisin A added to the coating was released after two days. Table 3 demonstrates the release of nisin from the coating and therefore theoretically entering the turkey bologna

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for the first 2 days of storage. The nisin changes confirmation when stored with turkey bologna, somewhere between day 2 and day 7. After day 7, the area of the peak was unable to be analyzed since it broke down into smaller peaks. Future research should investigate this change and whether it effects reduction in antimicrobial activity. The loss of nisin was first observed by Hirsch et al. (1951). Enzymes capable of degrading nisin include pancreatin, α-chymotrypsin, and subtilopeptidase inactivate (Xiao 2010, Heinemann, Williams 1966, Jarvis, Mahoney 1969).

According to (Marathe 2008) pectin shows better dissolving capability at low pH or below the pka of pectin. In this study the pH of the pectin coating was approximately 2.5, which is below the pka of the pectin (3.95) this allowed for the better release of nisin. The Marathe study concluded that if pH was above the pka, then there was not release of nisin. Even applying the knowledge from previous research the percentage of release of nisin leaving the pectin coating remained very low.

Viscosity can be reduced under low pH conditions. Viscosity increases with increasing pectin solution. Pectin solutions are viscous, but pectin is not particularly efficient as a thickener compound compared to other water soluble –polymers. The rheological properties of pectin solutions are very dependent on the presence of salts, calcium or similar non alkali metals, and on the pH. Other important facts are the chemical properties of pectin, including the degree of ester, and the average molecular weight. This study did observe release of nisin from the substrates but at low levels. It is hypothesized that if the viscosity were reduced by decreasing the pectin levels in the formulation then the nisin may release at a higher percentage from the coated substrate.

Hanusova, et al., (2010) measured migration from a dried natamycin solution on a coated LDPE film into water. In order to determine diffusion, the study utilized agar well diffusion method. Natamycin was released in amounts that inhibited microrganisms, however, the study indicated that the natmycin broke down after eight days of exposure to light.

Kim, et al., (2002) incorporated nisin into acrylic polymer and vinyl acetate ethylene co-polymer and coated onto paper. The study measured (via agar well diffusion) the release of nisin into different contact solutions, which included water, 2% sucrose solution, 2% citric acid solution, and a 2% NaCl solution; these solutions represented sweet, acidic, and salty foods, respectively. The study also measured the inhibition with against *Micrococcus flavus* in a nutrition broth medium; the study showed that there was inhibition against the microorganism.

Jin & Zhang (2008), in studies utilizing nisin as an antimicrobial in polylactic acid polymer, also measured the release of nisin. The standard curve was prepared by a seeded lawn overlay spot (also completed by (Siragusa, G.R., Cutter, C.N., Willett,J.L. 1999) with some modifications to the procedure. The nisin was heated (100º C for 5 minutes) and then cooled for 144 hours to obtain maximum release. The release kinetics from a PLA nisin matrix need further investigation, the study indicated, however their hypothesis follows a Fickian diffusion behavior.

<div align="center">

**Conclusion**

</div>

Diffusion was successfully measured by agar well diffusion method. It was determined that approximately 50% of the nisin remained entrapped in the pectin coating.

Microscopy was examined as a new method for tracking nisin diffusion in the food and films and found to be useful. Finally, HPLC results demonstrated that the nisin might have been breaking down when in contact with the turkey bologna. Future studies should examine the structural change in the protein nisin and if it has an effect on the antimicrobial activity of the protein.

## Acknowledgements

**Table 4.1. Summary of Coding for treatments.**

| Codes (1st Letter) | Substrates | Codes (2nd Letter) | Coating |
|---|---|---|---|
| P | Sealed Air laminate coextruded forming web with a polypropylene skin, a plastomer sealant and EVOH barrier | C | Control: No Nisin or Calcium Chloride |
| W | A wax parchment paper from Ingles | A | Coating with nisin |
|  |  | B | Coating with nisin and Calcium Chloride |

**Table 4.2. Release of nisin (measured by agar well diffusion method) from two substrates, two formulations with four different nisin concentrations (10,000IU/g, 20,000IU/g, 40,000IU/g and 80,000IU/g) overtime.**

| Treatment & Time | 10,000IU/mL | | 20,000IU/mL | | 40,000IU/mL | | 80,000IU/mL | |
|---|---|---|---|---|---|---|---|---|
| | Inhibition Zone (mm) | Nisin Release IU/mL | Inhibition Zone (mm) | Nisin Release IU/mL | Inhibition Zone (mm) | Nisin Release IU/mL | Inhibition Zone (mm) | Nisin Release IU/mL |
| **PA** | | | | | | | | |
| 30 | 0.0 | 0.0 | 0.00 | 0.00 | 2.2 | 1880.9 | 2.2 | 1880.9 |
| 5 | 0.0 | 0.0 | 1.8 | 1534.7 | 1.8 | 1534.7 | 3.3 | 3291.1 |
| 10 | 0.6 | 850.7 | 4.3 | 5472.9 | 3.2 | 3127.9 | 5.8 | 11736.6 |
| 24 | 1.4 | 1245.8 | 3.0 | 2825.4 | 5.4 | 9576.2 | 7.0 | 21607.4 |
| 48 | 1.4 | 1252.2 | 3.2 | 3127.9 | 5.8 | 11736.6 | 7.6 | 29317.8 |
| 72 | 1.4 | 1245.8 | 3.5 | 3643.5 | 6.7 | 18549.7 | 8.0 | 35932.3 |
| 7 | 2.9 | 2617.8 | 4.8 | 7057.7 | 7.0 | 21607.4 | 8.3 | 41855.2 |
| **WA** | | | | | | | | |
| 30 | 0.0 | 0.0 | 0.5 | 792.3 | 2.5 | 2191.0 | 2.8 | 2552.1 |
| 5 | 0.0 | 0.0 | 0.5 | 792.3 | 1.2 | 1131.1 | 4.0 | 4698.5 |
| 10 | 0.0 | 0.0 | 1.7 | 1458.6 | 2.0 | 1699.0 | 5.9 | 12349.0 |
| 24 | 1.1 | 1075.0 | 2.2 | 1880.9 | 2.2 | 1880.9 | 6.9 | 20535.9 |
| 48 | 1.6 | 1365.3 | 2.0 | 1699.0 | 3.6 | 3833.6 | 6.2 | 14384.6 |
| 72 | 1.1 | 1075.0 | 2.0 | 1699.0 | 3.2 | 3127.9 | 8.0 | 35932.3 |
| 7 | 1.1 | 1075.0 | 4.0 | 4698.5 | 6.5 | 16755.7 | 8.1 | 37807.1 |
| **PB** | | | | | | | | |
| 30 | 0.0 | 0.0 | 1.0 | 1021.7 | 3.8 | 4244.0 | 5.5 | 10075.8 |
| 5 | 1.5 | 1297.6 | 1.5 | 1317.5 | 3.1 | 2972.8 | 5.7 | 11154.6 |
| 10 | 2.8 | 2513.5 | 2.2 | 1880.9 | 5.6 | 10601.5 | 7.0 | 21607.4 |
| 24 | 2.8 | 2591.3 | 2.8 | 2552.1 | 5.4 | 9576.2 | 7.7 | 30847.5 |
| 48 | 2.7 | 2425.5 | 1.9 | 1614.7 | 6.9 | 20535.9 | 7.7 | 30847.5 |
| 72 | 2.7 | 2413.2 | 3.5 | 3643.5 | 7.0 | 21607.4 | 8.0 | 35932.3 |
| 7 | 2.0 | 1699.0 | 4.8 | 7057.7 | 7.0 | 21607.4 | 8.2 | 39779.7 |
| **WB** | | | | | | | | |
| 30 | 0.0 | 0.0 | 1.0 | 1021.7 | 4.5 | 6058.9 | 3.6 | 3833.6 |
| 5 | 0.0 | 0.0 | 3.5 | 3643.5 | 5.8 | 11736.6 | 7.3 | 25169.1 |
| 10 | 0.0 | 0.0 | 4.7 | 6707.7 | 5.4 | 9576.2 | 7.2 | 23921.0 |
| 24 | 0.0 | 0.0 | 5.0 | 7813.4 | 7.0 | 21607.4 | 8.0 | 35932.3 |
| 48 | 1.0 | 996.0 | 3.3 | 3291.1 | 7.0 | 21607.4 | 8.2 | 39779.7 |
| 72 | 1.7 | 1451.2 | 4.8 | 7057.7 | 7.0 | 21607.4 | 8.2 | 39779.7 |
| 7 | 2.0 | 1699.0 | 4.7 | 6707.7 | 7.0 | 21607.4 | 8.2 | 39779.7 |

[a] Formulation B has added Calcium Chloride for slow release compared to formulation A
[b] "WA" is wax paper substrate with formulation A
[c] PA" is polymer substrate with formulation A
[d] "WB" is wax paper substrate with formulation B
[e] "PB" is polymer substrate with formulation B

**Table 4.3. Diffusion of nisin leaving the coated substrates and entering the turkey measured by high performance liquid chromatography over 48 hours.**

| Day | Sample | Concentration in substrate | Concentration in Meat |
|-----|--------|---------------------------|-----------------------|
| 0 | PA | 59877.15 | 122.85 |
|   | WA | 60666.92 | -666.92 |
|   | PB | 60820.96 | -820.962 |
|   | WB | 59817.01 | 182.99 |
| 1 | PA | 54778.78 | 5221.22 |
|   | WA | 52528.01 | 7471.99 |
|   | PB | 50492.87 | 9507.13 |
|   | WB | 46410.34 | 13589.60 |
| 2 | PA | 31768.041 | 28231.96 |
|   | WA | 27812.97 | 32187.03 |
|   | PB | 26657.82 | 33342.18 |
|   | WB | 23737.29 | 36262.71 |

[a] Formulation B has added Calcium Chloride for slow release compared to formulation A
[b] "WA" is wax paper substrate with formulation A
[c] PA" is polymer substrate with formulation A
[d] "WB" is wax paper substrate with formulation B
[e] "PB" is polymer substrate with formulation B
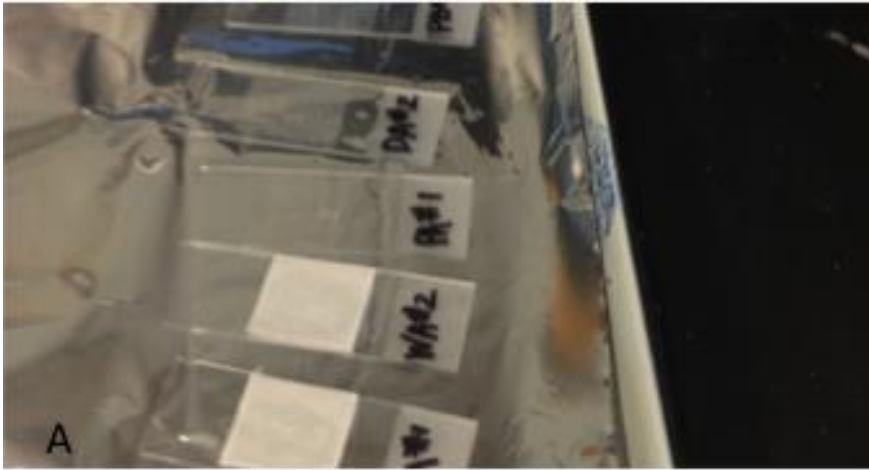
**Figure 4.1. A: Coated substrates glued to microscopy slides. B: Meat glued to microscopy slides. C: Combined substrate and meat slides vacuum packaged and stored at 4ºC between sampling.**
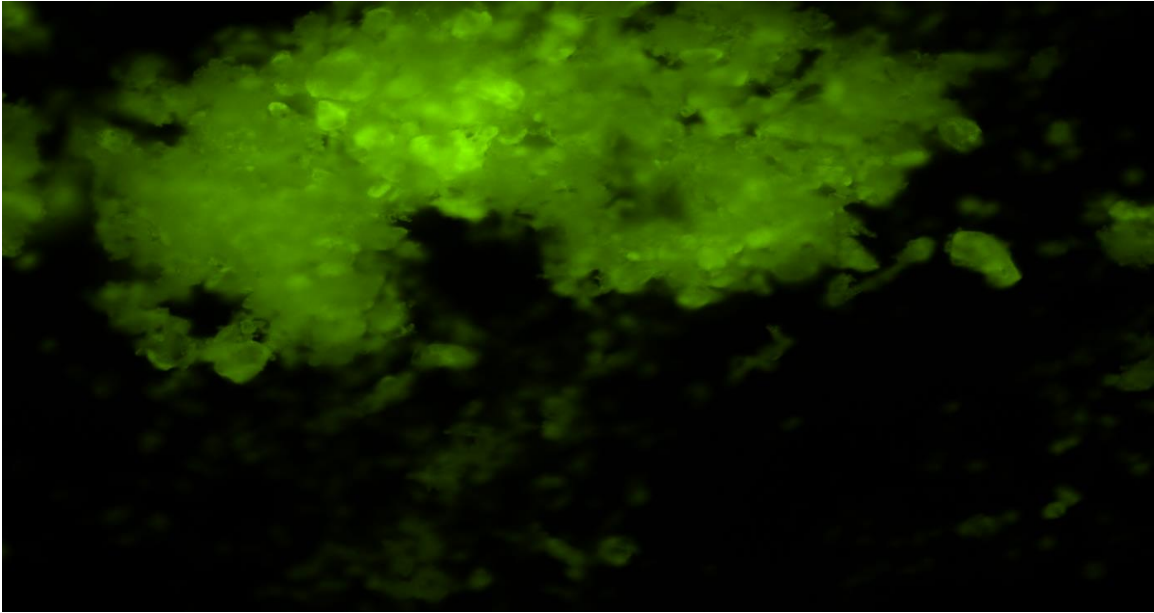
**Figure 4.2. Auto-fluorescence of Nisin A (>95%) (10x magnification).**



A (10X Magnification)

B (10X Magnification)
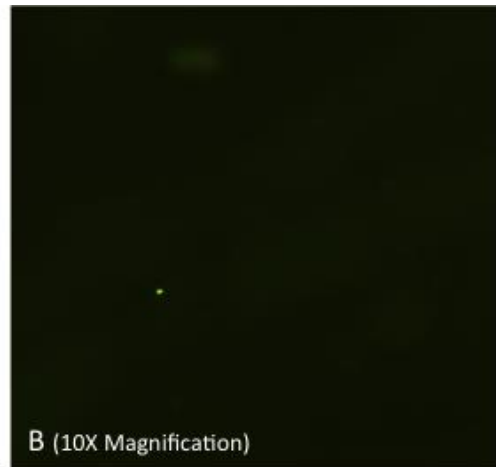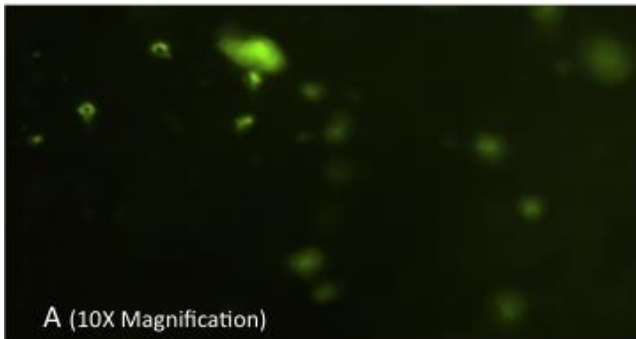
**Figure 4.3. A. Nisin A Fluorescence in the coated substrate (10x magnification) day 0 (before in contact with the meat). B: Nisin A Fluorescence in the coated substrate (10x magnification) after 63 days in contact with the meat.**
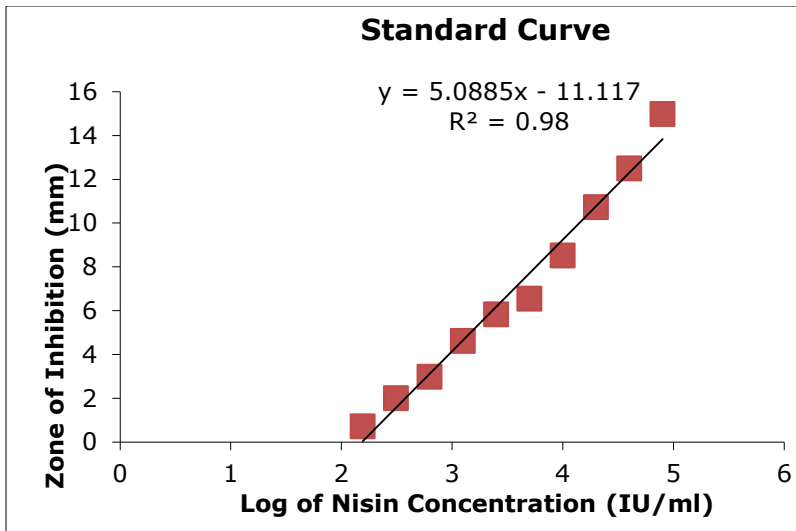
**Figure 4.4. Nisin A standard curve for agar well diffusion method.**
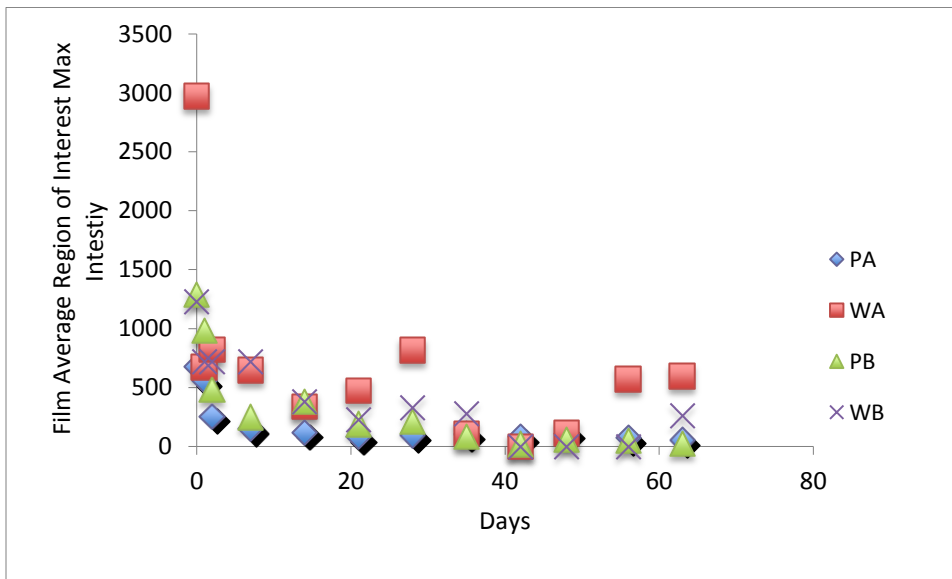


**Figure 4.5. Nisin A leaving the substrates over 63 days measured by reduction in fluorescence intensity.**

[a] Formulation B has added Calcium Chloride for slow release compared to formulation A
[b] "WA" is wax paper substrate with formulation A
[c] PA" is polymer substrate with formulation A
[d] "WB" is wax paper substrate with formulation B
[e] "PB" is polymer substrate with formulation B
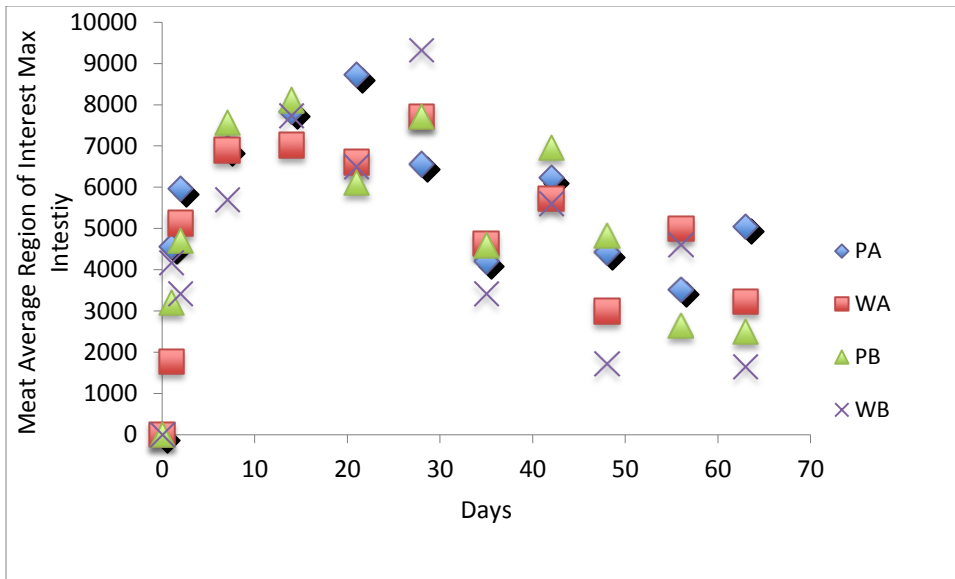
**Figure 4.6. Nisin A entering the meat over 63 days measured by fluorescence intensity.**

a Formulation B has added Calcium Chloride for slow release compared to formulation A

b "WA" is wax paper substrate with formulation A

c PA" is polymer substrate with formulation A

d "WB" is wax paper substrate with formulation B

e "PB" is polymer substrate with formulation B
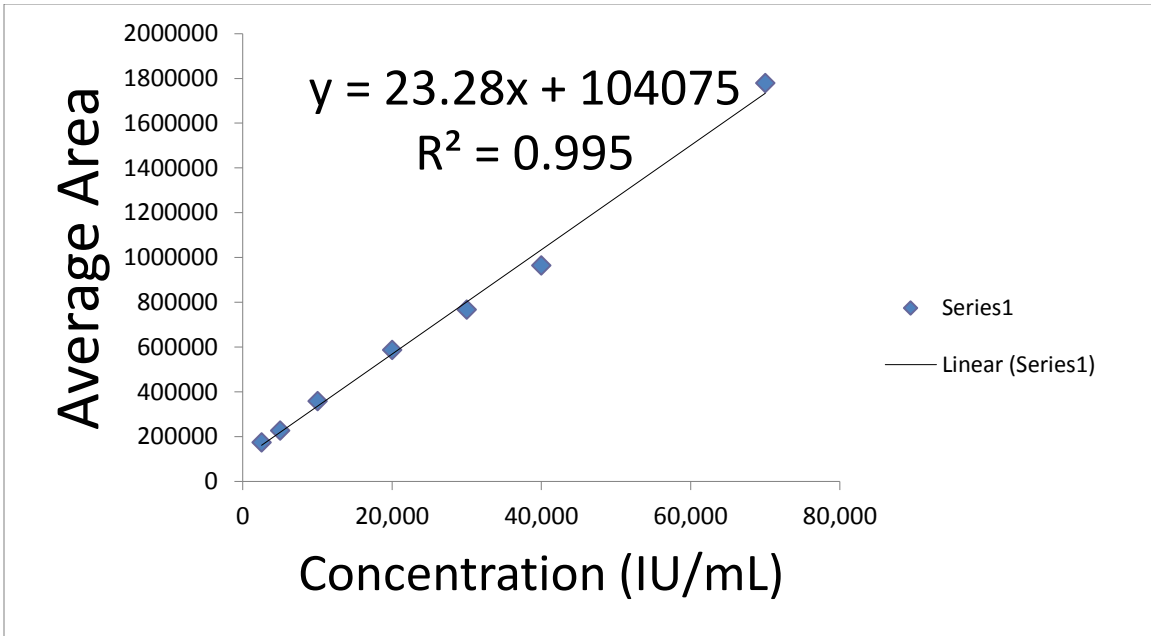
**Figure 4.7. High-performance liquid chromatography standard curve of nisin A (>95%).**

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CHAPTER V

THE EFFECT OF BOVINE ALBUMIN AND POLYMER TYPE ON NISIN Z

CONTAINING EXTRUDED FILM

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**Abstract**

Antimicrobial food packaging may extend shelf life, reduce spoilage, maintain

food quality and can eliminate foodborne pathogens in ready-to-eat deli meat. Nisin is a

polypeptide with natural antimicrobial activity against gram-positive microorganisms,

such as *Micrococcus luteus*. This food additive is generally recognized as safe (GRAS) in

the United States. Although nisin is thermally stable, it loses antimicrobial activity at

high extrusion temperatures. The researchers' objective was to investigate the effect of

bovine albumin and polymer type on nisin Z containing extruded films, such as ethylene

vinyl acetate, metallocene linear low density polyethylene and polypropylene. The

modified resins were produced using a Micro 15cc twin-screw compounder. In all cases

the resin was added first and melted at 170ºC. The barrel was then cooled to 140ºC before

additives were included. The batch was then continually mixed for five minutes before

passing through the film die. After extrusion, each film type was tested for antimicrobial

activity using a modified seeded lawn overlay assay. Statistical analysis was performed

using a two tailed, unequal variance T-TEST's in Excel. The results demonstrated that

there was significant increased inhibition of *M. luteus* when the bovine albumin was used in combination with the nisin Z. Also, there was a significant difference between the type of polymer and the amount of inhibition of *M. luteus*. This research is directed toward the development of an antimicrobial vacuum skin package (VSP) for ready-to-eat (RTE) meat. It provides new and necessary information on post-extrusion retention of the activity of the antimicrobial, nisin.

## Introduction

The Centers for Disease Control and Prevention estimates that approximately 48 million cases of foodborne illness occur each year in the United States including 128,000 hospitalizations and 3,000 related deaths [1]. Active packaging was introduced as a response to the demands of consumers for high quality, safe and extended shelf-life of food products; it has also suited the changes in retail and distribution practices, (for instance, online commerce and "retail superstores"), which have pressed logistic tensions, as distribution distances increase and require longer storage times [2,3,4]. Active packaging applications (including the incorporation of antimicrobials and subsequent release into the product), have received considerable attention as a means of extending the bacterial lag phase, leading to slower growth of microorganisms [5,6]. The direct addition of antimicrobials (organic acids, spice extracts, chelating agents, metals, enzymes or bacteriocins, such as nisin) have also shown a decrease in antimicrobial growth because of leaching into the food matrix, and cross-reaction with other food components such as lipids or proteins [7,8,9].

Nisin is a polypeptide produced from subspecies of *Lactococcus lactis*, which is a gram-positive lactic acid bacterium (LAB) and is used extensively in the production of various dairy products, including various cheeses, butter and sour cream [10,11]. Nisin has been approved for use as an antimicrobial in food by the Joint FAO/WHO Expert Committee on Food Additives (which is run by the United Nations); nisin has been given the food additive number 234, and is approved for use in over 50 countries [4]. Nisin is the most abundantly used of all bacteriocins [12], as it is an effective antimicrobial [10]. However, while nisin is GRAS (generally recognized as safe) by the FDA since 1988 [12], there is a concentration limit of 10,000 IU (international units) in food. Nisin is an effective antimicrobial against gram-positive microorganisms, such as *Micrococcus luteus*. *M. luteus* species are non-motile, non-spore forming, aerobic, gram-positive and oxidase-positive cocci coming from the genus Micrococcus and family *Micrococcaceae* [13] that can survive halophilic environments.

The process of extruding nisin has been examined previously, although minimally [14,15,16,17,18]. To the author's knowledge, research in comparing resin type and its effect on release of nisin has not been studied. Preliminary studies examined Low density polyethylene (LDPE), Linear low density polyethylene (LLDPE), Nexxstar[TM] Low EVA-00111 7.5 % Va content and Elvax 3120 EVA 7.5% Va content. However the main research focuses on resins that are co-polymers. Ultimately, this film could potentially be produced by co-extrusion or lamination. This would allow the innermost layer to contain the antimicrobial additives. The resins investigated in this study are Ethylene vinyl acetate (EVA) Elvax 3124, Exceed[TM] 1018CA Metallocene polyethylene (mLLDPE) and

Versifty<sup>TM</sup> 3200 polypropylene (PP). These resins were chosen for their low melting index suitable for nisin to retain antimicrobial activity during extrusion. Also, these resins are commonly used in ready-to-eat meat packaging. This study also examined the addition of bovine albumin, (BA) since previous research demonstrated an increase in thermal stability for other additives [19, 20].

## Materials and Methods

Nisin Z of 2.5% purity was supplied by Handary (Brussels, Belgium). The BA had a pH of 5.2 and was supplied by VWR (Radnor, Pennsylvania). Both were stored at refrigeration temperature until use. The resin Elvax 3120 EVA (Va content 9%) was supplied by DuPont (Fayetteville, North Carolina). The resins mLLDPE and PP were supplied by Dow Chemical (Houston, Texas). Preliminary studies in addition examined LDPE (Dow Chemical, Houston, Texas), LLDPE (Dow Chemical, Houston, Texas), Nexxstar<sup>TM</sup> Low EVA-00111 (7.5 % Va content) (ExxonMobil Chemical, Irving, TX) and Elvax 3120 EVA (7.5% Va content) (DuPont, Wilmington, DE).

*Culture storage and preparation.*

*Micrococcus luteus* ATCC® 4698™ was obtained from ATCC. The culture was grown aerobically in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, Mich.) at 30°C for 48 h. The culture was stored at -80°C in TSB and glycerol as stock cultures until needed for experiments. To prepare the culture for experiments, 0.1 mL volumes from frozen stock culture was transferred to 10 mL of fresh TSB at 37°C for 48 h. The culture was later transferred to Tryptic Soy Agar (TSA; Difco) plates, struck to isolation, and incubated at 30°C for 48 h. Finally, a single colony of the culture was taken from the

TSA plate and transferred to TSB and allowed to grow statically for 48 h at 30°C to obtain a cell concentration of ~6 $\log_{10}$ CFU/mL. This transfer process was done in triplicate.

*Preliminary single screw extrusion experiments*

The researchers saw promise in the thermal stability of the antimicrobial nisin based on the previous studies. The researchers wanted to determine if nisin would survive signal screw extrusion. A mixture of 200 grams of resin and 2 grams of Nisin Z (2.5%) were placed in the single screw extruder (Killion, Cedar Grove New Jersey) hopper. The operating parameters are shown in Table 1. The die was removed from the single screw extruder in these initial trials. This was to ensure back pressure would not build up, destroying the rupture disk in the machine. This was repeated with LLDPE using the same methods as well. Control film was also extruded for each run. Without the coat hanger die on the extruder the appearance of the extrudate can be seen in Figure 1. The extrudate was then cut into slices and heat pressed at 122ºC. All of the films were tested for antimicrobial activity against *Micrococcus luteus* ATCC 4698.

Single screw extrusion with the use of the coat hanger die was then conducted. The following polymers were used LDPE, LLDPE, mLLDPE and EVA (3 types). 200 grams of each of the resins was blended and extruded with 2g of Nisin Z (2.5%). The processing parameters can be found in Table 2, Table 3 and Table 4. Control films were made for each run and had similar processing parameters. All of the films were tested for antimicrobial activity against *Micrococcus luteus* ATCC 4698.

*Preparation of twin-screw extrusion*

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Resins were prepared using a batch process. A micro-15cc twin-screw compounder (DSM xplore Netherlands) was used with a total volume of 10 g. In all cases the resin was added first and melted at 170ºC. Then the barrel was cooled to 140ºC before incorporating the additive(s). The batch was continually mixed for 5 minutes before passing through the film die. The chill roll parameters were as follows: speed 370 m/min and torque 78. This was completed in triplicate for each treatment.

*Treatments*

This experiment has two sets of controls (one for each type of resin (EVA, mLLDPE &PP and one for each resin type plus BA). Treatment 1 is the resin type and nisin added without BA. Treatment 2 is the resin type, added nisin and BA (See Table 5).

*Preparation of antimicrobial testing*

After extrusion, each set of samples was tested for antimicrobial activity using a modified seeded lawn overlay assay [21]. TSA agar plates were overlaid with ~8mL of semisoft TSB agar (0.5% w/v agar) seeded with 1% of broth culture of *M. luteus.* The seed density was approximately 1x $10^6$ cfm ml$^{-1}$ of overlay. The corresponding control was always placed on the same overlaid agar plate. Plates were evaluated for zones of inhibition after 24-48 h incubation at 30ºC. The inhibition zones were measured on all four sides of the film. Each treatment was plated in duplicate.

*Statistical analysis*

Statistical analysis was performed to determine if there was a statistical difference ($p < 0.05$) for the inhibition of *M. luteus* when nisin was added compared to the control film using Control 1 (no nisin) to those with nisin (Treatment 1). Comparisons were also

made between nisin without BA (Control 2) and those with nisin and BA (Treatment 2) as well as between Treatment 1 and Treatment 2. The inhibition was measured on all four sides of the film. A caliper was used to measure from the edge of the film to the outer edge of the zone of inhibition. The analysis was performed using a two tailed, unequal variance T-TEST's in Microsoft® Excel® for Mac® version 11.5  (2004, Microsoft Corp. Redmond, WA). A p-value of less than 0.05 was considered significant. Every plate was also completed in duplicate. The films were tested in triplicate and then the average was used to conduct statistical analysis. A control film was always placed on each plate to ensure no inhibition was related to the film itself.

Statistical analysis was performed to compare the type of polymer used (EVA, mLLDPE or PP) to see if a significant difference in inhibition of *M. luteus* exists between polymer type. This was completed for Treatment 1 and Treatment 2 for each film type. The statistical analysis was performed using ANOVA in excel 2010. A p-value of less than 0.05 was considered significant. Then an individual two tailed, unequal variance T-TEST's was performed. The analysis was performed in Microsoft® Excel® for Mac® version 11.5  (2004, Microsoft Corp. Redmond, WA) in order to determine where the significant difference between polymers were occurring. This was completed for Treatment 1 and Treatment 2 for each film type. A p-value of less than 0.05 was considered significant.

## Results

First preliminary extrusion studies were conducted. Table 6 show the inhibition achieve for the single screw extrusion without the coat hanger die in place. Table 7

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displays LDPE, LLDPE, EVA and MLLDPE initial trials (with coat hanger die) and the inhibition achieved for each. Then the studies involving bovine albumin on the twin screw extruder were completed. Tables 8, shows statistical differences between controls and treatments by completion of a T-test. Table 9, shows statistical differences between polymer types for both treatment 1 and 2, by completion of an ANOVA. Table 10 shows how a T-test to demonstrates statistical difference for comparing type of polymers and achieved inhibition for treatment 1. Table 11 uses a T-test to demonstrate statistical difference for comparing type of polymers and achieved inhibition for treatment 2.

## Discussion

The researchers were not sure how nisin would behave when extruded under pressure. Since pressure was not investigated in studies from the literature the coat hanger die was removed for early experiments. The average inhibition zones for LDPE and LLDPE, which were extruded without the coat hanger die on the extruder, can be seen in Tables 6. LDPE did not demonstrate activity. LLDPE did demonstrate activity after extrusion up to temperature of 170ºC without the coat hanger die.

After this successful experiment, the coat hanger die could be used for future trials (Table 7). Once the coat hanger die was added the LLDPE no longer demonstrated antimicrobial activity, however. It should be noted that mLLDPE and EVA did display antimicrobial activity. For future studies both mLLDPE and EVA 3124 were selected because of the promising preliminary results.

In the preliminary results limited conclusions can be made with varying time, temperature and multiple polymer types (Table 7). It is difficult to compare each trial

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with so many changing variables. Also mixing and pressure changes were influential in the amount of inhibition achieved. Another concern was sampling bias, as variation existed between beginning, middle and end of each run. The goal was to achieve a homogenous extrudate with even inhibition throughout the entire run. Future studies were conducted on a twin-screw extruder with a smaller hopper and screw to help eliminate bias and control variables.

The researchers were able to produce an antimicrobial film with or without the addition of BA for all resin types (EVA, mLLDPE and PP). Antimicrobial activity was measured using modified seeded (with *M. luteus)* lawn overlay assay. The films analyzed were tested at the beginning, middle and end of the extrusion run. Table 11 summarizes the treatment and controls that were used in this study. There was a significant difference (p<0.05) in antimicrobial activity of Treatment 1 to Control 1 (See Table 8). In addition, there was also a significant difference (p-value < 0.05) in antimicrobial activity between Treatment 1 and Treatment 2 (See Table 8). Only EVA at the end of the run did not have a statistical difference (p-value of 0.051).

As expected, there was a significant difference (p<0.05) between Control 2 and Treatment 2. Therefore, it can be observed that BA is beneficial in aiding the nisin for inhibition of *M. luteus*. Nisin has been incorporated into film by extrusion in the past [14]. Nisin has also been encapsulated in order to increase the survival of the activity of the nisin after extrusion. The objective of this study was to investigate the addition of BA in addition to nisin during extrusion. BA is a protein that exhibits multiple mechanisms of denaturation [22]. A study by Aoki et al., [23] found that some fractions of BA are

denatured by heat; however, the amount of BA resistant to heat denaturation decreases at higher temperatures. Aoki et al., [23] also observed that in the presence of fatty acids the BA was even more resistant to heat. BA has been used to increase the viability and leaching during high-temperature extrusion of: oat hulls, soybean hulls, yeast extract, soybean flour and mineral salts [19].

It is possible that the BA is protecting the nisin during processing at 140ºC. It is known that nisin begins to degrade at high processing temperatures. [9] determined that extrusion above 140ºC resulted in denaturation of nisin and complete loss of antimicrobial activity. However, Siragusa et al., [24] extruded nisin using polyethylene resin at 120ºC and found that it had excellent antimicrobial activity of nisin. Although nisin may still retain some antimicrobial activity, that activity is reduced at higher temperatures. The researchers are suggesting that BA can help protect the nisin at extrusion temperatures, however, only within a narrow range (120-160ºC). Studies demonstrated loss of inhibition of *M. luteus* at extrusion temperatures above 160ºC with or without the presence of BA. Also, it was observed that with extrusion below 120ºC, BA does not provide added benefit when incorporated. This agrees with previous work showing nisin has a high percentage of activity below these temperatures [24].

This study also investigated the difference between resin type and antimicrobial activity of the film. In order to yield a proper comparison, the same parameters that were used during extrusion and the same concentrations of additives that were used were analyzed. Statistical analysis demonstrates that the type of resin does have a significant impact of the antimicrobial activity of the film. ANOVA results demonstrate a p-value of

0.025 for treatment 1 and a p-value of 0.04 for treatment 2 (See Table 9). This means at least one of the films has a statistical difference in the amount of inhibition occurring against *M. luteus*.

After further investigation, the significant difference in the achieved inhibition was occurring between mLLDPE and PP. Treatment 1 has a p-value of 0.004 (See Table 10) while treatment 2 had a p-value of 0.046 (See Table 11). There was not a significant difference between EVA and mLLDPE or EVA and PP with p-values of greater than 0.05. PP and mLLDPE are both nonpolar, EVA has 9% VA content (VA content is polar). This demonstrates that polarity is not the main reason for the significant difference in inhibition, as was expected. PP had the lowest melting temperature at 85ºC, while mLLDPE had the highest melting temperature at 117.7ºC, and EVA melting temperature was 99ºC. It is hypothesized that the PP achieved a more-even mixing (due to lower melting temperatures) compared to the mLLDPE and this accounts for the differences in inhibition.

Future research should be conducted to see if an encapsulation process of nisin in BA could be developed to increase the temperature range at which BA can protect nisin. Currently, there is a narrow window in which the BA is providing protection. The literature also suggests that adding fatty acids could aid in protection of the BA, possibly increasing the protection of the nisin at higher temperatures.

## Conclusion

In conclusion, BA can be used to protect nisin from temperatures in the extruder and help retain antimicrobial activity. The type of polymer that is used to incorporate the

antimicrobial during the extrusion process also plays a role. It has been demonstrated that lower melting resin is ideal for the extrusion of nisin and can significantly increase the inhibition achieved against *M. luteus*. The production of this antimicrobial film was able to extend shelf life, which will help sustainability. This study also provides new and necessary information on post-extrusion retention of the activity of the antimicrobial nisin.

## Acknowledgments

**Table 5.1. Processing Parameters for single screw extrusion without coat hanger die.**

| Polymer | LDPE Trial 1 | LLDPE Trial 2 | LLDPE Trial 3 | LLDPE Trial 4 |
|---------|--------------|---------------|---------------|---------------|
| Zone 1 | 256ºF/124.4ºC | 225ºF/107.2ºC | 225ºF/107.2ºC | 240ºF/115.5ºC |
| Zone 2 | 300ºF/148.9ºC | 265ºF/129.4ºC | 265ºF/129.4ºC | 310ºF/154.4ºC |
| Zone 3 | 311ºF/155.0ºC | 267ºF/130.5ºC | 266ºF/130.0ºC | 344ºF/173.3ºC |
| RPMs | 24.4 | 21.3 | 23.1 | 23.2 |
| Melt | 297ºF/147.0ºC | 253ºF/122.8ºC | 253ºF/122.8ºC | 323ºF/161.0ºC |

**Table 5.2. Processing parameters for single screw extrusion for low density polyethylene (LDPE) and linear low density polyethylene (LLDPE).**

| Polymer | LDPE | LDPE | LLDPE | LLDPE | LLDPE |
|---------|------|------|-------|-------|-------|
| Zone 1 | 245ºF/118.3ºC | 220ºF/104.4ºC | 220ºF/104.4ºC | 229ºF/109.4ºC | 230ºF/110.0ºC |
| Zone 2 | 300ºF/148.8ºC | 325ºF/162.8ºC | 325ºF/162.8ºC | 325ºF/162.8ºC | 320ºF/160.0ºC |
| Zone 3 | 370ºF/187.8ºC | 344ºF/173.3ºC | 344ºF/173.3ºC | 345ºF/173.9ºC | 343ºF/172.8ºC |
| Die | 366ºF/185.6ºC | 341ºF/171.7ºC | 341ºF/171.7ºC | 341ºF/171.7ºC | 342ºF/172.2ºC |
| Adaptor | 366ºF/185.6ºC | 340ºF/171.1ºC | 340ºF/171.1ºC | 339ºF/170.6ºC | 341ºF/171.7ºC |
| Pressure (psi) | 800 | 550 | 1270 | 1140 | 1320 |
| Screw RPMs | 110.9 | 35.6 | 35.6 | 35.7 | 35.0 |
| Melt | 369ºF/187.2ºC | 330ºF/165.6ºC | 333ºF/165.6ºC | 329ºF/165.0ºC | 335ºF/168.3ºC |
| F.P.M | 80.0 | 15.2 | 15.2 | 15.1 | 15.2 |
| Res. time | 44 sec | 2.5min | 2.5min | ~2.5mins | 2.5 min |

**Table 5.3. Processing parameters for single screw extrusion for Ethylene vinyl acetate (EVA).**

| Polymer | Nexxstar™ Low EVA-00111 7.5 % Va content | Nexxstar™ Low EVA-00111 7.5 % Va content | Elvax 3120 EVA 7.5% Va content | Elvax 3124 EVA 9.0% Va content | Elvax 3124 EVA 9.0% Va content |
|---|---|---|---|---|---|
| Zone 1 | 227ºF/108.3ºC | 230ºF/110.0ºC | 230ºF/110.0ºC | 230ºF/110.0ºC | 246ºF/118.9ºC |
| Zone 2 | 300ºF/148.8ºC | 300ºF/148.8ºC | 280ºF/137.8ºC | 280ºF/137.8ºC | 300ºF/148.8ºC |
| Zone 3 | 301ºF/149.4ºC | 301ºF/149.4ºC | 286ºF/141.1ºC | 287ºF/141.7ºC | 375ºF/190.6ºC |
| Die | 301ºF/149.4ºC | 301ºF/149.4ºC | 285ºF/140.5ºC | 285ºF/140.5ºC | 366ºF/185.6ºC |
| Adaptor | 300ºF/148.8ºC | 299ºF/148.3ºC | 285ºF/140.5ºC | 285ºF/140.5ºC | 368ºF/186.7ºC |
| Pressure (psi) | 1140 | 1140 | 1000 | 530 | 210 |
| Screw RPMs | 33.4 | 34.9 | 35.1 | 35.1 | 35.0 |
| Melt | 293ºF/145.0ºC | 292ºF/144.4ºC | 279ºF/137.2ºC | 279ºF/137.2ºC | 365ºF/185.0ºC |
| F.P.M | 6.1 | 4.2 | 3.5 | 3.5 | 3.5 |
| Res. time | 3 min 80 sec | 1min 45 sec | 2 min 28 sec | 2 min 17 sec | 2 min 17 sec |

**Table 5.4. Extrusion parameters for Metallocene polyethylene (mLLDPE).**

| Polymer | Exceed[TM] 1018CA mLLDPE | Exceed[TM] 1018CA mLLDPE |
|---|---|---|
| Zone 1 | 230ºF/110.0ºC | 230ºF/110.0ºC |
| Zone 2 | 300ºF/148.8ºC | 300ºF/148.8ºC |
| Zone 3 | 307ºF/152.8ºC | 304ºF/151.1ºC |
| Die | 301ºF/149.4ºC | 300ºF/148.8ºC |
| Adaptor | 300ºF/148.8ºC | 300ºF/148.8ºC |
| Pressure (psi) | 1780 | 1160 |
| Screw RPMs | 34.9 | 22.5 |
| Melt | 301ºF/149.4ºC | 297ºF/147.2ºC |
| F.P.M | 4.8 | 6.0 |
| Res. time | 2 min | 4 min 20 sec |

**Table 5.5. List of controls and treatments for bovine albumin experiment.**

| Control 1 Film: Resin Only | Control 2 Film: Resin + BA | Treatment Film 1: Resin + Nisin Z | Treatment Film 2: Resin + Nisin Z +BA |
|---|---|---|---|
| a. EVA (10.0 g) | a. EVA (9.95 g) + BA (0.05 g) | a.EVA (9.8 g) + Nisin Z (0.2 g) | a.EVA (9.75 g) + Nisin Z (0.2 g) + BA (0.05 g) |
| b.mLLDPE (10.0 g) | b.mLLDPE (9.95 g) + BA (0.05 g) | b.mLLDPE (9.8 g) + Nisin Z (0.2 g) | b.mLLDPE (9.75 g) + Nisin Z (0.2 g) + BA (0.05 g) |
| c.PP (10.0 g) | c.PP (9.95 g) + BA (0.05 g) | c.PP (9.8 g) + Nisin Z (0.2 g) | c.PP (9.75 g) + Nisin Z (0.2 g) + BA (0.05 g) |

BA: bovine albumin
EVA: Ethylene vinyl acetate 3124
mLLDPE: Exceed$^{TM}$ 1018CA Metallocene polyethylene
PP: Versifty$^{TM}$ 3200 polypropylene
Nisin Z: Nisin Z at 2.5 % purity

**Table 5.6. Inhibition zones for polymers extruded without the die.**

| Trial | Resin Type | Temperature ℃ | Average Inhibition (mm/in) Start | Average Inhibition (mm/in) Middle | Average Inhibition (mm/in) End |
|---|---|---|---|---|---|
| 1 | LDPE | 147.0 | 0 | 0 | 0 |
| 2 | LLDPE | 122.8 | 5.25 | 4.725 | 5.1 |
| 3 | LLDPE | 122.8 | 3.9 | 4.6 | 3.65 |
| 4 | LLDPE | 161.0 | 5.2 | 6.625 | 7.1125 |

**Table 5.7. Antimicrobial activity of films after extrusion for low density polyethylene (LDPE), Linear low density polyethylene (LLDPE), Ethylene vinyl acetate (EVA) and Metallocene polyethylene (MLLDPE).**

| Resin Type | Residence Time | Temperature (ºC) | Average Inhibition (mm/in) |
|---|---|---|---|
| LDPE | 2 min 30 sec | 165.6 | None |
| LDPE | 44sec | 187.2 | None |
| LLDPE | 2 min 33 sec | 168.3 | None |
| LDPE | 2 min 30 sec | 165.6 | None |
| LLDPE | 2 min 20 sec | 165.0 | None |
| EVA Nexxstar 7.5% | 2 min 40 sec | 145.0 | 2.1 |
| EVA Nexxstar 7.5% | 1 min 45 sec | 144.4 | 2.4 |
| EVA 3124 9% | 2 min 17 sec | 185.0 | None |
| EVA 3124 9% | 2 min 17 sec | 137.2 | 3.3 |
| EVA 3120 7.5% | 2 min 28 sec | 137.2 | 2.6 |
| mLLDPE | 4 min 20 sec | 147.2 | 3.4 |
| mLLDPE | 1 min 45 sec | 149.4 | 6.5 |

**Table 5.8. Statistical results (p-value) for differences in inhibition between controls and treatments.**

| Polymer | Control 1 compared to Treatment 1 | | | Control 2 compared to Treatment 2 | | | Treatment 1 compared to Treatment 2 | | |
|---|---|---|---|---|---|---|---|---|---|
| Sampling position | B | M | E | B | M | E | B | M | E |
| EVA | 0.000* | 0.000* | 0.012* | 0.000* | 0.000* | 0.001* | 0.013* | 0.034* | 0.051 |
| mLLDPE | 0.000* | 0.000* | 0.000* | 0.000* | 0.000* | 0.000* | 0.021* | 0.012* | 0.032* |
| PP | 0.000* | 0.000* | 0.000* | 0.000* | 0.000* | 0.000* | 0.007* | 0.000* | 0.021* |

EVA: Ethylene vinyl acetate 3124
mLLDPE: Exceed$^{TM}$ 1018CA Metallocene polyethylene
PP: Versifty$^{TM}$ 3200 polypropylene
Control 1: Resin Only
Control 2: Resin + Bovine Albumin
Treatment 1: Nisin Z + Resin
Treatment 2: Nisin Z + Resin + Bovine Albumin
B: Beginning of run
M: Middle of run
E: End of run
*: Statistically significant with a p value of <0.05

**Table 5.9. ANOVA results (p-values) for difference inhibition between polymers (EVA, mLLDPE & PP).**

| Treatment for each polymer type | P-value for difference inhibition between polymer type |
|---|---|
| Treatment 1 | 0.024* |
| Treatment 2 | 0.040* |

EVA: Ethylene vinyl acetate 3124
mLLDPE: ExceedTM 1018CA Metallocene polyethylene
PP: VersiftyTM 3200 polypropylene
Treatment 1: Nisin Z + Resin
Treatment 2: Nisin Z + Resin + Bovine Albumin
*: Statistically significant with a p value of <0.05

**Table 5.10. P-value Results for TTEST's comparing type of polymers achieved inhibition for treatment 1.**

|  | mLLDPE | PP |
|---|---|---|
| EVA | 0.405426128 | 0.074 |
| mLLDPE | ------------------ | 0.005* |

*: Statistical significant with a p value of <0.05
EVA: Ethylene vinyl acetate 3124
mLLDPE: ExceedTM 1018CA Metallocene polyethylene
PP: VersiftyTM 3200 polypropylene
Treatment 1: Nisin Z + Resin

**Table 5.11. P-value Results for TTEST's comparing type of polymers achieved inhibition for treatment 2.**

|  | mLLDPE | PP |
|---|---|---|
| EVA | 0.416348363 | 0.091 |
| mLLDPE | ------------------ | 0.047* |

*: Statistical significant with a p value of <0.05
EVA: Ethylene vinyl acetate 3124
mLLDPE: ExceedTM 1018CA Metallocene polyethylene
PP: VersiftyTM 3200 polypropylene
Treatment 2: Nisin Z + Resin + Bovine Albumin

**Figure 5.1. Extrudate from initial trials without the coat hanger die.**

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CHAPTER VI

FUTURE RECOMMENDATIONS

Developing an extruded antimicrobial film is challenging. However, the following are suggestions that the researcher was unable to attempt and may prove successful. The major challenges to overcome with respect to extrusion are diffusion and residence time in the barrel.

- A multi-layer blown film should be attempted to aid at the release of the nisin. The objective would be an extruded film with a thin layer of concentrated nisin on the inside or "food contact side" of the extruded blown film. In theory this would decrease the diffusion necessary for the nisin to exit the film.

- In addition, the nisin should be aided by use of a port to reduce the residence time in the barrel and achieve maximum retention of antimicrobial activity. Ideally, nisin would be added using a "wet port". This would allow the nisin to be mixed with a plasticizer and added in liquid form to the barrel. The plasticizer may aid in bringing the nisin to the surface of the film as it blooms, potentially increasing antimicrobial activity.

- Previous research demonstrates that PLA is the most ideal polymer for developing a nisin-extruded film. This is thought to be due to the breakdown of the PLA allowing the nisin to release from the film at a controlled manor.

- The researcher also suggests using polypropylene and stretching or orienting the film to create small pores. Finally, more research using bovine albumin could be

conducted in combination with the previous suggestions as it also creates pores in the film.

Although a successful antimicrobial coating was developed, more research is still necessary for commercialization.

- The concentration of pectin in the formulation could be decreased to aid in release of nisin and reduce cost. The current formulation uses double the legal limit of nisin (20,000 iu/ml versus limit of 10,000 iu/ml) because of how nisin was entrapped in the film. Caution should be taken when decreasing pectin concentration since it will also lower the viscosity of the coating and may limit possibilities for commercialization.

- Also, attempting other "food safe" carriers is suggested.

- Future research does not suggest adding calcium since it did not show a significant difference in release of nisin. The pectin alone entrapped too much nisin in the coating not allowing it to release.

If extrusion and coating prove to demonstrate limited success then a spray could be developed.

- The spray could be added immediately prior to packaging the food or directly after extrusion of the film.

- Future studies should exam the shelf life of nisin in warehouses as it may breakdown during storage. This research also raised questions about nisin itself. How is the nisin breaking down? Does it still retain its antimicrobial activity?

Will it only break down when in contact with the food? These questions are important in order for future research to measure diffusion accurately.

- Finally, nisin is a natural antimicrobial but may prove more successful if used in combination with other natural antimicrobials. Especially, if the additional antimicrobial added allows for inhibition of gram-negative pathogens. Some research suggests that a synergist effect will occur; yielding a higher inhibition than either antimicrobial alone would produce. This would allow for the use of less nisin in the formula and reduction of cost, which is an important factor in the commercialization of a process.

## Appendix

Nisin has been studied for years but traditionally its effect on foods. The literature has demonstrated that nisin can survive food-processing temperatures such as smoking. However, food processing temperatures are not as extreme as temperatures used in extrusion. Nisin is a protein and concerns of denaturation or loss of antimicrobial activity need to be examined.

The following experiments were conducted prior to extrusion or coating. First, thermal stability studies of nisin Z (2.5%) were conducted on a hot plate/stirrer (Fisher Scientific, USA). In a small vial, 1 mL of polyethylene glycol 400 was combined with 0.01 gram of nisin Z (2.5%). There were a total of 16 vials. Each vial was then heated in an oil bath to the target temperature of: 140ºC, 160ºC, 170ºC, 180ºC, 190ºC, 200ºC or not heated (control). This was completed in duplicate. The temperature ranges can be found in Table 1 and were monitored with a thermal couple. After heating, each vial was

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immediately cooled on ice to room temperature. See Figure 1 and 2 for the test-up. The solution was then tested for antimicrobial activity using the spot on lawn method against *L. monocytogenes* and *M. luteus*.

The heat stability results can be found in Table 2. However, conducting heat stability experiments is a complex system. It is not only heat that affects the stability of the nisin when extruded. Researchers believe it is a combination of temperature, time in barrel and pressure. This experiment only takes into consideration time and temperature. In addition, the microorganism examined also has an effect. For example *Micrococcus luteus* is more easily inhibited by nisin than a pathogen such as *Listeria monocytogenes.*

Heat press studies were then conducted to determine nisin's heat resistance in a polymer compared to the oil bath. 0.1 grams of Sigma Nisin (2.5%) was mixed with 20 grams of Low Density Polyethylene (LDPE) resin. The mixture was pressed and folded five times inside the carver heat press (Enerpac, USA) (See Figure 3). This was conducted at 130 ° C, 140 ° C, 150 ° C, 160 ° C, 170ºC and 180ºC temperatures. This was repeated in the same manner for Linear Low Density Polyethylene (LLDPE). Control films were pressed for each polymer as well. Control films did not contain nisin.

All of the heat pressed films were tested for antimicrobial activity against *Listeria monocytogenes* ATCC 15313. The heat pressed films were sampled in duplicate by cutting 1" by 1" squares from the center of the film. The squares were then placed under an ultra violet (UV) light (Zeta 7400 Loctite Corporation, Newington Connecticut) for 5 minutes. The UV treatment is to remove any microbial contamination on the film

(Growler et al year). The plate overlay method was used  (Siragusa, G.R., Cutter, C.N., Willett,J.L. 1999).

Heat pressed studies measure the effect of time and temperature with the addition of examining two types of nisin and multiple polymers. The average inhibition zone for the carver pressed heat samples can be found in Figure 4. LLDPE had a trend of having slightly larger inhibition zones. In addition nisin Z has determined to generally produce larger inhibition zones and better diffusion properties as mentioned in the literature  (De Vos et al. 1993).

Previous research demonstrated that nisin could maintain antimicrobial activity at the high temperatures that are needed to produce an extruded film. Then studies on the single-screw extruder were conducted (see 5.1). Although some success was achieve the researchers quickly realized that the powder nisin created a large dust cloud when it was dumped into the "hopper" of the extruder. In order to prevent loss of nisin during processing the nisin could not be added to the "hopper" as a powder. One method that was examined was compounding the powder nisin into the resin ethylene vinyl acetate (EVA).

A master batch was developed by combining 7g of EVA 3124 resin with 7g of Nisin Z (2.5%). The mixture was added to a Micro 15cc twin-screw compounder (DSM xplore Netherlands) (See Figure 5) at the processing temperature of 140ºC.The mixture was continually mixed for 10 minutes and then extruded with a round die.  The master batch was pelletized by using HAAKE PP1 Pelletizer (Figure 6). A second formulation was developed for a master batch. This included 7.5 grams of EVA 3124 and 2.5 grams

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of Nisin Z (2.5%). In this trial the EVA 3124 was placed in the twin-screw compounder by itself and melted at 160℃. Then the twin screwed was cooled to 140℃. Then the nisin was added and mixed for 5 minutes. Then it was extruded without any die and pelletized as described above. All the processing parameters can be seen in Table 3.

The 4 grams of the master-batch #1 was then combined with 200 grams of EVA 3124. This was placed in the hopper of the single screw extruder and extruded. See Table 3. The film was then tested for antimicrobial activity as described above. A control film was also tested. In addition, the master-batch was tested for antimicrobial activity before additional processing.

Master batch #2 7.6 grams were combined with 192.4 grams of EVA 3124 and extruded using the single screw extruder. The process parameter used can be seen in Table 4. The film was then tested for antimicrobial activity as described above. A control film was also tested. In addition, the master-batch was tested for antimicrobial activity before additional processing.

Many trials were conducted with varying time, temperature and multiple polymer types. It is difficult to compare each trial with so many changing variables. Also mixing and pressure changes were influential in the amount of inhibition achieved. Another concern was sampling bias, as variation existed between beginning, middle and end of each run. The goal was to achieve a homogenous extrudate with even inhibition throughout the entire run. In order combat this challenge compounding and pelletizing was investigated.

Compounding targeted the mixing challenge but added an additional heat treatment to the nisin. Master-batch #1 did not demonstrate inhibition after extrusion. However, before extrusion it demonstrated an average of 16.1 mm/in of inhibition (completed in triplicate). Master-batch #2 did not demonstrate inhibition after extrusion against *L. monocytogenes.* However, inhibition was observed (Figure 7) against *M. luteus* (average inhibition 6.79mm/in). Before extrusion inhibition was 16 mm/in. The added heat reduced the antimicrobial activity of the nisin.

Encapsulation of nisin prior to extrusion was attempted to help create a more homogenous film without addition of heat. The researchers also thought it could help with the feasibility of adding the nisin to the hopper. Without encapsulation the nisin created a power cloud when added, resulting in loss of nisin. The other theory was the protection of heat in the barrel with the nisin being encapsulated. The nisin was encapsulated with beta cyclodextrin (Siro, I., Fenyvesis, E., Szente, L., De Muelenaer, B., Devlieghere, F., Orgovanyi, J., Senyi, J., Barta,J. 2006). The process was time consuming and the yield was low. However, the encapsulation was successful and extrusion was attempted. It was determined that a more homogenous blend made it more difficult for the nisin to release from the extrudate. Also, the encapsulation did not protect the nisin from the heat in the barrel. It produces an "ugly" film: the encapsulated nisin was sized too large, creating holes in the film and edge tears.

The next steps included using alternative methods to help the nisin release from the extrudate. Such techniques such as addition of plasticizer were used. However, the cast extruder available for experimentation did not have the necessary port that would

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help in metering the plasticizer. Another challenge with the plasticizer was slip. The screws in the barrel were not pushing the polymer forward due to the oily plasticizer. Therefore, the plasticizer addition was not feasible to use without a port.

The literature demonstrated that polylactic acid proved more successful because it degraded overtime-creating pores for the nisin to release from the film. Other methods such as addition of bovine albumin, salt or sugar were attempted to create small pores to help with diffusion. Although bovine albumin demonstrated promise, the thickness of the film was correlated with its success. Thick film produced on the cast extruder did not show a significant difference in diffusion when bovine albumin was added. If a thin film were produced the film would have defects such as large holes in the film and edge tears. The same phenomenon existed when the concentration of nisin was increased.

Finally, addition of ethylenediaminetetraacetic acid (EDTA) another antimicrobial was added to the formulation to see if a synergist effect could be achieved. EDTA is a liquid and could possibly act as a plasticizer. It was used in low concentration to prevent slip. However, the flash point of the EDTA was too low and was unable to be extruded.

The challenges in coating were minor in comparison to extrusion. The major issue was premature gelling when calcium was added. It was important to keep the solution warm when coating. Also slow addition of ingredients was necessary to prevent clumping. Finally, since the nisin was transparent when dissolved in acidified water it was difficult to measure when a homogenous mixture was achieved in solution.

**Table 6.1. Temperature Ranges**

| Target Temperature ºC | Temperature Range ºC |
|---|---|
| 140 | 135-147 |
| 160 | 156-170 |
| 170 | 169-177 |
| 180 | 176-181 |
| 190 | 189-200 |
| 200 | 198-205 |
| No Heating (control) | None |

**Table 6.2. Nisin thermal stability results**

| Temperature (ºC) | L. monocytogenes Average Inhibition zones (mm/in) | M. luteus Average Inhibition zones (mm/in) |
|---|---|---|
| 140 | None | 14.6 |
| 160 | None | 14.4 |
| 170 | None | None measurable but spotty |
| 180 | None | None measurable but spotty |
| 190 | None | None |
| 200 | None | None |
| No heating | 3.7 | 11.4 |

*2 minutes 30 sec at each temperature*

**Table 6.3. Master-batch Processing Parameters**

| Polymer | Master- batch #1: Elvax 3124 EVA 9% Va content (7g) | Master-batch #2: Elvax 3124 EVA 9% Va content (7.5g) |
|---|---|---|
| Nisin | Z (2.5%) (7g) | Z (2.5%) (2.5g) |
| Rear Zones | 140/140/139ºC | 140/140/139ºC |
| Front Zones | 140/141/148ºC | 140/141/148ºC |
| Force | 5700-10,000 | 3000 |
| Melt | 130ºC | 140ºC |
| Residence Time | 10 mins | 5mins |

**Table 6.4. Processing parameters for master batch extrusion**

| Polymer | Elvax 3124 EVA 9.0% VA content (200g) | Elvax 3124 EVA 9.0% VA content (192.4g) |
|---|---|---|
| Master-batch | #1 (4.0g) | #2 (7.6g) |
| Zone 1 | 230ºF/110.0ºC | 230ºF/110.0ºC |
| Zone 2 | 280ºF/137.8ºC | 300ºF/148.8ºC |
| Zone 3 | 286ºF/141.1ºC | 303ºF/150.6ºC |
| Die | 285ºF/140.5ºC | 301ºF/149.4ºC |
| Adaptor | 285ºF/140.5ºC | 301ºF/149.4ºC |
| Pressure (psi) | 1090 | 520 |
| Screw RPMs | 35.1 | 35.1 |
| Melt | 277ºF/136.1ºC | 290ºF/143.3ºC |
| F.P.M | 3.4 | 4.2 |
| Res. time | 2 min 17 sec | 1 min 45 sec |

**Figure 6.1. (A) Heat Stability Apparatus (B) Thermocouple measuring the temperature of the vial during heating.**
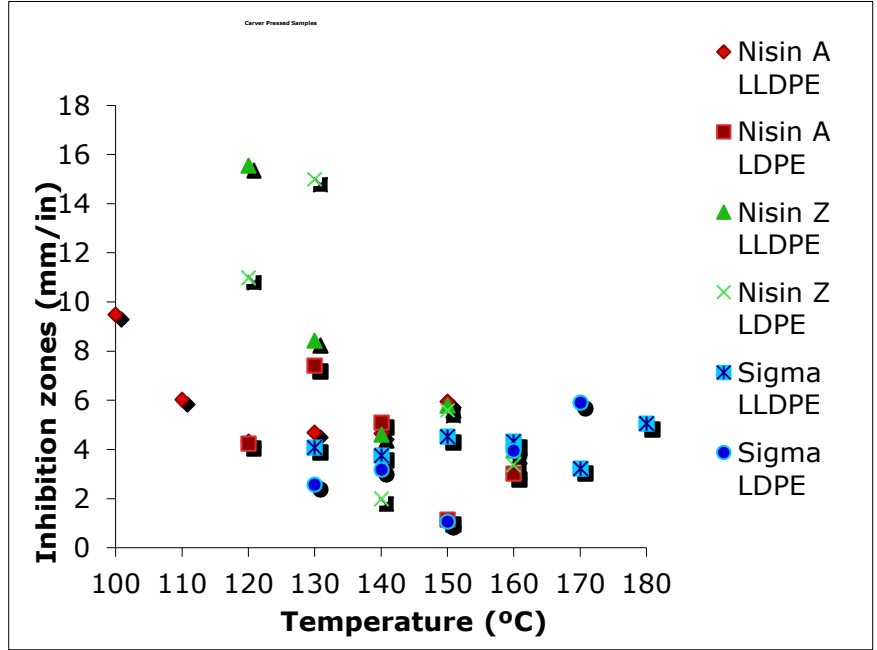


**Figure 6.2. Carver Heat Press**

**Figure 6.3. Carver heat pressed inhibition results for LDPE and LLDPE containing nisin.**



**Figure 6.4. Twin Screw Compounder seen in the open position.**

**Figure 6.5. PP1 Pelletizer**



**Figure 6.6. Plate overlay assay with film demonstrating antimicrobial activity**

# References

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