Material Properties of Xanthan Coating Applied to Poly(lactic acid) Films and Antimicrobial Effects of Xanthan Coating Containing Bacteriophages

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A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements for the degree of
Master of Science
in
Food Science

Guelph, Ontario, Canada
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ABSTRACT

MATERIAL PROPERTIES OF XANTHAN COATING APPLIED TO POLY(LACTIC ACID) FILMS AND ANTIMICROBIAL EFFECTS OF XANTHAN COATING CONTAINING BACTERIOPHAGES

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Antimicrobial active packaging systems utilizing biobased materials are of growing research interest. A biobased film of xanthan coated PLA was developed and characterized. Viscosity and moisture sorption of the coating were evaluated, in addition to water permeability, thermal and surface properties of the coated film. Microscopic analyses indicated that repeatable coatings could be created using a draw-down casting approach. The addition of bacteriophage preparation to xanthan solution did not significantly affect water barrier or coating consistency on PLA films. The *Listeria* phage A511 and *Salmonella* phage were immobilized in xanthan coatings, and the antimicrobial activity of the xanthan-coated PLA films evaluated.

Antimicrobial films were tested on cooked sliced turkey breast resulting in 1 to 2 log-reductions over the study period, with greater reduction in *Listeria* inoculated samples. The active packaging film can be applied as a separator or antimicrobial layer for sliced RTE meat products.

**Key Words:** Poly(lactic acid), xanthan, coating, active packaging, bioplastic, biocomposites, bacteriophage, ready-to-eat foods, food preservation, *Listeria, Salmonella*.
Acknowledgements

My sincere thanks to my advisor, Dr. Loong-Tak Lim, for his advice, guidance, and wisdom have allowed me to realize my fullest potential. I will be forever grateful for his patience, understanding, and sharing of knowledge academic and beyond. I’m certain the skills and life lessons I’ve garnered as a member of your team will stay with me for the rest of my life. I also would like to sincerely thank to my committee members, Drs. Keith Warriner and Manju Misra. I am very appreciative for all their advice and support given throughout this project.

Studies presented in section 6.0 were conducted in collaboration with Dr. Devon Radford and Dr. Sampathkumar Balamurugan of Agriculture and Agri-Food Canada’s Guelph Research and Development Centre. Many thanks for their contribution, assistance and expertise, without which it would have not been possible to complete such a large trial in a food system using xanthan-coated PLA system. Many thanks as well to the technicians at Agriculture and Agri-Food Canada, Philip Strange, Rafath Ahmed, who’s efforts also helped make this work possible. Additionally, I must thank Dr. Sandy Smith, for her kindness, expertise and instruction in SEM and Dr. Fernanda Svaikauskas, for her help with DSC analysis.

A special thanks to the Ontario Ministry of Agricultural, Food, and Rural Affairs (OMAFRA) for their financial support. Their support of this novel research has allowed for unique discoveries and has created opportunities for continued research within and outside our lab group and for this I’m very grateful.

I am also immensely grateful and appreciative for the friendship and support of my colleagues in our Food Packaging and Biomaterials Group, past and present. In the past three
years, I have had the pleasure of meeting and working alongside so many intelligent minds. My thanks to you all: Khalid Moomand, Xuiju Wang, Hanie Ahmadi, Roc Chan, Wenjing Wang, Wenyu Huang, Hans Tee, Apratim Jash, Jessey Cowell, Kanwarjeet Kaur, Maryam Bahram-Parvar, Yanan Zhang, Yasumi Horimoto, Linlin Wang, Matthew Zwicker, Caihua Shi, Zheng Zhang, Barbara Biduski, Gloria Wang, and everyone else I’ve crossed path with in my studies as a Graduate student, especially our amazing secretaries.

Thanks to my friends and colleagues in the Department of Food Science whom I’ve known since I started my Undergraduate degree, you have made the past 8 years memorable in countless ways. To my Mom and Dad, for their love, support, inspiration and shared devotion to food industry. You truly lead by example, as parents and professionals. To my sister, Brianna for her support and being there, and especially to my love, Judith, for your unconditional support, encouragement, and helping me stay positive throughout this journey. Thank you everyone.
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<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
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<tr>
<td>HHP</td>
<td>High hydrostatic pressure</td>
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<tr>
<td>HPMC</td>
<td>Hydroxypropyl methylcellulose</td>
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<tr>
<td>LDPE</td>
<td>Low density polyethylene</td>
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<tr>
<td>LLDPE</td>
<td>Linear low density polyethylene</td>
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<tr>
<td>MAP</td>
<td>Modified atmosphere packaging</td>
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<tr>
<td>MC</td>
<td>Methylcellulose</td>
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<tr>
<td>MMT</td>
<td>Montmorillonite</td>
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<tr>
<td>nAg</td>
<td>Nanosilver</td>
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<td>NanoTA</td>
<td>Nano thermal analysis</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OLA</td>
<td>Oligomers of lactic acid</td>
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<tr>
<td>PA</td>
<td>Polyamide</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
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<tr>
<td>PEVA</td>
<td>Poly(ethylene vinyl acetate)</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SM buffer</td>
<td>Sodium-magnesium buffer</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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<tr>
<td>WVTR</td>
<td>Water vapor transmission rate</td>
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1.0 Introduction

Enhanced pathogen control and product safety can minimize foodborne illness and associated wastage of contaminated food products. Food waste at retail and consumer levels remains an issue in developed countries such as Canada, USA and much of Europe. It is estimated up to one-third of food produced in some developed countries, such as those mentioned, may be thrown away (Muth, Kosa, Karns, & Buzby, 2007). Food wastage is especially prevalent in perishable products, such as meats. On per capita basis, meat consumption has grown 3% annually since the mid-1990s in developing and emerging markets in developing due to the rise of the middle class (Nigatu & Seeley, 2015). Fresh and ready-to-eat (RTE) meat products are susceptible to post-processing contamination by pathogens that may occur at the food production facility, retail level, and consumer’s home. As the demand for quality meat continues to increase with increasing global population, issues surrounding the safety and preservation of processed meat products is expected to become more prevalent. Accordingly, there is an increased need for meat packaging systems utilizing enhanced safety features, as well as sustainable packaging materials that minimize the impacts of production and distribution.

Conventional plastic packaging plays a vital role in delivering meat products to consumers. However, there is concern about the long-term impact of petroleum-based polymers, resulting in a growing demand for research focused on sustainable packaging materials derived from renewable resources (Reddy, Vivekanandhan, Misra, Bhatia, & Mohanty, 2013). Despite this demand, there are some challenges that must be addressed when implementing biobased materials to replace the conventional petroleum-based materials.
Biobased materials are sourced from renewable resources, in contrast to fossil resources which are generally considered non-renewable, given their renewal time of millennia. Poly(lactic acid) (PLA) is a biobased polymer comprised of lactic acid that is produced in the controlled fermentation of biomass. A growing area of research is to explore ways of improving and modifying the material properties of biobased packaging materials, since some properties may differ from petroleum based counterparts.

Active packaging contains supplementary components, included within or on the surface of the packaging structure, to enhance its protection function (Lim, 2011; Robertson, 2013). To address product shelf life and safety concerns, researchers have developed antimicrobial active packaging systems. Various antimicrobial constituents have been incorporated in the design of active packaging to extend shelf-life, improve quality, and ensure safety.

Bacteriophages have recently been approved as a processing aid in North America and Europe. Studies to date on the application of bacteriophage as a biocontrol agent indicate bacteriophages may be suited as part of a hurdle approach to ensure food safety. For example, bacteriophages have shown positive effects in controlling pathogen growth and have been applied as a spray or wash during pre- or post-slaughter/evisceration (Soon, Chadd, & Baines, 2011), direct incorporation into processed meat (Hussain et al., 2015), and as a spray applied onto raw meat (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Sukumaran, Nannapaneni, Kiess, & Sharma, 2015). Some research has been conducted on the immobilization of phage on polymeric surfaces for biomedical applications (Kim, Jin, Salieb-Beugelaar, Nam, & Stieglitz, 2014; Smerkova et al., 2013), or embedding phage using other
encapsulation technology systems (Tang et al., 2013; Vonasek, Le, & Nitin, 2014). To date, there is a limited, yet a growing amount of research on the use of bacteriophages in food packaging.

The following literature review details the two biobased materials of research interest in this project, PLA and xanthan. Active packaging approaches currently under research which integrate biobased materials are discussed and evaluated for enhancing shelf-life in meat products. Lastly, bacteriophages are introduced as a biocontrol agent, and those approved for use in food products and how they might be applied to meat products is investigated. This project is focused on improving the safety of fresh and RTE meats through the development of novel antimicrobial packaging in which bacteriophages have been embedded on the surface of PLA film using a xanthan coating.
2.0 Literature Review

2.1 Biobased Materials in Food Packaging

Maintenance of product quality, and safety throughout the food distribution chain are essential in providing consumer satisfaction. Furthermore, as consumers and manufacturers are becoming more knowledgeable on safety and sustainability throughout the food supply chain, the demand for packaging materials derived from renewable resources will increase. Biobased materials are made using substances derived from renewable resources, typically living or once-living organisms. The overarching theme of this project is to develop biobased antimicrobial packaging for RTE meat products, and demonstrate proof of concept in the packaging of cooked turkey breast. The biobased materials used to develop this packaging are PLA and xanthan gum.

2.1.1 Poly(lactic-acid)

Among commercially available biopolymesters, PLA has the greatest commercial use and is manufactured on a large scale. Worldwide production of PLA was approximately 320,000 tons in 2013 (Research In China, 2015). Ninety percent of this amount came from six large manufacturers, NatureWorks™ LLC being the largest with an annual capacity of 150,000 tons and 45.2% market share (Tiwari, 2016). PLA is a linear aliphatic polyester polymerized from lactic acid monomers obtained from controlled fermentation of glucose derived from corn, wheat starches, or whey lactose. The PLA has been successfully converted into clamshell packaging for fruit and vegetables, thermoformed cups for yogurt, bottles for beverages,
flexible films for pouches, and others (Carper, 2011; Lim, Auras, & Rubino, 2008; NatureWorks, 2017; Seglina et al., 2013). In the packaging of meat products, PLA has also been used to create expanded, thermoformed trays for fresh meat packaging (Schut, 2007) and films for packaging fresh or processed meat (Cannarsi, Baiano, Marino, Sinigaglia, & Del Nobile, 2005; Tawakkal, Cran, & Bigger, 2014).

One of the drawbacks preventing PLA from wide-range application in food products is its poor barrier to water vapor, as water vapor transmission (WVTR) values of PLA often higher than polyethylene (PE) and other packaging films. Methods of improving barrier in PLA film include biaxial orientation, altering ratio of L- and D-enantiomers, and use of nanomaterials as a filler or coating. Nano-scale materials, such as montmorillonite (MMT) layered silicate have been added to PLA as a filler to create nanocomposite packaging structures, given the thickness of MMT platelets can range 1-3 nm (Matsumura et al., 2008). The presence of these impermeable particles in PLA matrix increases the torturous path of permeant molecules, thereby reducing their overall transmission rate through the composite. To efficiently leverage this phenomenon for enhancing the barrier properties of PLA, homogeneous dispersion of MMT platelets in the polymer matrix is essential. Native MMT is hydrophilic and therefore incompatible with typical packaging polymers that are relatively hydrophobic. Therefore, the layered silicate is often modified, where sodium, lithium, calcium, etc. cations within the gallery of the layered silicates are exchanged with a surfactant (e.g., trimethyl stearyl ammonium, octadecylamine, and methyl dihydroxyethyl hydrogenated tallow ammonium). The exchange of cations within MMT with surfactants allows polymer chains or monomers to intercalate into the galleries in the MMT. By applying adequate shear during melt processing, the expanded MMT can be
exfoliated into separate silicate layers, the dispersion of which in a polymer matrix is critical to increase molecular tortuosity essential to enhance the barrier properties of the composite structure (Lim, 2011b; Sengwa & Choudhary, 2012; Singala, Mungray, & Mungray, 2012). The enhanced barrier properties of MMT nanocomposite, as compared with the neat polymer, can also be attributed to the MMT’s physical anchorage effects on the polymer molecules, which limits the segmental chain mobility. Though not tested in-depth in the current work, the addition of MMT and other means of barrier enhancement for PLA and other biobased films, could be promising for further research.

2.1.2 Xanthan

Xanthan gum is a polysaccharide derived from the controlled fermentation of glucose, sucrose, or lactose by *Xanthomonas campestris*. Xanthan is an approved food additive that has been used as an edible coating in many products, such as pre-cut potatoes, pears and peeled and trimmed baby carrots (Kim, Min, Lee, & Lee, 2012; Mei, Zhao, Yang, & Furr, 2002; Sharma & Rao, 2015). The application of a xanthan coating supplemented with cinnamic acid significantly reduced oxidative browning, resulting in less degradation of phenolics compared to uncoated pears (Sharma & Rao, 2015). Control pears browned quicker than xanthan-cinnamic acid coated pears as determined from sensory studies due to higher polyphenol oxidase activity. Mei, et al., (2002) investigated xanthan solutions, supplemented with α-tocopherol or calcium gluconate, as coating formulations for peeled baby carrots. Packaged peeled baby carrots were dipped into 0.3% (w/w) xanthan solution containing either 5% (w/w) calcium gluconate or 0.2% (w/w) α-tocopherol for 30 s then dried under ambient conditions for 1 hour. With the exception of
having a slight slippery surface, it was found the coated carrots had improved surface colour as compared with the control, without impacting taste, texture, aroma and overall flavor attributes over a 3-week storage period (Mei et al., 2002).

These studies demonstrate the feasibility of using xanthan as a carrier of antimicrobial agents for fruits and vegetables. In this present research, a delivery method is proposed by applying xanthan solution with bacteriophages on PLA film, allowing it to dry, forming an antimicrobial coating. The approach has several advantages. For one, moisture from the food product will be used to hydrate the xanthan layer on the food contact side of the film, causing the xanthan coating to swell and dissolve and thereby activating the release of the bacteriophage. Secondly, the resulting films could be used in conjunction with other antimicrobial controls used in the product, or with processes such as high hydrostatic pressure (HHP) treatments. These treatments are common for mitigating risk from pathogens, such as *L. monocytogenes*, especially in RTE meat and cheese products. Secondly, packaging converters can manufacture pathogen-specific antimicrobial films by incorporating antimicrobial agents that will target only specific microorganisms unique to specific food products. For example, raw meats are often at risk due to contamination by *Pseudomonas sp.*, *E. coli*, *Clostridium perfringens*, or *Salmonella sp.*, whereas cooked meats are susceptible to *L. monocytogenes* contamination. The method proposed in this work would facilitate packaging converters’ ability to create tailor-made antimicrobial films for mitigating microbiological risks a specific food product’s surface may be exposed too.
2.2 **Active Packaging in Meat products**

Active packaging contains supplementary components incorporated within or on the surface of the packaging structure to enhance the protective function of the packaging system (Lim, 2011a; Robertson, 2013). Many active components have been studied, some of which have been commercially exploited for active packaging systems, including antioxidants, antimicrobials, desiccants, absorbents, and so on. In this section, current advances in antimicrobial active packaging areas are reviewed.

2.2.1 **Antimicrobial Active Packaging Systems**

Polymers used in active packaging films vary widely and may include biobased variants such as PLA and polyhydroxybutyrate (PHB), or conventional polymers such as PE, polypropylene or multilayered composites. Antimicrobials can include plant extracts, essential oils, organic acids; as well as, antimicrobial ions and peptides. Thymol is a natural extract of thyme known to have antimicrobial effects on pathogens and spoilage organisms in meat products; as well as, provide antioxidative effects (Fratianni et al., 2010; Jayasena & Jo, 2013). Tornuk et al. (2015) investigated active linear low-density polyethylene (LLDPE) films dispersed with eugenol, carvacrol, or thymol-loaded halloysite for controlling discoloration, lipid oxidation and *E. coli* O157:H7 growth in fermented sausage and fresh beef. Fresh beef color was maintained up to 4 d using the active nanocomposite films and bacteriostatic/bactericidal effect was observed on *E. coli* O157:H7 up to 7 d of storage. Similarly, bacteriostatic/bactericidal effects were also observed in fermented Turkish-style sausage over a 30 d storage period at 4 °C due to the same treatments (Tornuk, Hancer, Sagdic, & Yetim, 2015). Coutinho de Oliveira et al. (2012)
extracted essential oils from winter savory (*Satureja montana* L.) for the preservation of mortadella style processed meats. They reported applying concentration above 15.60 μL/g adversely affected product colour, but a reduction in thiobarbituric acid value was still observed in treatment with 7.80 μL/g savory essential oil, the lowest concentrations tested without the addition of nitrite. These results show that the combined use of essential oils, with minimal or without the use of chemical preservative in cured meat products could be promising in extending the shelf-life of processed meat products (Coutinho de Oliveira et al., 2012).

In another study, MMT nano-clay and polyphenolic extract from brewery byproduct were incorporated into poly(ethylene vinyl acetate) (PEVA) and low-density polyethylene (LDPE) films during extrusion (Barbosa-Pereira, Angulo, Lagarón, Paseiro-Losada, & Cruz, 2014). Antimicrobial activities of the active films were evaluated using ASTM E 2149-01 standard test method under dynamic contact conditions. This test method is designed to test materials that will not readily leach or dissolve into an aqueous solution by applying constant agitation of the test specimen during the test period, and is hence dynamic, rather than static or stationary as in other testing methods for antimicrobial films. PEVA film with 6% w/w phenolic extract alone demonstrated antimicrobial activity against *E. coli* after incubation in the dynamic system for 48 h and onward until the end of the 6 d study period. The same film showed activity throughout the 6 d incubation period against *Staphylococcus aureus*. All PEVA active films with MMT-functionalized phenolic extract showed antimicrobial activity against *S. aureus* during 6 d incubation (Barbosa-Pereira et al., 2014). Studies by Tornuk et al. (2015) and Barbosa-Pereira et al. (2014) suggest there is an advantage in functionalizing MMT or halloysite with low molecular weight antimicrobials such as phenolic compounds to facilitate their release. Other
plant-derived anti-oxidative antimicrobial compounds for preserving fresh and processed meats include grape seed extract, green tea, rosemary, pomegranate, nettle and cinnamon (Shah, Bosco, & Mir, 2014).

2.2.2 Antimicrobial Proteins and Peptides

Lysins, also known as endolysins or murein hydrolases, are enzymes produced by bacteriophages that disrupt a host's cell wall during the final stage of the lytic cycle. Lysins are highly evolved enzymes that can target one of the five bonds in peptidoglycan (murein), the main component of bacterial cell walls, allowing the release of progeny virions from the lysed cell. Researchers have investigated these enzymes as biocontrol agents in food industry due to their effectiveness and specificity (Mahony et al. 2011; Garcia, Rodriguez, et al. 2010). Lysins may be a preferable biocontrol agent compared with antibiotics, because the indiscriminate use of the latter can result in bacterial resistance (Alanis, 2005). Lysins have shown to have a synergetic effect with nisin on reducing S. aureus growth in pasteurized milk (Garcia, Martinez, et al. 2010). Future studies should determine if synergistic effects can also be demonstrated in fresh or RTE meat products.

Nisin is a polycyclic antibacterial peptide produced by the bacterium Lactococcus lactis, which is used as a food preservative. Nisin has primarily been applied to meats and cheeses to control growth of gram-positive spoilage and pathogenic bacteria. It is common to use nisin in foods at levels ranging from ~1 to 25 ppm, depending on the food type and regulation in the jurisdiction of production. The effect of nisin combined with different packaging treatments on L. monocytogenes growth in RTE meat (turkey bologna) has been evaluated (Naas, Martinez-
Dawson, Han, & Dawson, 2013). Here, bologna was inoculated with *L. monocytogenes* and exposed to 100% CO₂, air, or vacuum MAP, each with and without nisin. *L. monocytogenes* counts were 1 to 2 log cfu/cm² lower for each treatment with nisin. Packaging with 100% CO₂ prevented outgrowth throughout 42 d of storage, whereas a 1 to 2-log increase in populations during storage was observed for air and vacuum packaged. Reportedly, nisin (500 IU/mL) combined with 100% CO₂ was the most effective in preventing the growth of *L. monocytogenes* on bologna during 42 d of refrigerated storage (Naas et al., 2013). More recently, another study has focused on assessing the bacterial communities in beef burgers stored in nisin-coated LLDPE packaging, and highlighted the effectiveness of this strategy in prolonging beef burger shelf life (Ferrocino, Greppi, Lastoria, Rantsiou, & Ercolini, 2016). In this study, the researchers evaluated beef from two different production lots. Control samples showed a significantly higher abundance of bacteria taxa sensitive to nisin, such as *Kocuria rhizophila*, *Staphylococcus xylosus*, *Leuconostoc carnosum*, and *Carnobacterium divergens*, as compared to the control samples. However, the nisin-treated batch exhibited a significantly lower amount of nisin-sensitive bacteria found on treated samples (Ferrocino et al., 2016). These studies demonstrate the effectiveness of nisin under different meat packaging configurations for inhibiting the growth of both spoilage and pathogenic bacteria.

### 2.3  Bacteriophage Use in Meat Packaging and Preservation

Information in the literature related to immobilization on or dispersion of bacteriophages into packaging film is growing, with research activities in this area expected to rise as manufacturers explore options to improve and ensure pathogen control in the increasing number of clean label products on the market. Modification techniques reported in the medical
literature to immobilize phage to a surface are typically based on exploiting genetic engineering tools to create novel bacteriophages with uniquely specific expressed proteins (Pavoni, Vaccaro, D’Alessio, De Santis, & Minenkova, 2013; Zanghi, Sapinoro, Bradel-Tretheway, & Dewhurst, 2007). Previous studies have investigated ground red meat (beef and pork) and poultry, inoculated with *Salmonella* at 7 log CFU/g, and treated with commercial bacteriophages S16 and Felix-O1 during meat tumbling (Yeh et al., 2017). Overall, the bacteriophage application resulted in 1.0 and 0.8 log CFU/g reduction of *Salmonella* in ground beef and pork, and 1.1 and 0.9 log CFU/g reductions in ground chicken and ground turkey, respectively (Yeh et al., 2017). Galarce et al. (2016) evaluated the effectiveness of a bacteriophage cocktail containing five lytic bacteriophages at reducing *Salmonella enterica* growth in cooked turkey ham and chicken sausage, as well as in cured sausage Italian salami and barbecue sausage. Samples of each product (n=25 for each) were inoculated with *S. enterica*, followed by treating with a phage cocktail, and then incubated for 10 d at 18 °C or 4 °C. The phage titers used were $10^8$ PFU ml$^{-1}$ and $10^9$ PFU ml$^{-1}$ to reach a multiplicity of infection of $10^5$ when applied to the milled food samples directly as a 2.5 mL aliquot. A significant reduction in bacteria, ranging from 0.5 to 2.1 log CFU/g depending on the product and temperature, was found for all products with the exception of the Italian salami (Galarce et al., 2016). Current approaches being tested in packaging film include embedding phage in a carrier matrix for delivery to a surface, as in the work by Vonasek et al. (2014), and Gouvêa, Mendonça, Soto, & Cruz, (2015). In this project, the approach of embedding phages was also adopted. In Vonasek et al., the antimicrobial activity of T4 bacteriophages immobilized in whey protein isolate films was tested on lettuce leaves. Via growth inhibition assay, a 5 log CFU/mL
reduction in counts of *E. coli* BL21 with respect to negative control was observed, as was the release of a microbiologically significant amount of immobilized phage from coating to leaf surface after 3 h contact time. In Gouvêa et al. (2015), bacteriophages with infectivity against *Salmonella Typhimurium* ATCC 14028 were immobilized in acetate cellulose films. Inhibition zones were produced in Muller-Hinton agar, and in growth curve analysis performed in liquid medium showed increased lag phase and slower growth of pathogens was observed in the presence of active cellulose acetate films compared to control. These studies indicate that immobilization of bacteriophage in an edible material may provide an effective means of antimicrobial delivery and reduce pathogen growth when applied to the surface of high moisture foods.

2.3.1 Approved Bacteriophages and Their Use in Food Systems

Listex™ P100 bacteriophage, which is considered as GRAS (Generally Recognized as Safe) by the USDA, specifically targets and lyses cells of pathogenic *L. monocytogenes* (USDA, 2006). For bacteriophage, the mode of antimicrobial activity on the surface of food products is due to ‘lysis from without’ (Abedon, 2011). This is the effect whereby multiple phage are bound to the surface of cells and viron adsorption occurs en masse without phage production. The lytic action on bacteria by exogenously supplied phage lysin brings about this phenomenon as well (Abedon, 2011). Listex™ P100 phage have been evaluated for inactivating *Listeria* by Chibeu et al. (2013) on RTE cooked turkey and roast beef products. They reported that at $10^7$ PFU/cm$^2$ dosage, *L. monocytogenes* was reduced by 2.1 log CFU/cm$^2$ for cooked turkey and 1.7 log CFU/cm$^2$ for roast beef, compared to the untreated meat samples. Listex™ also exhibits
listericidal effect on other meat products, including fresh sausages (Rossi et al., 2011) and fresh salmon fillets (Soni, Nannapaneni, Soni, & Nannapaneni, 2010). Other approved phage preparations that may be applied to meat product include Salmonellex™ and SalmoFresh™ (Micreos; Wageningen, Netherlands) targeting *Salmonella* spp. and ListoShield™ (Intralytix; Baltimore, Maryland), targeting *Listeria* spp. bacteria.

2.3.2 Extension to Meat Packaging

To extend the use of bacteriophages to packaging systems, current research has investigated means of maintaining phage stability and activity. Firstly, phage must be incorporated in a fashion that enables them to access the target pathogens. In the case of lambda phage, this would necessitate orientation to a film such that the head of the bacteriophage is attached to the film, and the tail facing outward towards the food system (see Fig. 2.1). Alternatively, the phage particles may be randomly dispersed within a polymer matrix which swells or dissolves in the presence of moisture, thereby releasing the phages to the food system. Secondly, the stability of the phage during storage conditions and exposure to processing environments (e.g., heat, pressure) must be investigated. In one study, researchers tested the virulence of three *Shigella flexneri* and a single *Vibrio cholerae* phage cocktails on salmon and mussels subjected to high hydrostatic pressure (HPP) (150-450 MPa for 5 and 9 min, 300 MPa for 13 min, or 550 MPa for 5 min) conditions (Ahmadi, Anany, Walkling-Ribeiro, & Griffiths, 2015). Complete inactivation of *S. flexneri* and *V. cholerae* (P < 0.05) was achieved at 550 MPa for 5 min or, more efficiently, at 350 MPa for 5 min followed by the addition of phages. Therefore, use of HHP and virulent bacteriophages proved more effective as combined
hurdle technologies then when used as stand-alone treatments for meat and seafood processing.

**Figure 2.1:** Bacteriophages aligned A) randomly and B) aligned with tail facing away from the packaging surface

From an end-use standpoint, long-term stability of the phage in the packaging material is important to ensure antimicrobial efficacy. This is of concern considering food packaging materials are usually being stored for a prolonged period in typical commercial settings. A few methods have been investigated to prolong phage stability. These include freeze drying and encapsulation. With respect to freeze drying, bacteriophages selective for *S. aureus* (strains 9563 and 8588, NCIMB, respectively) and *P. aeruginosa* (strain 217M) have been freeze-dried from sodium-magnesium (SM) buffer with or without the addition of gelatin (Puapermpoonsiri,
The lytic activity of freeze dried phage was compared against control samples stored in a tris-buffer (1 M Tris–HCl, 0.1 M NaCl, 8 mM MgSO₄, pH 7.5) with and without 0.1 g/L gelatin, and using sucrose or PEG 6000 as a stabilizer. Following lyophilization, phage samples were stored at 4 °C in sealed containers with silica gel and lytic activity was tested for up to 30 d. Bacteriophages encapsulated in freeze-dried formulations with sucrose and no gelatin were more stable than those in with PEG, while gelatin addition was found to have a slightly detrimental effect on phage stability (Puapermpoonsiri et al., 2010). In addition to freeze drying, encapsulation methods may also be useful in enhancing the stability of bacteriophages. For example, alginate or pectin biopolymers have been utilized to encapsulate CA933P phage (lytic for EHEC serotypes of E. coli) in microspheres (Dini, Islan, De Urraza, & Castro, 2012). Microspheres of emulsified pectin provided high encapsulation efficiency and protection against acidity, with phages remaining active after 30 min pH 1.6 exposure, and protecting the phage from pepsin activity (4.2 mg/mL). On the other hand, free phages were completely inactivated at pH 1.6 and with pepsin (0.5 mg/mL) after 10 min exposure. Another study developed a bacteriophage encapsulation system utilizing liposomal phospholipid bilayers for efficient phage delivery to the site of infection by multi-drug resistant strains of bacteria (Singla et al., 2016). In their study, liposome prepared with 1.5% (molar percent) lipid using soy phosphatidylcholine with Tween 80, and protamine sulphate was most stable at 4 °C, compared to formulations with a lower lipid content or with dicetyl phosphate in place of tween. The liposomes from phosphatidylcholine with Tween 80, and protamine sulphate showed insignificant reduction (0.096 log PFU/mL) in the number of entrapped phages during 9 weeks of storage. The phage liposomes were less stable at room temperature, and highly unstable at
37 °C (Singla et al., 2016). These findings from studies on encapsulation and freeze drying of phage show that while these methods are beneficial in protecting phage from enzymatic activity or enable delivery to tissues via the gastric system, refrigerated storage is still often required to maintain the bacteriophage in its protected state. Methods of stabilizing phage for inclusion in various meat products warrants further investigation.

Comparisons have also been made between storage of bacteriophages in dry versus wet/moist environments. Felix O1 phage encapsulated in microspheres of wet 0.8% (w/w) alginate and 5% (w/w) whey protein remained viable for up to 6 weeks at 4°C (Tang et al., 2013). Tang et al. (2013) also found that immersing alginate-whey microspheres with Felix-O1 in 40% w/w maltodextrin solution overnight, prior to drying the microspheres at 22 °C for 30 h under a laminar flow hood, imparted stabilizing effects maintaining phage viability over the 16 d storage period. Comparable results were observed in another study on bacteriophages encapsulated in chitosan coated alginate microspheres for oral delivery (Ma et al., 2012). In another study, the antimicrobial efficacy and stability of phage in whey protein isolate have been characterized using E. coli growth inhibition assay and plaque forming unit determination (Vonasek et al., 2014). The researchers reported that the edible films with stabilized phages during storage at 22 °C on a light exposed laboratory bench, and in dark, 4 °C conditions without significant loss in phage infectivity for greater than one month.

These studies demonstrate that encapsulation technologies are promising to enhance the stability of bacteriophages, to ensure that they survive the thermal and pressure treatment processes essential to achieve the required lethality on the target pathogens. This will give
meat processors the flexibility to add approved bacteriophages as an in-process treatment and as an additional hurdle prior to packaging of finished product.

3.0 Objectives

The use of biopolymers in sustainable antimicrobial active packaging require continued investigation and research before implementation in the marketplace. The overall goal of this research is to develop an antimicrobial packaging film having immobilized bacteriophages within a xanthan coating for direct food contact applications. The specific objectives of this research are to:

1. Develop an edible coating for PLA film and evaluate material properties (including permeability, coating viscosity, thermal properties and topography) of the resultant composite.

2. Evaluate the potential of the xanthan coated PLA film as a carrier for bacteriophages to reduce bacteria growth in vitro and in a model food system of cooked turkey breast.

3.1 Background

In section 4.0, different xanthan coating formulations are evaluated and a final coating formulation of 2% (w/w) xanthan with 1% (w/w) glycerol was evaluated further using DSC, AFM, water barrier, and contact angle. A manuscript with results from this section has been prepared for submission to the Journal of Biobased Materials and Bioenergy. Prior to this testing, a series of preliminary studies were conducted to determine a compatible coating material for antimicrobial delivery on PLA film. A complete listing of materials tested and observational results can be found in Table 8.1 of the Appendix. Materials evaluated for their
coating and PLA compatibility included chitosan, poly(ethylene oxide) (PEO)-alginate blends, waxy and non-waxy corn starch, corn zein, and ethylcellulose/methylcellulose blends. Compatibility of these coating polymers with PLA was evaluated via roll-casting and draw-down casting the respective polymer solutions on extrusion cast PLA films. Preliminary experiments led to the development of xanthan formulation and draw-down coating methods, which were used for further investigation in this section. Some of the key findings from the preliminary studies on xanthan coated PLA are highlighted in Table 8.2 of the Appendix.

In section 5.0, xanthan gum and chitosan coatings are evaluated for their ability to act as a carrier for bacteriophage. Antimicrobial effects of PLA with either chitosan or xanthan-coatings, with and without the addition of bacteriophage in the coating formulations are tested on nutrient and selective agars. Collaborating with researchers at Agriculture and Agri-Food Canada (AAFC), Guelph, methods of testing the efficacy of antimicrobial xanthan-coated PLA were developed and carried out in section 6.0, using plate culture and RTE cooked turkey breast model system. Microbial assays were conducted at AAFC’s Guelph Research and Development Center. A manuscript based on the methodology developed and results obtained in this section has been published in the Journal of Food Microbiology, and the paper authored by Devon Radford, Brandon Guild, Loong-Tak Lim, and Sampathkumar Balamurugan.
Material Properties of Xanthan Coated Poly(lactic acid) film

4.1 Introduction

During the formulation of commercial foods, product safety and stability are of increasing concern to manufacturers. To the retailer, longer product shelf life is essential to ensure sales. Maintaining product stability throughout the food chain is essential in providing consumer satisfaction. Many plant-derived polysaccharides possess film-forming properties for edible applications. Edible coatings derived from plants are sustainable and low cost solutions for enhancing shelf-life of ready-to-eat foods such as sliced meats, cheeses, and pre-cut fruits and vegetables. For example, xanthan gum is a polysaccharide derived from controlled fermentation of glucose, sucrose, or lactose by Xanthomonas campestris. It is an approved food additive that has been used as a coating on many products, such as potatoes, pears and carrots (Kim et al., 2012; Mei et al., 2002; Sharma & Rao, 2015).

The primary functions of packaging are containment, convenience, protection and preservation; as well as, communication (Robertson, 2013). Active packaging refers to packaging systems in which constituents have been added to provide benefits supplementary to the primary functions packaging. Active packaging enhances the protection aspect of packaging, such as moisture control, barrier enhancement, oxygen scavenging, and antimicrobial (Mihindukulasuriya & Lim, 2014; Robertson, 2013). Antimicrobial active packaging constitutes a large and growing subcategory of active packaging. These packaging systems are of recent interest because natural bioactive agents, such as plant extracts and bacteriophages, when incorporated into packaging material, can have preservative effects on the food product and enhancing shelf life.
Increased environmental consideration has led to the development of new materials which abstain from the use of petroleum-based matrices and synthetic additives for food packaging. Among the biobased polymers, poly(lactic acid) (PLA) is the one of the most widely adopted compostable polymers for packaging of food products, such as chips, processed sliced meats, salads, beverages, yogurts, and more (Dukalska et al., 2013; Guo, Jin, Wang, Scullen, & Sommers, 2014; Llana-Ruiz-Cabello et al., 2016; NatureWorks, 2017). Researchers have adopted strategies to impart antimicrobial properties to enhance the end-use performance of biobased polymers, such as applying a coating material with bioactive components, or embedding such components directly into the polymer matrix during processing. For example, in RTE meats, edible antimicrobial coatings comprise of chitosan, lauric arginate ester, and nisin have been developed (Guo, Jin, & Yang, 2014). The coating solutions were prepared by dissolving 2-5% (w/w) chitosan in an acid solution containing 2% acetic, 2% lactic, and 2% levulinic acids. RTE, pre-sliced turkey samples were directly coated with the solutions, or brushing the bioactive coating on PLA film followed by drying in a vacuum oven. Antimicrobial coatings of chitosan with LAE achieved greater log-reduction in *Listeria innocua* growth than films with nisin. No significant difference in effectiveness was observed between direct coating on the meat samples and coating on the PLA film.

In another study, oligomers of lactic acid (OLA) (a plasticizer) and carvacrol (essential oil in oregano) were incorporated in PLA-PHB blends (Burgos et al., 2017). The composite film of PLA+PHB+OLA+carvacrol exhibited improved barrier to water vapor and oxygen compared to PLA alone, but this improvement diminished as the OLA content increased, due to plasticizing effect of OLA. Antioxidative and antimicrobial (*Staphylococcus aureus* and *Escherichia coli*)
activities of the films were also increased with increasing carvacrol content (Burgos et al., 2017). Catechins are tea extracts known to possess bioactive properties as antioxidants in the cells of organisms, and can also reduce lipid oxidation in foods, with beneficial effects on shelf life (Lante & Friso, 2013). The antioxidant catechin has been incorporated into biobased composites of PHB and PLA made with acetyl(tributyl citrate) as a plasticizer (Arrieta et al., 2014). The addition of the plasticizer increased catechin release from the composite film. Thermal analysis using differential scanning calorimetry (DSC) showed that onset oxidation temperatures for all formulations increased due to the addition of catechin, indicating enhanced film thermal stability.

While these studies have evaluated the material properties of bioactive PLA, none have utilized edible soluble coating, such as xanthan, as a carrier to deliver bioactive species to contacting food surfaces. The objective of this work is to evaluate the material properties of xanthan coated PLA film for antimicrobial active food packaging applications. Here, a xanthan coating is developed with desirable coating attributes with respect to viscosity and surface wetting properties. The finalized xanthan coating formulation was then characterized using microscopic and analytical methods.
4.2.1 Materials

PLA film was prepared from 4032D polylactide resin (NatureWorks™ LLC, Minnetoka, MN, USA) using a lab-scale extruder (Microtruder RCP-0625, Randcastle, NJ, USA). This extruder has four heating zones; the first three zones line the barrel with the fourth zone controlling heat at the lip die fitted to an interchangeable cartridge section at the base of the extruder. The first zone was set at 300 °F, zones 2 and 3 at 360 °F, and zone 4 at 300 °F. Chill roll temperature was set to 15 °C, and 25 rpm. The slit height of the lip die was adjusted to a maximum gap of 1.17 mm (0.046 inches). The screw had a diameter of 15.875 mm (0.625 inches) with an L/D ratio of 24:1. Prior to extrusion, the PLA resins were dried in a vacuum oven overnight at 60 °C and 20 inHg. The dried resins were loaded into the hopper which was sealed off from the environment to prevent moisture absorption.

Xanthan gum from *Xanthomonas campestris* was obtained from Sigma Aldrich (St. Louis, MO). Glycerol was from Fisher Scientific (Fair Lawn, NJ). *Listeria* phage A511 phage was donated by the Institute of Food, Nutrition and Health (Eidgenössische Technische Hochschule; Zurich, Switzerland) and was used in previous works by Klumpp et al., (2008). It was re-propagated at Agriculture and Agri-Food Canada’s Guelph Food Research Center by the method described in Radford, Ahmadi, Leon-Velarde, & Balamurugan, (2016). Briefly, host cultures of *Listeria* were propagated on the surface of bacterial lawns grown under refrigeration conditions in which *Listeria* and their A511 phages grow in equilibrium. Room temperature sterile sodium-magnesium (SM) buffer was added to the lawn surface in 5 mL aliquots and plates incubated at 4 °C. After 16-24 h, as much as possible of the surface liquid was recovered from plates with *Listeria* lawns flooded with the 5 mL buffer. The recovered suspension consisting of free phage
and cell debris was then filter-sterilized using a 0.2 mm syringe filter. Phage was suspended in SM buffer (pH 7.5), prepared from the following components: 100 mM NaCl (Fisher Scientific; Fair Lawn, NJ), 10 mM MgSO$_4$ (EMD Millipore; Etobicoke, ONT), and 50 mM Tris-HCl (Sigma Aldrich; St. Louis, MO). For water vapor transmission testing, desiccant salts (LiCl, MgCl$_2$ and NaCl), were obtained from Fisher Scientific, (Fair Lawn, NJ).

4.2.2 Methods

4.2.2.1 Coating of PLA Film

The extruded PLA films had an average thickness of 30 ± 2 µm, measured using a digital micrometer (Model 49-70, TMI New Castle, DE). To prepare the xanthan coating solution, xanthan polymer at 2% (w/w) was dispersed in ultrapure water, added with 1% (w/w) glycerol, and mixed in a beaker using magnetic stir bar. To prepare the coating solutions, Listeria phage A511 phage in SM buffer was added to the xanthan solution at 1% (w/w) in a centrifuge tube and then vortexed to ensure the phage solution was evenly dispersed. PLA films were coated using a 15.24 cm (6 inch), 12.7 µm (0.5 mil) gap clearance drawdown applicator (BYK Gardner, Alt, GA, USA). Films were dried in an environmental chamber at 23 °C for 24 h and equilibrated at 0% relative humidity (RH) in a desiccant chamber. For atomic force microscopy (AFM) examination and water sorption testing, an additional drying step of at least 12 h in a vacuum oven at 20 inHg and 45 °C for 24 h was performed on xanthan coated films prior to equilibration at 0% RH. Film coating formulations used in viscosity and moisture sorption testing are indicated in Table 4.1.
Table 4.1: Different Xanthan coatings evaluated for water sorption testing and capillary viscosity

<table>
<thead>
<tr>
<th>Glycerol (G), % (w/w)</th>
<th>0.5</th>
<th>1*</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthan (X), % (w/w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1X-0.5G</td>
<td>1X-1G</td>
<td>1X-2G</td>
</tr>
<tr>
<td>2</td>
<td>2X-0.5G</td>
<td>2X-1G</td>
<td>2X-2G</td>
</tr>
<tr>
<td>4</td>
<td>4X-0.5G</td>
<td>4X-1G</td>
<td>4X-2G</td>
</tr>
</tbody>
</table>

*For capillary viscosity testing, 1, 2 and 4% (w/w) concentrations of xanthan were tested, all with 1% (w/w) glycerol plasticizer

4.2.2.2 Viscosity of Coating Solutions

Viscosity values of coating solutions were determined using a shear capillary rheometer mounted on an Instron Universal Testing Machine (Model 1122; Instron, Norwood, MA, USA). Tests were performed at different crosshead speeds (10, 12.5, 15, 17.5, and 20 mm/min). The length of the capillary was 155 mm and the inner diameter was 2 mm. The area of the plunger was 5.3 cm². The viscosity was measured by examining the relationship between the pressure drop from the solution side to the open side of the capillary (related to shear stress) and the flow rate through the capillary (related to shear rate) (Alborzi, Lim, & Kakuda, 2010; Carreau, De Kee, & Chhabra, 1997; Moomand & Lim, 2015). The shear stress, \( \sigma \) (Pa), was calculated as follows:

\[
\sigma = \frac{F}{4A_p \left(\frac{L_c}{D_c}\right)}
\]
where $F$ is the force on plunger (kgF), $A_p$ is the plunger area ($m^2$), $L_c$ is the length of the capillary (m), and $D_c$ is the capillary diameter (m). The Rabinowitsch correction was applied as follows when calculating the shear rate, $\dot{\gamma}$ (s$^{-1}$):

$$\dot{\gamma} = \frac{3n + 1}{4n} \times \frac{8V d_p^2}{d_c^3}$$  \hspace{1cm} (2)

where $n$ is the flow behavior index (dimensionless), $V$ is the crosshead speed (m/s), $d_p$ is the diameter of plunger (m), and $d_c$ is the capillary diameter (m). Regression analysis was used to determine the $n$ value from the shear data for each of the xanthan concentrations. This $n$ value was cross-checked by fitting a power law model to a plot of shear stress vs. shear rate for each coating solution. Subsequently, the apparent viscosity, $\mu$, expressed in Pa.s was calculated as follows:

$$\mu = \frac{\sigma}{\dot{\gamma}}$$  \hspace{1cm} (3)

And the power law model was as follows:

$$\mu = m \dot{\gamma}^{n-1}$$  \hspace{1cm} (4)

where $m$ is the consistency coefficient (Pa.s$^n$). An $n$ value of one is considered as Newtonian fluid and less than one a shear thinning solution. Formulations of xanthan tested for viscosity tested were 1, 2 and 4% (w/w) xanthan, with 1% (w/w) glycerol added to each formulation as a plasticizing agent.
4.2.2.3 Water Sorption

Moisture sorption behaviors of oven-dried film samples with xanthan coating of different formulations were studied gravimetrically during storage at 75% RH and 4 °C. Weight changes for three replicates of each film treatment (Table 4.1) were measured over a 24 h period, recorded by measuring the total weight of the sample unit (film and aluminum tray) over time using an analytical balance (Mettler Toledo, model XPE 205). RH of 75% was maintained using saturated NaCl solution in air-tight glass jars as per ASTM E104-05. Changes in moisture content of the dried films was modelled using the first order kinetics equation below:

\[
\frac{M_e-M}{M_e-M_o} = Ae^{-kt}
\]

where \(M_o\) and \(M_e\) are the initial and equilibrium moisture contents, respectively and \(M\) is the measured moisture content dry basis at given time point. \(K\) is the rate constant for moisture sorption and \(A\) is a constant. Parameters in Eq. 5 were estimated using the Solver add-In function in Microsoft Excel™.

4.2.2.4 Water Permeability

Water vapor transmission rate (WVTR) analysis of coated films was performed at 11, 33 and 75% RH using saturated salt solutions per ASTM E104-05. Film samples were cut into circles (22 mm radius) and sealed to disposable 38 mm inner diameter flanged aluminum dishes (Fisher Scientific Canada, Mississauga, Canada) using wax. An equivalent amount (4 g) of
desiccant (Drierite™, Hammond, WA) was placed in each aluminum dish through a slot cut on the side wall of the aluminum dish and then sealed with two layers of foil tape (Fig. 4.1).

Four film replicates were tested for each treatment (control, coating faced to humidity side, coating faced to desiccant side). Experiment was conducted at 4 and 21 °C, three levels of RH at each temperature. Film thickness for each treatment was calculated as the mean value of five measurements using a digital micrometer (Model 49-70, TMI, DE). The WVTR value was calculated from the slope \( K \) of a linear regression of weight loss versus time plot, using Eq. (6) and converted to water vapor permeability \( P \) in kg.m/m².s.Pa using Eq. (7):

\[
\text{WVTR} = \frac{K}{A} \quad (6)
\]

\[
P = \frac{\text{WVTR} \times l}{\Delta P} \quad (7)
\]

where \( l \) is the film thickness; \( A \) is the film exposed area (determined by \( \pi r^2 \)), \( \Delta P \) is the difference in water vapor partial pressure across the film at 4 or 21°C.
4.2.2.5 Differential Scanning Calorimetry

A differential scanning calorimeter (Model 2910; TA Instruments, New Castle, DE, USA) was used to study the thermal properties of the xanthan coating caused by the exposure to different relative humidity conditions. Xanthan coating was scrapped from coated PLA film samples and was conditioned at different RH levels for at least 24 h in open, in unsealed DSC pans. After 24 h, samples of 2-3 mg xanthan were weighed accurately and sealed in flat, aluminum DSC pans prior to measurement. All tests were conducted under a nitrogen flow rate of 25 mL/min and a heating rate of 5°C/min. Indium was used for the heat flow and temperature calibration. Data was graphed and midpoint \( T_g \) was determined using Universal Analysis 2000 software (TA Instruments; New Castle, DE).
4.2.2.6 Scanning Electron Microscopy

Morphology and cross sectional interface of films were examined using a scanning electron microscope (Model S-570; Hitachi High Technologies Corp., Tokyo, Japan) at an accelerating voltage of 10 kV. Coated film samples were cryo-frozen with liquid N₂, then fractured to review the bulk morphology. Film samples were coated with 10 nm of gold using a sputter coater (Model K550, Emitech, Ashford, Kent, England) before imaging in the microscope.

4.2.2.7 Atomic Force Microscopy

Atomic force microscopy was performed using an afm+™ system (Anasys Instruments, Santa Barbra, CA) to examine the surface topography of the coating. Scans were performed in tapping mode with phase, height and amplitude channels used for data collection. Nano thermal analysis (nanoTA™) was also used to evaluate the thermal properties of xanthan-coated PLA, xanthan coated glass and neat PLA films. Data collected was analyzed using Analysis Studio version 3.12 (Anasys Instruments, Santa Barbra, CA). To provide a complete comparative assessment, three distinct types of film were scanned; neat PLA, 2% (w/w) xanthan with 1% (w/w) glycerol coated on neat PLA, and the same formulation of xanthan coated on a glass microscope slide.
4.2.2.8 Contact Angle Assessment

A goniometer (Model A-100, Ramé-Hart Inc.) was used to study the wetting properties of xanthan-coated and uncoated PLA films. Contact angle measurements of Milli-Q filtered water on xanthan coated PLA (2% w/w xanthan with 1% w/w glycerol) and plain PLA films were taken in 5 min intervals over the span of 1 h. The procedure was repeated three times for each treatment.

4.2.2.9 Statistical Analysis

To determine statistical significance and apply post-hoc testing for coating viscosity, water adsorption, and water permeability data, SPSS statistics ver.24 (IBM, Armonk, New York, U.S.) was used. AFM data was analyzed using Analysis Studio (version 3.12; Santa Barbara, California).

4.3 Results and Discussion

4.3.1 Viscosity of Coating Solutions

All xanthan coating formulations exhibited shear thinning flow behaviors (Fig. 4.2). Allouche et al. (2015) reported a similar viscosity trend on xanthan in their studies, although their xanthan solution was tested at a lower concentration of 0.08% (w/w) over a broader range of shear rate, ranging from $10^{-4}$ to $10^{3}$ s^{-1}. Also, in agreement with work by Hsia and Smith, (1992) the consistency coefficient increased with increasing xanthan concentration. In
the present study, shear rate tested ranged from $1 \times 10^2$ to $3 \times 10^3$ s$^{-1}$. This corresponds to the shear rate range of medium viscosity solutions typically used in draw-down or brushing applications (Duffy, 2016). Increasing xanthan concentration from 2 to 4% (w/w) resulted in dramatic increase in apparent viscosity for a given shear rate, nearly 4 times greater than 1 or 2% (w/w) xanthan concentrations. During the draw-down application, the 2% (w/w) xanthan solutions resulted in uniform coating layer. However, the 1% (w/w) xanthan solutions tended to bead-up from the PLA surface, resulting in less uniform coating. As such, the 2% (w/w) xanthan solution plasticized with 1% glycerol was chosen for PLA coating in WVTR analysis and microscopy. At 4% (w/w) concentration, the xanthan solutions could not be spread consistently on the PLA film surface due to high viscosity.

The effects of divalent cations on the physical properties of a xanthan solution and the release properties of a bioactive drug has been investigated (Baumgartner, Pavli, & Kristl, 2008). The researchers observed initial slow release of the drug pentoxifylline from the xanthan tablets, which was governed by polymer relaxation. In the presence of calcium ions, the rate of drug release increased, as the mass transport became diffusion controlled. This phenomenon was explained by the charge screening of ionized groups on xanthan side chains, lowering the inter-molecular repulsion. In their study, the xanthan to calcium chloride dihydrate ratio ranged from 12:1 to 2:1 based on weight. In the present study, a much greater amount (2% w/w) of xanthan was used, compared to the 10 mM MgSO$_4$ present in the SM buffer that was added at 1% (w/w) level. Therefore, the effect of Mg$^{2+}$ on the solubility and rheological properties of xanthan solution is expected to be minimal.
4.3.2 Water Sorption

Changing the amount of glycerol in film formulations increased moisture sorption properties of the xanthan coatings. As shown in Figs. 4-6, equilibrium moisture content ($M_e$) values for coating with 2% (w/w) glycerol content were significantly higher than those with 1 and 0.5% (w/w) glycerol concentrations ($p<0.05$). The increased equilibrium moisture contents can be attributed to the increased plasticization and hydrophilicity of the coating layer. Similar effects of glycerol on other biopolymer have been reported in the literature (Arik Kibar & Us,
2013; Imran, El-Fahmy, Revol-Junelles, & Desobry, 2010; Lim, Mine, & Tung, 1999). No significant differences were found between treatments for the A values (p<0.05). On the other hand, films prepared with 1% (w/w) xanthan had a larger rate constant (k) for hydration than those with xanthan concentrations of 2 or 4% (w/w) (p<0.05). This could be related to the lower density of material on 1% (w/w) coatings, which could be more readily hydrated by water vapor molecules. A previous study carried out over a longer duration (~189 d) concluded that an equilibrium moisture isotherm cannot be derived for PLA once the irreversible hydrolysis of ester linkages has taken place after exposure to elevated temperature and high humidity, with these conditions resulting in moisture uptake (Holm, Ndoni, & Risbo, 2006). Therefore, future work should aim to perform moisture testing over a longer period at elevated RH, on neat PLA and xanthan coated PLA films to determine the effect of xanthan coating on the moisture sorption for the composite structure. Another study reported that the dry basis moisture content in melt-pressed PLA films stored at 40 °C were in the range of 0.6 to 0.8 %, slightly higher than equilibrium moisture content seen in this research (Cairncross, Becker, Ramaswamy, & O’Connor, 2006). The \( M_e \) values reported in Table 4.2 are slightly below previously reported values for PLA sorption, likely due to lower experimental temperatures at 4°C.

The rapid rate of water sorption that plasticized the coating matrix may be beneficial to induce the release of the bacteriophages – a phenomenon that can potentially be exploited for the preservation of high moisture food products. Other works have investigated the release of phage from xanthan gum in a cell culture and ready to eat cooked turkey breast (Radford et al. 2017). Their findings showed 99% of bacteriophages incorporated into xanthan coated PLA
prepared by the methods herein could be released onto the surface of ready-to-eat cooked turkey breast after 30 min contact with the food surface.

From the viscosity and moisture sorption testing, the 2% (w/w) xanthan with 1% (w/w) glycerol formulation was the most suitable coating formulation and was applied for further evaluation in permeability, DSC and microscopic evaluation.
Table 4.2: Mean and standard deviation values of moisture sorption kinetic parameters
(Equation 5) for xanthan coatings at 4°C and 75% RH. $M_e$ is the equilibrium moisture content, dry basis; $A$ and $k$ are pre-exponential and rate constants

<table>
<thead>
<tr>
<th></th>
<th>$M_e$ (%)</th>
<th></th>
<th>$A$ (%)</th>
<th></th>
<th>$k$ (1/h)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1% (w/w) Xanthan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% (w/w) glycerol</td>
<td>0.137%</td>
<td>± 0.046</td>
<td>100.48a</td>
<td>± 0.00841</td>
<td>7.20E-01a</td>
<td>± 0.00698</td>
</tr>
<tr>
<td>1.0% (w/w) glycerol</td>
<td>0.374%</td>
<td>± 0.238</td>
<td>99.99a</td>
<td>± 0.00110</td>
<td>7.24E-01a</td>
<td>± 0.18218</td>
</tr>
<tr>
<td>2.0% (w/w) glycerol</td>
<td>0.487%</td>
<td>± 0.167</td>
<td>100.11a</td>
<td>± 0.00161</td>
<td>6.86E-01a</td>
<td>± 0.16332</td>
</tr>
</tbody>
</table>

| **2% (w/w) Xanthan** |           |          |          |          |            |          |
| 0.5% (w/w) glycerol | 0.392%    | ± 0.118  | 99.95a   | ± 0.03321| 5.22E-01b  | ± 0.20266|
| 1.0% (w/w) glycerol | 0.305%    | ± 0.177  | 102.12a  | ± 0.02147| 4.43E-01b  | ± 0.07261|
| 2.0% (w/w) glycerol | 0.565%    | ± 0.222  | 100.90a  | ± 0.01259| 6.00E-01b  | ± 0.33947|

| **4% (w/w) Xanthan** |           |          |          |          |            |          |
| 0.5% (w/w) glycerol | 0.338%    | ± 0.034  | 98.15a   | ± 0.03039| 4.60E-01b  | ± 0.07647|
| 1.0% (w/w) glycerol | 0.511%    | ± 0.235  | 102.26a  | ± 0.01929| 4.60E-01b  | ± 0.09780|
| 2.0% (w/w) glycerol | 0.698%    | ± 0.075  | 101.10a  | ± 0.01522| 4.87E-01b  | ± 0.22845|

*N=3 for each film treatment
**Means in a column for a variable with the same lower case letters are not statistically different at $\alpha=0.05$
Figure 4.3: Moisture sorption by 1% xanthan coated PLA film coatings with different levels of glycerol during 24 h storage at 75% RH and 4°C

Figure 4.4: Moisture sorption by 2% xanthan coated PLA film coatings with different levels of glycerol during 24 h storage at 75% RH and 4°C
4.3.3 Water permeability

PLA films, with and without additional phage in the xanthan coating, had significantly higher permeability values at 21°C than at 4°C (p < 0.05). No significant differences were observed due to coating orientation (i.e., coating faced down towards desiccant or up towards the environment). The coated films had significantly higher permeability values when tested at 75% and 33% RH than those evaluated at 11% RH (p < 0.05). The addition of 1% (w/w) of phage to the xanthan coating formulation did not significantly impact the permeability. The orientation of the coating did not affect significantly the water vapor permeability of the coated film, likely due to the relatively thin layer of the coating and high water transmission rate of the base PLA film. The results support the observation that mass transfer must be governed by the bulk PLA layer, measuring 30 µm in thickness compared to 2-3 µm coating. Results in Table 4.2
indicate that the effect of RH on water permeability was minimal. This observation agreed with data reported in the literature that water permeability values of PLA values do not change considerably with RH (Auras, Harte, & Selke, 2004).

On the other hand, if the xanthan layers were to be thicker, the water permeability value would be expected to increase as RH increased due to increased swelling of the polymer matrix, as in many biopolymers. Kristo and Biliaderis (2007), investigated water absorption isotherms, kinetics and barrier properties of nanocomposite films of sorbitol plasticized pullulan filled with 0–40% (w/w) starch nanocrystals. Water absorption led to chain increased chain mobility and water diffusivity. They observed that the aw level on each side of a film played an important role in water permeability and impacted the driving force of molecules across the film (Kristo & Biliaderis, 2007).

Whether the improvement over PLA provided by the xanthan coating could enhance product shelf life in a dry film application of composite matrix would require further research. Other biobased materials such as methyl cellulose and hydroxypropyl methyl cellulose are known to have efficient O₂ and lipid barrier properties while also exhibiting film forming ability (Robertson, 2013; Umaraw & Verma, 2015). It would be of interest to investigate if a composite or nanocomposite coating could be developed using these materials.
Table 4.3: Effect of relative humidity on water permeability (kg.m/m².s.Pa) for xanthan coated ploy(lactic acid) films at different temperatures with and without addition of Felix-O1 bacteriophages to coating formulation

<table>
<thead>
<tr>
<th></th>
<th>11% RH</th>
<th>33% RH</th>
<th>75% RH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td>No phage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>4.23E-15&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>1.38E-15</td>
<td>5.11E-15&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>21°C</td>
<td>1.52E-14&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>7.21E-16</td>
<td>3.97E-14&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>With phage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>4.12E-15&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>5.59E-16</td>
<td>4.54E-15&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>21°C</td>
<td>3.83E-15&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>3.30E-16</td>
<td>3.95E-14&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*N=12 each temperature and RH combination

**Means in a row for a given temperature with the same lower case letters (c, d) are not statistically different at α=0.05

***Means in a column for a given RH are statistically different (a, b) for temperature at α=0.05

4.3.4 Differential Scanning Calorimetry

As in other hydrophilic polymers, the exposure of xanthan to elevated RH increased the moisture content of the biopolymer, resulting in a depression of glass transition temperature ($T_g$). Xanthan coating dried in a vacuum oven and stored at 0% RH had a midpoint $T_g$ of -13.4 ± 1.9 °C (Figs. 4.6 and 4.7). After conditioning at 33% RH, midpoint $T_g$ decreased to -19.4 ± 1.4 °C. This observation can be attributed to the plasticizing effect of water on xanthan polymer, leading to increased free volume and a weakening of the inter-chain interactions. The plasticizing effect of water on $T_g$ is well documented. Basu et al. reported that the $T_g$ of xanthan gum (dry, as received from supplier) held at a range of RH (11 to 84%) until equilibrium was reached decreased from - 16.4 to - 23.3°C as $a_w$ increased from 0.11 to 0.84 (Basu, Shivhare, &
Mujumdar, 2007). Similarly, Lazaridou and Biliaderis observed that the incorporation of sorbitol (10 and 30% d.b.) on the moisture sorption in chitosan, chitosan–starch and chitosan–pullulan films resulted in significant depression of $T_g$ due to plasticization of the polysaccharide film matrices (Lazaridou & Biliaderis, 2002). Studies aimed at determining a model for $T_g$ vs. food system composition have described this effect in greater chemical detail (Matveev, Grinberg, & Tolstoguzov, 2000).

Figure 4.6: DSC thermograms for xanthan coating conditioned at different relative humidity. Heat flow normalized and thermograms overlaid.
Figure 4.7: DSC thermograms for xanthan coating conditioned at different relative humidity, showing depression of midpoint $T_g$.

4.3.5 Scanning Electron Microscopy

SEM micrographs showed that the films surface was smooth for neat and uncoated PLA (Fig. 4.8). For xanthan coated films, the surface had a rough but consistent morphology. Further magnification at the interface, shows that the coating of xanthan was approximately 2-3 µm in thickness.
4.3.6 Atomic Force Microscopy

Tapping mode scans of film surfaces are presented in Figs. 4.9-4.11. Tapping mode scans of xanthan coating applied on PLA and glass slide showed beaded surface morphologies, with 1.0-1.5 μm by 2-2.5 μm long surface features as seen in Fig. 4.9a and 4.9b. By contrast, these structures were not observed on the uncoated, neat PLA (Fig.4.10). Similar regularity and smoothness of uncoated PLA was observed in previous studies observing neat PLA film using
AFM scanning (Rocca-smith et al., 2016). In the latter study, AFM was used to assess the effect of corona treatment on surface roughness of PLA film. Film samples were 17 to 20 µm thickness and obtained from Taghleef Industries (Nativia NTSS, San Giorgio di Nogaro, Udine, Italy). It was found that corona treatment increased surface roughness of neat PLA by 100%, while the amplitude range for uncoated PLA was much lower than in the coated samples measured in their study.

Nano thermal analysis (NanoTA) uses an modified AFM probe equipped with a resistive heater (Olmos, Martínez, González-Gaitano, & González-Benito, 2011). In NanoTA measurements, deflection signal is recorded as a function of temperature after bringing the heated probe into contact with the specimen, in this case a polymer film. Force feedback is disabled in this mode, and when probe temperature is increased, the polymer film thermally expands. With this technique, the temperature of transition events (such as $T_g$ or $T_m$) can be determined. NanoTA analysis of xanthan coated PLA (Fig. 4.12b) shows expansion of xanthan layer leading to eventual melting around 400 °C. NanoTA analysis of neat PLA (Fig. 4.12a) showed glass transition occurring around 65°C, with crystallization near 110 °C, which is consistent with published works investigating the thermal properties of PLA using nanoTA (Miloaga, Hosein, Rich, & Drzal, 2010). The $T_g$ reported in the latter study is also comparable to the midpoint $T_g$ observed for PLA films in this study determined using DSC (data not shown), but much greater than the midpoint $T_g$ observed for the xanthan coating that was scraped-off PLA and tested using DSC in section 4.3.3.
Figure 4.9: Tapping mode scans of xanthan-coated side of PLA film. a) height; b) amplitude; c) phase

Figure 4.10: Tapping mode scans of neat PLA; a) height; b) amplitude; c) phase

Figure 4.11: Tapping mode scans of 2% (w/w) xanthan coated glass microscope slide; a) height; b) amplitude; c) phase
Figure 4.12: Nano thermal analysis (NanoTA) for a) xanthan coated PLA; b) neat PLA film. Plots are deflection as a function of heating temperature.
4.3.7 Contact Angle assessment

Application of xanthan coating led to an improvement in the wetting of PLA film by water (Fig. 4.13). Neat PLA exhibited negligible wetting over one hour as only slight change in contact angle was observed after 10 min. After 40 min, no measurement could be made for water on the xanthan coated samples. The change in angle of water on xanthan coated PLA is effected by spreading of water droplet across the film surface due to the interfacial tension between water and the xanthan coated surface. The absorption of water into the xanthan coating due to the hydrophilic nature of xanthan also contributed to the decreasing contact angle over time.

![Graph showing contact angle measurements as a function of time for water on plain PLA and xanthan-coated PLA](image)

Figure 4.13: Contact angle measurements as a function of time for water on plain PLA and xanthan-coated PLA
4.4 Conclusion

Viscosity measurements and visual observations indicated that 2% (w/w) xanthan and 1% (w/w) glycerol produced the most acceptable coating on PLA amongst the formulations tested. Drying of the coating produced a hydrophilic layer that readily absorbed water in elevated RH environments. Absorption of moisture had a $T_g$ depression effect and increased water permeability at 21°C. The addition of *Listeria* phage A511 did not significantly impact the material properties tested. Compatibility of bacteriophages with other food-grade antimicrobials such as other bacteriophage strains, lysins or other antimicrobials currently used in processed meats such as sodium diacetate, may be explored in future work. AFM and SEM showed roughness of coated PLA in comparison to the uncoated PLA. Continued testing in fresh and processed meat products is also recommended. The xanthan coated PLA film created in the present study has novel applications for industry. It could allow for the fabrication of pathogen-specific or product-specific antimicrobial edible film for application to food systems such as meat and cheeses. It could also be applied as a separator for sliced product, or as an outer-layer in direct contact with the surface of skin-sealed product.
5.0 Bioactive coatings of chitosan and xanthan with bacteriophages on PLA

5.1 Introduction

There are a variety of antimicrobial materials and coatings that may be integrated for active packaging of meat products. In preliminary experiments, PLA-compatible coating polymers were investigated, including cellulose derivatives, chitosan, and xanthan. Exploratory research in the cellulose derivatives methyl cellulose (MC) and hydroxypropyl methyl cellulose (HPMC) is detailed in Appendix 8.3.

Chitosan is a linear polysaccharide composed of (1,4)-linked D-glucosamine and N-acetyl-D-glucosamine, and is a deacetylated derivative of chitin, and is obtained from shellfish wastes (Aider, 2010). The resultant deacetylated chitosan is a powerful cell membrane disrupter (Jeon, Oh, Yeo, Galvão, & Jeong, 2014) and thereby imparts antimicrobial effects. Xanthan is a long-chain polysaccharide and approved food additive from that has been used as to deliver antimicrobials such as cinnamic acid to pre-cut pears and as a carrier of Vitamin E and Calcium to peeled and trimmed baby carrots (Mei, Zhao, Yang, & Furr, 2002; Sharma & Rao, 2015). In this section, xanthan and chitosan were evaluated as carriers for bacteriophages on PLA films. The antimicrobial films were tested on *Listeria innocua* and *Salmonella* WG49 using a lawn-clearing approach on nutrient and selective agar, with and without the addition of phage to the coating on PLA.

5.2.1 Materials

Selective culture media for *L. innocua*, was prepared from Oxford Agar (Sigma Aldrich, St. Louis MO), and *Salmonella* WG49 were cultured on XLD agar (Sigma Aldrich, St. Louis MO).
Nutrient agar from Fisher Scientific (Fair Lawn, NJ) was also used to culture each organism. Low molecular weight chitosan, MC (25 cP), HPMC (4060 cP), xanthan gum was obtained from Sigma Aldrich (St. Louis MO). Glycerol, poly(ethelyne glycol) (PEG) and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ) and 95% ethyl alcohol from Commercial Alcohols (Toronto, ON). ListoSheild™ and SalmoFresh™ bacteriophage preparations were obtained from Intralytix (Baltimore, MD).

5.2.2 Methods

5.2.2.1 Film preparation

Plain PLA films were prepared as described in Section 4.2 using a Randcastle Microtruder. Chitosan solution was prepared by adding glycerol at 1.75% (w/w), and then chitosan (3.5 % w/w) to deionized water while stirring at approximately ~50 °C until dispersed. Ethyl alcohol was then added at 26.25 % (w/w), stirring for 5 min, followed by the addition of the phage in SM buffer at 1% (w/w) and then stirred for another 5 min. Finally, acetic acid was added at 3.5% (w/w), causing the solution to thicken. Stirring continued until the solution was homogeneous and clear, cooled to room temperature, and then used for coating PLA film.

To prepare xanthan coating solution, glycerol was added at 1% (w/w) to deionized water while stirring at approximately ~50 °C. Xanthan at 2 % (w/w) was then dispersed in 5 mL of ethyl alcohol then added gradually to deionized while stirring with a magnetic stir bar. Heat was then turned off, and solutions were stirred for at least 1 h to ensure the xanthan polymer was
fully dissolved. Once cooled to room temperature, bacteriophage preparation was added to the xanthan solution at 1% (w/w) and stirred for another 15 min.

All coating solutions were applied at 21 °C. An aliquot of 3 mL of coating solution was pipetted onto centerline of each PLA film strip (1.5 cm wide by 8 cm long) and drawn down using a 0.5 mil applicator (BYK Gardner USA, Columbia, MD). Coated film samples were taped at the edges onto aluminum foil, placed on trays, and dry in environmental chamber at 45 °C, 30% RH for 30 min until the surface appeared dry, followed by placing the samples in a 0% RH desiccant chamber overnight until testing the following day. For comparison, a 2-mil wire-wound applicator rod (BYK Gardner USA, Columbia, MD) was also tested. To facilitate visual inspection of the coating, the coating was dosed with food dye.

5.2.2.2 Bacterial Culture Preparation

Nonpathogenic, surrogate strains of *Salmonella* WG49 and *L. innocua* were obtained from American Type Culture Collection and isolated by streaking on nutrient agar. Using a sterile wire loop under a laminar flow hood, a single colony of bacteria was removed from the surface and incubated in sterile tryptic soy broth (TSB) overnight at 30 °C. The next day, microbial suspensions were centrifuged at 5000 rpm for 10 min using Fisher Scientific IEC Marathon model 21000 centrifuge (Fisher scientific, Pittsburg MA). Spent TSB was poured off, and the centrifuged cell masses were suspended in saline. Optical density for cell suspensions was measured at 600 nm to determine cell concentrations using a Bio Rad SmartSpec Plus® digital spectrometer (Bio Rad Laboratories in, Philadelphia, PA) calibrated with a known absorption concentration curve for these bacteria. Serial dilutions were performed to create
bacterial concentrations of $10^9$ and $10^8$ cells/mL for *Salmonella* WG49, and $10^6$ and $10^5$ cells/mL for *L. innocua* in sterile saline.

5.2.2.3 Evaluation of Antimicrobial Effects

Agar was prepared per manufacturer’s instructions. An aliquot of 15 mL of molten agar at 65 °C was poured into sterile petri dishes under a laminar flow-hood. XLD (selective) agar and nutrient agar were used for *Salmonella* WG49 tests, while Oxford (selective) agar and nutrient agar were used for *L. innocua* tests. Bacterial suspension, 0.5 mL, was spread plated on the surface of prepared agar to create a bacterial ‘lawn’. Cut-outs of antimicrobial film (1 cm by 1 cm) were placed on the agar surface in contact with the spread-plated bacteria. For films with antimicrobial coating, the coated side of the film was placed in direct contact with the microbial lawn. Plates were incubated at 30 °C. Observations were recorded at 0, 24, and 48 h. Growth was rated on a scale of 1 (little or no growth under film cut-out) to 5 (much growth under film cut-out). Testing was performed in triplicate for each film orientation at a given concentration. Experiments performed are summarized in Table 5.1. A schematic of antimicrobial testing is depicted in Figure 5.1.

For each experiment in Table 5.1, the following tests were conducted: (1) control without film treatment; (2) uncoated PE as a negative control; (3) uncoated PLA; (4) coated PLA without bacteriophages; and (5) coated PLA with SalmoFresh™ or ListSheild™ bacteriophage preparation added at 5% (w/w) level. Performing a plaque assay for both SalmoFresh™ and
ListSheild™ preparations validated potency of bacteriophages. The procedure for this assay can be found in Appendix 8.2.

Table 5.1: Experiments conducted for *Salmonella WG49* and *L. innocua* using agar disc diffusion tests

<table>
<thead>
<tr>
<th>Coating</th>
<th>Tests against <em>Salmonella WG49</em></th>
<th>Tests against <em>L. innocua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthan (2% w/w) with and without SalmoFresh™</td>
<td>Xanthan (2% w/w) with and without ListShield™</td>
<td></td>
</tr>
<tr>
<td>XLD selective agar at $10^9$, $10^8$ cells/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient Agar at $10^9$, $10^8$ cells/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mediums used

<table>
<thead>
<tr>
<th>Coating</th>
<th>Tests against <em>Salmonella WG49</em></th>
<th>Tests against <em>L. innocua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous Ethanolic Chitosan (3.5% w/w) with and without SalmoFresh™</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqueous Ethanolic Chitosan (3.5% w/w) with and without ListShield™</td>
<td></td>
<td></td>
</tr>
<tr>
<td>On XLD selective agar at $10^9$, $10^8$ cells/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>On Nutrient Agar at $10^9$, $10^8$ cells/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Antimicrobial film coupon

Spread-plated bacteria

Bottom solid agar layer

Zone of inhibition; measured by distance from the center of the film coupon

Figure 5.1: Method for testing antimicrobial effectiveness using film overlay on a spread-plated lawn of bacteria
5.4 Results and Discussion

In all treatments (Table 5.1), including the control samples, no growth was observed for films tested on nutrient agar. For brevity, only uncoated PLA, coated PLA and coated PLA + bacteriophage samples are shown in Fig. 5.2 and 5.3. This finding is unexpected, especially the absence of growth for the control films. The reason for this observation is unknown. However, the fact that the bacterial colonies tended to concentrate around the edges of the film samples suggested that the specimens did not elicit antimicrobial properties. Otherwise, clear zones would have been formed surrounding the test specimen films. It is also possible that the antimicrobial constituent did not readily diffuse out of chitosan or xanthan by the culture methods used. The black colonies under PLA films on XLD agar can be attributed to the hydrogen sulfide produced by Salmonellae when they metabolize thiosulfate.

Table 5.3: pH of antimicrobial coating solutions

<table>
<thead>
<tr>
<th>Coating formulation</th>
<th>pH</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% (w/w) Xanthan with 1% (w/w) Glycerol</td>
<td>4.96</td>
<td>0.071</td>
</tr>
<tr>
<td>Aqueous Ethanolic Chitosan</td>
<td>3.51</td>
<td>0.087</td>
</tr>
<tr>
<td>MC-HPMC (no nisin)</td>
<td>4.70</td>
<td>0.076</td>
</tr>
<tr>
<td>MC-HPMC (with nisin)</td>
<td>3.74</td>
<td>0.038</td>
</tr>
<tr>
<td>UltraPure H20</td>
<td>4.60</td>
<td>0.090</td>
</tr>
</tbody>
</table>
Figure 5.2: Antimicrobial testing of Xanthan and Aqueous Ethanol Chitosan coated PLA films with and without ListShield™ bacteriophage preparation. For row numbers: 1 = Blank sample no films; 2 = uncoated PLA; 3 = coated PLA; 4 = coated PLA with ListShield™ bacteriophage preparation at 1% (w/w). For columns: a = xanthan coating treatment on nutrient agar; b = xanthan coating treatment on Oxford; c = chitosan coating treatment on nutrient agar; d = chitosan coating treatment on Oxford.
Figure 5.3: Antimicrobial testing of Xanthan and Aqueous Ethanolic Chitosan coated PLA films with and without SalmoFresh™ bacteriophage preparation. For row numbers: 1 = Blank sample no films; 2 = uncoated PLA; 3 = coated PLA; 4 = coated PLA with SalmoFesh™ bacteriophage preparation at 1% (w/w). For columns: a = xanthan coating treatment on nutrient agar; b = xanthan coating treatment on XLD; c = chitosan coating treatment on nutrient agar; d = chitosan coating treatment on XLD.
Inhibition of bacterial growth by control films cannot be explained based on oxygen depletion under the film due to the barrier properties of the film, because both _L. innocua_ and _Salmonella_ WG49 strains used are facultative anaerobic. The leaching of materials, short chain oligomers and organic acid, particularly lactic acid, from the PLA matrix may have imparted antimicrobial effects underneath the film. However, this alone could not explain why clearing zone around the edges of the film was not observed. The third potential cause for absence of growth under film coupons might have been caused by the displacement of the liquid inoculant by the test film specimens. It is probable the leaching of limited lactic acid from the PLA ay have also been adequate to destroy the microorganism.

5.5 Conclusions

From the experiments, it was determined that chitosan coating formulations are not optimal for coating applications with bacteriophages due to the presence of ethyl alcohol, phage, acetic acid addition, and chitosan. These constituents can elicit antimicrobial properties, but can also have the potential to deactivate the bacteriophages. Previous works by Kumar et al, (2006) have shown that slightly low pH is important for the mechanisms of cell entry, extremely low pH, such as that in the chitosan solution could have other negative effects on the phage. Bacteriophage recovery and enumeration from these films after 24 and 48 h would have to be assessed to determine if this type of bacteriophage, or others, are able to survive the low pH and alcoholic conditions of the aqueous ethanolic chitosan solution prepared. Additionally, ethyl alcohol concentrations above 20% (v/v) have been shown to have profound effects on reducing phage activity (Lee, Eschenbruch, & Waller, 1985).
6.0 Antimicrobial Properties of Salmonella Phage Felix O1 and Listeria phage A511 Embedded in Xanthan Coatings on Poly(lactic acid) Films

6.1 Introduction

The pathogenic bacteria *Salmonella* sp. and *Listeria monocytogenes* are known to cause severe illness, and even death among at risk populations, such as the elderly, infants, pregnant women, and other immunocompromised individuals (Denes et al., 2014; Woolston et al., 2013). *L. monocytogenes* can also thrive under lower temperatures and pH levels than other foodborne pathogens, making it of particular concern for growth in refrigerated, RTE meat products (Chibeu et al., 2013). Considering the negative impact of foodborne outbreaks on consumers, the burden placed on healthcare system, and economic impact on companies, the control and prevention of Listeriosis and Salmonellosis is of great research interest.

Packaging systems for meat products range from over-wrap for short shelf-life products to vacuum and modified atmosphere packaging for longer-term storage. As discussed in Section 2.2, active packaging enhances the preservation and protection aspects of packaging to maintain product sensory properties, extend shelf life, and to inhibit the growth of spoilage/pathogenic microorganisms, thereby ensuring product quality.

While many antimicrobials have been investigated in active meat packaging, bacteriophages are under-investigated as an antimicrobial in food packaging. One advantage of bacteriophages are is that it only infects specific species, strains or life stages of bacterial hosts, while leaving other bacteria, such as native flora on fermented meat, relatively unaffected (Khalifa et al., 2015; Radford et al., 2016). When no hosts are present, bacteriophages
demonstrate good stability. Moreover, several bacteriophages tested have the GRAS status for use in food products by regulatory bodies, including Health Canada and the United States Department of Agriculture (Chibeu et al., 2013; Knobler and Gelbart, 2009; Vonasek et al., 2014). These specificity, stability and safety characteristics make bacteriophages particularly attractive as antimicrobials in applications where broad spectrum antimicrobials are undesirable or inappropriate (Chibeu et al., 2013; Hooton, Atterbury, & Connerton, 2011; Khalifa et al., 2015; Radford, Ahmadi, Leon-Velarde, & Balamurugan, 2016).

Combining antimicrobial treatment of bacteriophage, with conventional packaging in a hurdle approach is expected to have a pronounced impact on reducing product spoilage and preventing foodborne illness by inhibiting spoilage/pathogenic microorganisms and enhancing post-processing protection. To date, there has been considerable challenges in developing delivery system for controlled release of bacteriophages to the surface of packaged food product without losing antimicrobial activity or stability (Chibeu et al., 2013; Gouvêa et al., 2015; Vonasek et al., 2014). In this study, methods to apply edible xanthan coating, embedded with bacteriophages specific for L. monocytogenes and Salmonella sp.

The overall goal of this chapter is to evaluate the effectiveness of xanthan coated PLA films as carriers for embedded bacteriophages in an active packaging system. Antimicrobial effects will be tested first in plate culture and then applied to inoculated cooked turkey breast samples stored under different temperatures under aerobic or vacuum packed conditions.
6.2.1 Materials

Information on the bacterial and viral strains tested are summarized in Radford et al., (2016). Bacteria cocktails of *L. monocytogenes* or *Salmonella* serotypes were applied on RTE cooked turkey breast as prescribed by the Health Canada method for accessing antimicrobial treatments in refrigerated RTE foods (Health Canada, 2011, 2016). The five *L. monocytogenes* strains used represent common serotypes associated with listeriosis. Similarly, the six Salmonella chosen are related to Salmonellosis. *L. monocytogenes* strains were propagated in 5.0 mL sterile brain heart infusion (BHI) broth and incubated for 24 h at 30 °C, thereby generating stationary phase cultures according to previously established methods (Radford et al., 2016). Salmonella strains were similarly grown in 5.0 mL of TSB aliquots and incubated for 24 h at 37 °C.

Sliced cooked turkey breast, stored at 4 °C prior to use, was obtained from a nearby supermarket for antimicrobial testing. Sodium chloride, potassium chloride, and vinegar were listed as ingredients and likely bacteriostatic agents. Experiments were performed in triplicate, with two independent packages of cooked turkey, sampling from one package of turkey for each bacterial cocktail.

6.2.2 Methods

6.2.2.1 Antimicrobial Film Preparation

PLA films were prepared from resin type 4032D from NatureWorks™ (Minnetoka, MN, USA). Resin was vacuum-oven dried at 60°C and 20 in Hg overnight, then extruded into films (30 ± 2 μm) using a lab-scale extruder (Microtruder RCP-0625, Randcastle, NJ, USA). Edges of
extruded PLA films were trimmed with a film cutter to produce rectangular sheets with 10 cm \times 14 cm in dimension. PLA sheets were coated with 2\% (w/w) xanthan solution with 1\% (w/w) glycerol as a plasticizer, with bacteriophage preparations with concentration 10^{12} PFU/mL in SM buffer added to treatment films at 1.0\% (w/w), producing a 10^{10} PFU/mL coating solution. Coatings were initially applied using the draw-down approach as described in Chapter 4. However, because the resulting phage density that was too low to impart a significant microbial activity, a pour casting method was adopted instead. In this approach, a 25 mL of xanthan coating solution containing 10^{10} PFU/mL of *Listeria* A511 was pour over PLA films with an area of 140 cm$^2$. The phage loading is calculated according to equation (8):

$$
4.8 \times 10^{12} \frac{PFU}{mL} \times \frac{1}{100} \text{ dilution} \times \frac{25 mL \text{ coating}}{1 \text{ film}} \div \frac{140cm^2}{1 \text{ film}} = 8.57 \times 10^9 \frac{PFU}{cm^2}
$$

The coated PLA films were dried at 45 °C, 30\% RH for 4 h in an environmental chamber, and then stored in a chamber maintained at 0\% RH until testing.

### 6.2.2.2 Plate Assays for Inhibition of Bacterial Growth

Antimicrobial effects of xanthan coated PLA films were evaluated spectroscopically using 12-well plate reader system. Disks of 7 cm$^2$ test films were placed into the wells with the coating facing up. Control wells were left empty to measure bacterial growth rate without the PLA treatment. Wells with and without disks were inoculated with 2.0 mL of TSB or BHI 0.2 \% (w/v) agar, along with 20 μL of *S. Typhimurium* DT104 or *L. monocytogenes* ATCC 19115 in the early log phase. Hourly changes in optical density in each sample well at 600 nm (OD600) over a 20 h period was recorded at 25 or 37 °C, using an automated plate reader (BioTek
Prior to each assay, optical density measurements were made at nine sites within each well to account for variation within cultures.

Optical density measurements were normalized at 600 nm to account for the inherent opacity of the coated PLA specimens and the reader wells, by subtracting the average initial OD600 of the well from that of the subsequent reading in the presence of the film sample. Differences in optical densities at a given time point for each treatment were tested for statistical significance using a two-sample T-test for samples of unequal variance (Ruxton, 2006) using Microsoft Excel™. Differences in growth curve data for plate culture treatments was evaluated for statistical significance by applying Kolmogorov–Smirnov tests using the statistical software, R (R Foundation; Vienna, Austria).

6.2.2.3 Treatment of Precooked Turkey Breast With Phage-Embedded Xanthan Coating on PLA

Cocktails of six Salmonella species, in equal concentration, were evaluated using the coatings embedded with the Salmonella phage: S. Typhimurium DT104, S. Heidelberg ATCC 8326, S. Typhimurium 19485A96 SGI1, S. Enteritidis ATCC 4931, S. Newport ATCC 6962 and S. Typhimurium 13311. These cultures, at the stationary phase, were isolated from streak plate colonies grown at 37 °C on tryptic soy agar, and then mixed and diluted to an equal concentration to yield uniformly mixed Salmonella cocktails as initial inoculums. On the other hand, to prepare the cocktails of five L. monocytogenes strains (L. monocytogenes FSL F6-367, ATCC 19115, 08-5578, C6-0003, and LI 0512), fresh cultures of each strain were grown following
a method from Chibeu et al. (2013) in BHI media at 30°C. Culture were isolated and diluted to produce ~100 CFU/mL to produce initial inoculums.

To maintain sterility, all sample preparations were carried out in a biosafety cabinet. The precooked sliced turkey breast samples were uniformly prepared by cutting 10 cm² circular stacks of sample, avoiding the slices’ outer edge, using an ethanol- and flame-sterilized 10 cm² cork-borer. To ensure that the samples are free from contamination, prior to inoculation, they were tested for Salmonella sp. and L. monocytogenes using the BAX® System (Hygiena, Camarillo, California) Real-Time PCR Assay (Health Canada, 2011, 2016). To inoculate, circular stacks of sample were separated into three slices using sterile forceps, and placed onto an expanded polystyrene foam tray (Dyne-A-Pak Inc., Laval, QC Canada). The surface of each sample was inoculated with 100 µL of Salmonella sp. or L. monocytogenes cocktail, and allowing the inoculums to be absorbed into samples for 3 min in a biosafety hood. Three inoculated samples were enumerated at 0 d to determine starting bacterial concentrations. Remaining inoculated samples were overlaid with either control film (uncoated PLA), xanthan coated PLA without bacteriophages, or coated PLA film with corresponding bacteriophages. For coated films, active side of the film was placed in direct contact with the inoculated surfaces on cooked turkey samples. Trays were then transferred to either oxygen permeable deli bags to mimic aerobic storage, or into commercial barrier bags (oxygen transmission rate 40–50 mL/ m².d.; Winpak Ltd., Winnipeg, MB Canada), and vacuum packaged (Multivac AGI, KnudSimonsen Industries Ltd, Rexdale, ON Canada) (Balamurugan et al., 2016; Chibeu et al., 2013). The schematic of the experimental setup and flow diagram are presented in Fig. 6.1a and 6.1b.
Figure 6.1a: Set-up of a sample triplicate for each treatment in expanded polystyrene trays. One triplicate per treatment was evaluated on a given sampling day.

Figure 6.1b: Experimental design for testing antimicrobial efficacy of active packing from bacteriophage-embedded xanthan coating on PLA film in cooked turkey breast. List = Listeria inoculation; Salm = Salmonella inoculation; Xan = Xanthan at 2% (w/w); Gly = Glycerol at 1% (w/w); Phage = Phage addition at 1% (w/w); A = Aerobic packaging; Vac = Vacuum packaging.
6.2.2.4 Enumeration of Pathogens and Bacteriophages

Samples were stored at 4 °C to mimic typical refrigeration conditions, or at 10 °C to simulate temperature abuse. Three replicates of each of the 12 treatments in Fig. 6.1b were removed after 30 min, 1, 3, 7, 10, and 14 d. For vacuum packaged samples, additional sampling was conducted at 21 and 30 d. Sample units were opened aseptically and *Salmonella* sp. or *L. monocytogenes* counts (CFU/cm²) were enumerated as follows. Meat samples were placed into stomacher bags and homogenized in 18 mL of sterile SM buffer. *Salmonella* sp. were enumerated by plating a dilution series from stomached samples onto XLT4 agar, then incubating at 37 °C for 24 h. Daily *L. monocytogenes* counts were enumerated by plating a similarly prepared dilution series onto Oxford agar and incubating at 30 °C for 48 h. To enumerate the remaining bacteriophages in the xanthan coated PLA, sections of the test film were placed in petri-dishes, flooded with 2.0 mL of sterile SM buffer, and left overnight. The solutions were then filtered aseptically using 0.2 µm syringe filters, prior to enumeration of phage recovered from bioactive PLA by performing overlay assays (Radford et al., 2016). Phage and bacterial counts at each time point were evaluated for statistical significance using Welch’s two-sample T-tests for samples of unequal variance using Analysis ToolPak in Microsoft Excel™.
6.3 Results and Discussion

In plate culture assays, inclusion of bacteriophages had a significant effect on reducing pathogen growth compared to coated and uncoated films, and blank control wells at the end of the incubation period at 37 °C (p<0.05) (Figs. 6.2 and 6.3). A greater reduction in final bacterial concentration was observed for *S. Typhimurium* DT104 than for *L. monocytogenes*. This observation is likely due to the optimal growth temperatures for these mesophilic bacteria.

When tests for *Salmonella* were repeated at 25°C (data not shown), the effect of bacteriophage treatment was no longer significant (p>0.05). This observation, as well as the results from tests conducted at 37°C, support the hypothesis that bacteriophages are most effective on host cells under ideal growth conditions. It is known that the ability of Felix-O1 to infect *Salmonella* is dependent on N-acetylglucosamine expression (Whichard, 2000). This monosaccharide is present in the lipopolysaccharide of cell walls, and is down-regulated at lower temperatures, making it harder for Felix-O1 to bind to host cells as temperature decreases. The apparent dip in optical density is due to the decreased absorbance of peptidoglycan; as the *Salmonella* cells are lysed and their cell wall breaks down, the absorbance of peptidoglycan is decreased due to the lytic activity of phage. Plateauing effects in phage treated samples may be due to the competing cell growth and infection and lytic activity of phage in growth culture tests over the study period.

Reduction in *L. monocytogenes* growth was significant at the end of incubation at both 37 °C and 25 °C (p<0.05). Therefore, unlike application of Felix-O1 for *S. Typhimurium*, bacteriophage A511 remained effective against *L. monocytogenes* at lower temperatures. This
likely due to the psychrotrophic nature of *Listeria sp.* which allows it to survive and grows at low temperatures, and thereby also enabling phage infection.

In both cases, some growth reduction was caused by PLA and coated PLA controls during the early growth phases, and in the case of *Listeria* treatments, this effect was significant from 3-17 h onwards (*p*<0.05). This may be due to leaching of lactic acid from the extruded PLA films. Xanthan may have also influenced the metabolic activity of pathogens, acting as a poor carbon source. Xanthan coating may have also imparted controlled release effects to any free lactic acid able to leach from films. Though noteworthy, these effects are nominal since stationary phase and endpoint concentrations after 20 h incubation at 37 °C are significantly lower for both bacteriophage-pathogen treatments compared to respective controls.
Figure 6.2: Plate culture assay for the growth of *S. Typhimurium* DT104 at 37 °C for 20 h with different antimicrobial film treatments
Figure 6.3: Plate culture assay for the growth of *L. monocytogenes* at 37 °C for 20 h with different antimicrobial film treatment
Under both vacuum and aerobic packaging conditions, over 99.9% of bacteriophages were recovered from the surface of cooked turkey samples after 30 min contact film containing Felix-O1 bacteriophages (Fig. 6.5). Multiplicity of infection (MOI) applied in this system was in the order of $10^5$ magnitude. The MOI is the ratio of infectious agents (in this case bacteriophages) to infection targets. This can be derived knowing an amount of phage in the order of $10^9$ PFU/cm$^2$ were applied to the food system which has been inoculated with pathogens at $10^4$ CFU/mL. For packages of turkey breast inoculated with *Salmonella* cocktail in Fig. 6.4, approximately 1 log, and 0.5 log reductions in growth verses controls had occurred for vacuum and aerobic storage conditions respectively (p<0.05) at 10 °C. The bactericidal effect of Felix-O1 containing films on growth of the *Salmonella* cocktail was less effective at 4 °C. No significant difference was found between aerobically stored treatments at 4 °C for *Salmonella* inoculated samples (p>0.05), and a small, but significant reduction was observed in vacuum stored samples after 30 d at 4 °C (p<0.05). These findings may have been impacted by the mesophilic nature of *Salmonella* and the need of growth phase host cells for Felix-O1 to induce antimicrobial effects.

For meat samples treated with *Listeria* cocktail, bactericidal effects of A511 phage in xanthan coated film was stronger than Felix-O1 application in the *Salmonella* treated system. A 2 log reduction in pathogen counts was recorded at the end of the 14 d study period for aerobically stored samples at 4 °C and 10 °C (Fig. 6.6). For vacuum sealed samples, a 2-3 log reduction was observed for samples stored at 4 °C compared to PLA and coated controls, while a 2 log reduction was observed at 10 °C. As in salmonella treated samples, over 99.9% recovery of bacteriophages from meat samples that received the coated film with bacteriophage A511
(Fig. 6.7). Growth reduction for *Listeria* inoculated meat samples is supported by the refrigerated temperatures. Since *Listeria* are psychotropic and reproduce well at the lower study temperatures, it is not surprising that bacteriophages had a stronger effect than compared to the *Salmonella* system. Since growth factor expression is required for a bacteriophage to bind to a host, inject viral DNA, produce progeny, and lyse host pathogens, understanding of temperature control, storage conditions, and appropriate phage application is necessary to reduce pathogen growth.

A simplified mechanism of the antimicrobial activity of xanthan coating PLA films with bacteria is proposed in Figure 6.8. In this figure, the bioactive film surface encounters moisture on food surface. The hydrophilic film coating is hydrated, the xanthan matrix becoming plasticized, increasing the free volume in the coating. This plasticization effect has been previously observed on protein and polysaccharides (Chan, 2012; Matveev et al., 2000), and can allow bacteriophage to diffuse more readily from the hydrated coating to the product surface. The antimicrobial activity can be derived from two phenomena. Initially, lysis from without (ie: from the outside of bacteria cell) may have occurred due to high initial ratio of active phage from the film coating which may bind to the pathogen surface at once, resulting in cell death without phage reproduction (Abedon, 2011). The second, more prevalent form of antimicrobial activity in systems where MOI is not maintained at an elevated level is caused by phage replication within the bacteria cell via the lytic cycle. Virulent phages inject their genetic material into the cell, commandeering cell machinery, and reproduce via the lytic cycle, resulting in phage progeny being released into the system. However, diffusion and interaction with other components in a complex food system can limit phage activity, hindering their ability
to replicate and to bind host over time. The activity of phage in food and other systems after immobilization in a carrier film has been previously reported (Gouvêa et al., 2015; Vonasek et al., 2014).
Figure 6.4: Relative survival of *Salmonella* sp. cocktail on precooked sliced turkey at 4 °C and 10 °C when packaged aerobically or anaerobically with uncoated PLA, or xanthan coated PLA with *Salmonella* phage Felix O1 or without phages.
Figure 6.5: Active *Salmonella* phages recovered over time from xanthan coating or the sliced turkey stored at 4 °C or 10 °C when packaged aerobically or in vacuum. Surviving *Salmonella* sp. present on the sliced turkey packaged with active packaging also compared to *salmonella* sp. enumerated from turkey breast packaged with uncoated PLA.
Figure 6.6: Relative growth of *Listeria monocytogenes* cocktail on precooked sliced turkey at 4 °C and 10 °C when packaged aerobically or in vacuum barrier bags with uncoated PLA, or xanthan coated PLA with or without *Listeria* phage A511.
Figure 6.7: Active *Listeria* phages recovered over time from xanthan coating or the sliced turkey stored at 4 °C or 10 °C when packaged aerobically or in vacuum. Surviving *Listeria* sp. present on the sliced turkey packaged with active packaging with phages are compared to *Listeria* sp. enumerated from turkey breast packaged with uncoated PL.
6.4 Conclusion

Both *Salmonella* Felix-O1 and *Listeria* A511 phages could delay the growth of host bacteria in plate culture, resulting in lower cell densities at the end of 20 h incubation period. The application of phage-embedded xanthan coated PLA to inoculated cooked Turkey breast samples generally produced greater reductions in pathogen growth at the end of study period for meat samples inoculated with *Listeria* cocktails than for *Salmonella* inoculated samples. A hurdle approach is necessary to prevent pathogen growth in processed meat products, combining other antimicrobial control measures to obtain reductions greater than the 2 log reduction observed in this study. Xanthan coatings proved to be a versatile coating allowing for rapid release of viable phage within 30 min contact time with meat samples.
antimicrobial films could be integrated into a hurdle methodology by meat processors in their efforts to reduce post-processing contamination of food products. Our findings suggest direct contact applications, such as separating inserts for sliced product, or as the outer layer in skin packaging would be most effective. Follow up studies should be performed to validate film effectiveness after prolonged storage of active films, using other bacteriophage or lysins, as well as assess sensory qualities of the packaged meat samples.
7.0 Conclusions and Recommendations

Growing interest into the development of biodegradable films as alternatives to petroleum plastics has bolstered research and development of more versatile biobased materials. Control over foodborne illness and pathogens are also crucial elements to consider when developing new packaging technologies. Each section of this study contributed to the development of a novel, biobased active packaging film that can reduce pathogen growth to prevent foodborne illness when applied as part of a hurdle approach.

In section 4.0, after much initial exploratory work, it was determined that xanthan gum had significant research potential as an effecting coating for PLA film. Viscosity measurements and visual inspection showed that a formulation of 2% (w/w) xanthan and 1% (w/w) glycerol produced the most acceptable coating on PLA amongst the formulations tested. Absorption of moisture in this xanthan coating on PLA had a depression effect on coating $T_g$. The addition of *Listeria* phage A511 had no effect on water vapor permeability. AFM and SEM showed roughness of xanthan coated PLA in comparison to the uncoated PLA. The atomic force microscopy analyses revealed the unique surface topography of the coating compared to neat PLA. Improvement to coating adhesion, and increased speed of processing could be accomplished by integrating a film pre-treatment such as corona treatment or plasma enhanced chemical vapor deposition. The effects of plasma pretreatment on PLA were investigated using the OpernAir™ plasma deposition system at PlastmaTreat© in Ancaster, Ontario. Full procedure and results from this experiment can be found in the Appendix.
In section 5.0, it was determined chitosan coating was not suitable for phage delivery due to the acidic and alcoholic nature of this coating formulation. Differences in roll-coating and draw-down coating validated the use of drawn-down coating as the more effective means of applying highly viscous coating solutions on a polymer film substrate. In section 6, bacteriophages embedded in xanthan based active packaging coatings were shown to significantly reduce *L. monocytogenes* growth on aerobically and anaerobically packaged RTE cooked turkey. The survival of *Salmonella* sp. also decreased at 10 °C over a 14 d sample period for aerobically packaged meat, and for 30 d for vacuum packaged samples at both temperatures. Reduction in *L. monocytogenes* growth was near 2 logs relative to uncoated controls. A reduction of this magnitude suggests that application of this technology as a hurdle conjunction with other control measures, as the most robust approach to preventing *Salmonella* and *L. monocytogenes* contamination on the surface of processed meats. As a carrier, xanthan coatings provide versatility and perform as well as, or better than previously published embedded phage packaging systems. The versatility of the bilayer film created in the present work stems from the potential to pre-fabricate pathogen-specific and/or product specific antimicrobial films for different food systems such as meat and cheeses, applied as a separator for sliced product or as a top-layer in direct contact with the surface of the product.

Recommendations for future work are outlined as follows:

- Testing using afm+™ systems provided valuable inside into the surface morphology and thermal analysis of xanthan coated PLA films. Future works using this technology could explore this tool as a means of in-depth compositional analysis.
• Utilization of OpenAir™ plasma treatment systems could be used to apply other coatings to PLA that may otherwise not be able to adhere to the film. Testing with plasma activation could also be coupled with afm+ analysis to determine effects of plasma treatment on surface roughness and thermal properties.

• Addition of MMT or other modified nanoclay to the PLA portion of the proposed xanthan coated, active packaging film could enhance barrier and improve other material properties. Investigation of polymer blending with or without nanomaterial addition may be trialed as a means for developing a biobased film able to withstand HHP processing of packaged RTE meats.

• Sensory and consumer testing must be carried out to further validate the efficacy of the xanthan layer as a suitable edible coating for meat or cheese products. Evaluating any changes in favor, appearance or texture imparted by the xanthan are crucial factors that must be accounted for prior to commercial implementation. Since the xanthan coating will be transferred to the food product, it would have to be added to the product label, which may impact consumer perceptions of ‘natural’ and ‘clean’ with respect to the product ingredients. While present in some emulsified cheese products, xanthan gum is not typically present in meat products. Therefore, it would be necessary to determine any negative impacts on consumer perceptions in applying this system.

• The long-term stability of film and preservation of its quality are important considerations in future work. Though the film tested in this project was stored for up to one month and validated for efficacy, longer periods of storage are likely in commercial
settings. Furthermore, specific storage conditions and means of maintaining film cleanliness during prolonged storage should be investigated in greater detail.

- Lysins are hydrolytic enzymes used by bacteriophages to cleave and rupture a host's cell wall during the final stage of the lytic cycle, resulting in the release of phage progeny. Lysins are smaller protein molecules, and due to their simpler structure, may exhibit greater survival under pressure and over prolonged storage. Since bacteriophages are known to be sensitive to the high pressures applied in HHP, lysin substitution for bacteriophages in active packaging films intended to withstand HHP treatment should be investigated. Additionally, phage or lysin compatibility with food-grade antimicrobials such as other bacteriophage strains, nisin, sodium diacetate and others currently used in processed meats should be investigated to determine if there are any synergistic or deleterious effects.
8.0 Appendix

8.1 Preliminary experiments in PLA coating

Following the method by Torlak & Sert (2013), chitosan was a promising coating material as it formed a very even coating on PLA film. However, because of the acetic acid solution used as a solvent, the resulting films had a pungent acetic odour. Therefore, the coating formulation may not be suitable for processed meat applications due to negative impact on product sensory attributes. Moreover, acidic and alcoholic conditions may impact the phage stability, as discussed in section 5.5.

Table 8.1: Summary of coating materials tested in preliminary experiments

<table>
<thead>
<tr>
<th>Coating material</th>
<th>Formulations tested</th>
<th>Application method</th>
<th>Observations</th>
<th>Worked cited (if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>2% (w/w) chitosan in 1% aqueous acetic acid, pH = 4.01</td>
<td>BYK roll-bar</td>
<td>No adhesion; solution beaded off readily. Attempted mimicking effects of plasma by holding film near Bunsen burner, but had no success.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 parts (by volume) 2% (w/w) chitosan in 1% (w/w) acetic acid; 30 parts 95% (v/v) ethyl alcohol</td>
<td>BYK roll-bar</td>
<td>Addition of alcohol to formulation improved spreading</td>
<td>Torlak and Sert, 2013</td>
</tr>
<tr>
<td></td>
<td>70 parts 2% (w/w) chitosan in 2% (w/w) acetic acid; 30 parts 95% (v/v) ethyl alcohol; tested with and without addition of Felix-O1</td>
<td>BYK roll-bar</td>
<td>In plate culture showed clearing, but not able to discern if clearing of bacteria was due to either: 1) acidic nature of coating; 2) smothering of bacteria; 3) chitosan in film; or 4) phage itself</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 parts 2% (w/w) Chitosan in 2% (w/w) acetic acid; 30 parts 95% ethyl alcohol; mixture plasticized with 10% (w/w) glycerol</td>
<td>BYK roll-bar</td>
<td>Chitosan layer peeled off and formed a coherent, standalone film without many visual defects</td>
<td></td>
</tr>
<tr>
<td>Alginate</td>
<td>84% (w/w) distilled water, 1% (w/w) NaCl, 10% glycerol, 5% alginate</td>
<td>BYK roll-bar</td>
<td>Alginat flaked off easily, SEM showed de-lamination of alginate layer</td>
<td>Harper et al., 2014</td>
</tr>
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<td>2% (w/w) alginate solution cast onto on PLA placed in petri dishes, followed by cross linking with CaCl₂ solution (25 g CaCl₂ in 70 mL H₂O)</td>
<td>Solution casting</td>
<td>Thick films that peeled easily from PLA. Drying at room temperature took several hours</td>
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<td>Poly(ethylene oxide)</td>
<td>Test 1 - 2% (w/w) PEO (900,000 Da), 0.5% (w/w) Alginate; Test 2 - 2% (w/w) PEO (100,000 Da), 0.5% (w/w) alginate; pipette CaCl₂ (25 g CaCl₂ in 70 mL H₂O) solution on polymer</td>
<td>BYK roll-bar</td>
<td>High molecular weight PEO (900,000 Da) adhered and spread better than the low (100,000 Da) PEO with less beading. Coating tended to peel at edges</td>
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<td>Test 1 - 2% (w/w) PEO (900,000 Da), 0.5% (w/w) alginate; Test 2 - 2% (w/w) PEO (100,000 Da), 0.5% (w/w) alginate; pipette CaCl₂ (25 g CaCl₂ in 70 mL H₂O) solution on polymer</td>
<td>Electrospinning</td>
<td>Lots of flaking and salt crystals due to CaCl₂ flooding; lower concentration of CaCl₂ should applied.</td>
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<td>Corn zein</td>
<td>10% zein in 50% (w/w) ethanol solution; 10% (w/w) zein and 10% (w/w) glycerol in 50% ethanol solution</td>
<td>BYK roll-bar</td>
<td>Zein without glycerol flaked off readily. Addition of 10% glycerol improved the adhesion. Colour imparted by zein was not desirable.</td>
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<td>10% (w/w) zein in 50% (w/w) ethanol solution</td>
<td>Electrospinning</td>
<td>This zein concentration required a greater concentration of ethyl alcohol to dissolve, but may inactivate the phage</td>
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<tr>
<td>Corn starch</td>
<td>Dispersed 9.5 g of regular (TEST1) or waxy (TEST2) corn starch in 60 mL distilled water. Heated and stirred until gelled/ 5 mL glycerol and 5 g citric were added when starch begins to gel</td>
<td>Dragged starch paste on PLA film</td>
<td>Corn starch coating curled at edges and was brittle after drying. Addition of citric acid created a lumpy texture.</td>
<td>Nancy L. García, et al., 201</td>
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</tbody>
</table>
Table 8.2: Summary of experimental findings during xanthan coating development

<table>
<thead>
<tr>
<th>Formulations tested</th>
<th>Application method</th>
<th>Drying</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1 - Xanthan at 1.0% (w/w) with glycerol at 10% (w/w); Test 2 - Xanthan at 1.0% (w/w) with glycerol at 20% (w/w); Control B - Xanthan at 1.0% (w/w), no glycerol; Control A - uncoated PLA</td>
<td>Pour-casting vs. roll casting</td>
<td>Dry in 45 °C 30% RH environmental chamber overnight</td>
<td>Pour casting was promising, but the amount of glycerol used was high (10 g and 20 g in 90 mL and 80 mL of distilled H2O respectively.</td>
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<td>Test 1 - Xanthan at 1.0% (w/w) with glycerol at 0.5% (w/w); Test 2 - Xanthan at 1.0% (w/w) with glycerol at 1.0% (w/w); Test 3 - Xanthan at 1.0% (w/w) with glycerol at 3.0% (w/w); Test 4 - Xanthan at 1.0% (w/w) with glycerol at 5.0% (w/w); Control B - Xanthan at 1.0% (w/w), no glycerol; Control A - uncoated PLA</td>
<td>Poured 15 g onto each film 10 cm X 14 cm; roll coating</td>
<td>Dried in 45 °C 30% RH environmental chamber overnight</td>
<td>Test 1 - xanthan flaked off when scraped with small stir stick; Test 2 - same as 1 but slightly stronger hold; Test 3 - more wet / sticky properties of glycerol-xanthan mixture became apparent; Test 4 - many bubbles and sticky surfaces; all films were flexible enough to bend into a hollow cylinder without visible cracking.</td>
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<td>Test 1 - Xanthan at 1.0% (w/w) with glycerol at 1.0% (w/w) with 1% (w/w) Felix-O1 phage; Test 2 - Xanthan at 1.0% (w/w) with glycerol at 2.0% (w/w) with 1% (w/w) phage; Control C - Xanthan at 1.0% (w/w), 1% (w/w) glycerol, no phage; Control B - Xanthan at 1.0% (w/w), no glycerol, no phage; Control A - Uncoated PLA</td>
<td>Poured 15 g onto each film 10 cm X 14 cm; roll coating</td>
<td>Dried in 45 °C 30% RH environmental chamber overnight</td>
<td>Control flaked off readily; TEST1 and TEST2 resulted in stronger coating; adhesion stronger for TEST2. Formulation used in subsequent immobilization of Felix O1 bacteriophages in xanthan with 2.0% glycerol</td>
</tr>
<tr>
<td>Test 1 - Xanthan at 1.0% with glycerol at 1.0% (w/w) with 1% (w/w) Felix-O1 phage; Test 2 - Xanthan at 1.0% (w/w) with glycerol at 2.0% (w/w) with 1% (w/w) phage; Control C - Xanthan at 1.0% (w/w), 1% (w/w) glycerol, no phage; Control B - Xanthan at 1.0% (w/w), no glycerol, no phage; Control A - Uncoated PLA</td>
<td>Poured 15 g onto each film 10 cm X 14 cm; roll coating</td>
<td>Dried in 45 °C 30% RH environmental chamber overnight</td>
<td>Strong adhesion for 1% (w/w) and 2% (w/w) glycerol. Conducted preliminary antimicrobial testing. Pour casting provided thicker film with higher loading of phage</td>
</tr>
</tbody>
</table>
Test 1 - xanthan plasticized with DATEM; (diacetyl tartaric acid ester of mono- and diglycerides) TEST2 - xanthan plasticized with 1% glycerol but held down under sides of cast frame; Control - uncoated commercial PLA film from Bi-Ax International Inc. (Wingham ONT).

Poured 15 g onto each film 10 cm X 14 cm; roll coating Dried in 45 °C 30% RH environmental chamber 24 hours DATEM not soluble in water. Waxy film formed by Test 1. Holding down TEST 2 under the sides of the frame did not improve coating appearance; uneven

Re-evaluated 2% xanthan coating using red food colouring to aid in visualizing coating appearance. 2% Xanthan dissolved in deionized water using ~10 mL ethyl alcohol to aid dispersion. 2:1 xanthan: glycerol ratio

Poured 15 g onto each film 10 cm X 14 cm; roll coating Dried 5-10 min after roll-casting Streaking more apparent than before. May be due to incompatibility between the roller and the coating or surface properties of PLA.

Re-evaluated 2% xanthan coating. No food colouring. Only one test. 2% Xanthan dissolved in deionized water using ~10 mL ethyl alcohol to aid dispersion. 2:1 xanthan: glycerol ratio.

Poured 15 g onto each film 10 cm X 14 cm; draw-down coating bar Dried 5-10 min after roll-casting, but placed in Environmental chamber at 45 °C 30% RH. Draw down applicator produced a more evenly coating than roller. Coating appears even after drying overnight in environmental chamber. This formulation will be used in continued material property testing

8.2 Spot assay procedure for bacteriophage activity

- Host cell incubated in nutrient broth for 30 min at 37 °C. This is to produce logarithmic growth phase bacteria. Tryptic soy agar (TSA) prepared according to manufactures directions. Soft agar prepared from 2 % (w/v) TSA agar powder added to 100 mL nutrient broth (TSB; Fisher Scientific, Whitby ON). This overlay of agarose keeps the cells stable, and creates a uniform host concentration. On the base of the petri dish bottom, mark the dilution series (10^0 to 10^{-11} or end of the dilution series).
• To obtain purified bacteriophage suspension, host cultures of *Listeria* or *Salmonella* were propagated on the surface of bacterial lawns grown under conditions in which host cell and their phages grow in equilibrium. Room temperature sterile sodium-magnesium (SM) buffer was added to the lawn surface in 5 mL aliquots and plates incubated at 4 °C. After 16-24 h, as much as possible of the surface liquid was recovered from plates with *Listeria* lawns flooded with the 5 mL buffer. The recovered suspension consisting of free phage and cell debris was then filter-sterilized using a 0.2 μm syringe filter. From this phage suspension, a dilution series of phage from $10^0$ to $10^{-11}$ was created using sterile saline or SM buffer or peptone water in 1 ml aliquots.

• To create the bacterial lawn, 500 μL of bacteria dispersed throughout 5 mL soft agar. Solid agar poured first and allowed to solidify for 20 min. Mix host culture and soft, low percentage agar after top agar has cooled to ~50 °C to prevent heat killing of culture. Mix thoroughly before pouring onto solid bottom agar. Bottom agar can be warm but not liquid; it must be solid. Soft agar with host bacteria is then poured over top and allowed to solidify for another 20 min. Using sterile pipette tips, spot 10 μL of each phage dilution onto the soft agar layer in the appropriate place according to the markings made on the base of the petri dish.

• Allow the spotted bacterial dilutions to dry to the soft agar layer in a biosafety cabinet for 30 min or until no signs of wet dilution remain. It is very important that the spots do not ‘run’ or spread when inverted as this will compromise the test results. Incubate in an inverted position at 37 °C for 24 hours.
During the incubation period, phage will bind to host, viral DNA is injected and reproduced, then host cells lyse releasing phage progeny. These released phages infect neighboring cells and eventually cause a visible clearing of the cell lawn, referred to as a plaque. Plaque forming units (PFU) are enumerated as follows:

\[
\frac{PFU}{mL} = \frac{\text{number of plaques}}{\text{mL spotted}} \times \frac{1}{\text{dilution factor}}
\]

8.3 Preliminary work on MC-HPMC films with nisin

8.3.1 Introduction

Coatings of Methyl Cellulose (MC) and Hydroxypropyl Methyl Cellulose (HPMC) and other modified cellulose blends have been investigated by Imran, El-Fahmy, Revol-Junelles, & Desobry, (2010) and Takala, Vu, Salmieri, Khan, & Lacroix, (2013). Imran et al. (2010) prepared HPMC films incorporated with glycerol as a plasticizer and nisin as an antimicrobial agent. Films were prepared via solution casting onto petri dishes, then dried at 20 °C and ~50% RH for 24–48 h. Films were evaluated on their mechanical, barrier (O\(_2\) and H\(_2\)O), optical, and antimicrobial properties. Antimicrobial activity was detected by measuring growth inhibition caused by the diffusion of antimicrobial into a lawn of bacteria grown on nutrient agar. The films exhibited antimicrobial activity in decreasing order against \( \text{Listeria} > \text{Enterococcus} > \text{Staphylococcus} \).

Transparency and water permeability of the films were negatively affected by the addition of nisin. On the other hand, Takala et al. (2013) prepared bioactive MC films using two antimicrobial formulations. Formulation A consisted of an organic acid mixture (DuraFresh™, by
Kerry ingredients and flavors; Monterey, Tennessee, USA) rosemary extract, and an Asian spice essential oil (BSA Food Ingredients s.e.c./l.p. Montreal, Canada) while formulation B consisted of the same organic acid mixture, rosemary extract, and Italian spice essential oil (BSA Food Ingredients s.e.c./l.p. Montreal, Canada) Antimicrobial activity of these films was tested on broccoli florets inoculated separately with *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella typhimurium* to have a final microbial concentration of 5 logs CFU/g sample. In their test, the antimicrobial MC films were sealed in air in Winpak® bags (PA/PE) stored at 4°C and evaluated for a 4 d period. They reported that both films displayed inhibitory properties against the growth of *L. monocytogenes* and *E. coli*, and that formulation A was more effective than formulation B. The volatile nature of these bioactive mixtures could be promising when applied in MAP meat products, especially in packaging where there is headspace between packaging and product. To this end, the volatile compounds must be encapsulated within a carrier matrix prepared using methods such as casting, coating, spraying, electrospinning, and so on.

For applications involving direct contact of food with antimicrobial packaging, non-volatile antimicrobial species have been investigated. Imran, El-Fahmy, Revol-Junelles, & Desobry (2010) prepared HPMC films incorporated with glycerol as a plasticizer and nisin as an antimicrobial agent. Films were prepared via solution casting onto petri dishes, then dried at 20°C and ~50% RH for 24–48 h. Films were evaluated on their mechanical, barrier (O₂ and H₂O), optical, and antimicrobial properties. Antimicrobial activity was detected by measuring growth inhibition caused by the diffusion of antimicrobial into a lawn of bacteria grown on nutrient agar. The films exhibited antimicrobial activity against the test pathogens, in the following decreasing efficacy: Listeria > Enterococcus > Staphylococcus. Transparency and water
permeability of the films were negatively affected by the addition of nisin. Additionally, cellulose has been shown to be a promising carrier for bacteriophages for antimicrobial active packaging (Gouvêa, Mendonça, Soto, & Cruz, 2015). These researchers demonstrated the antimicrobial effect of cellulose acetate films embedded with solution of isolated bacteriophages (native to poultry exudate and poultry and swine feces) against *Salmonella Typhimurium* ATCC 14028 in plate cultures.

### 8.3.2 Materials and Methods

MC-HMPC coating was prepared following a similar procedure as described by Grower, Cooksey, & Getty (2004). Briefly, 0.375 g MC and 0.875 g HPMC were dry blended, then dispersed using continuous stirring with a magnetic stir bar into 25 mL of deionized water and 25 mL of 95% ethyl alcohol. PEG 400, at 0.75 g, was added as a plasticizer. The solution was acidified by adding 1.25 mL of 0.02 N acetic acid and mixed using a stir bar. The solution was then split in two equal volumes of 25 mL, and nisin added at 3.75% (w/w) and 5.0% (w/w) levels respectively. Another volume of 50 mL of MC-HPMC solution without nisin was also prepared as a control coating.

With nisin containing-MC-HPMC coating, the coated PLA films were tested on soft nutrient agar layer (0.4%, w/v) in which $10^8$ CFU/mL dilution of *L. innocua* had been dispersed, and poured on top of a solid agar layer. *L. innocua* was selected here to evaluate the efficacy of active packaging films with nisin since it is effective against gram positive bacteria species, due to its ability to disrupt membrane potential across the singular membrane in gram positive cells (Gao, Abee, & Konings, 1991; Ruhr & Sahl, 1985).
8.3.3 Results and Discussion

Some thickness variations and irregularities were observed with MC-HPMC coating when applied with the roller, while the draw-down applicator produced a more even coating. The pH of coating solutions can be seen in Table 5.3. Chitosan coating had the lowest pH of all coatings applied. Addition of nisin reduced the pH of MC-HPMC coatings, likely due to the native solubility of nisin being in the acidic range, with optimal solubility near pH 4 to 5 (Liu & Hansen, 1990) or due to the denatured milk solid present in this product having. MC-HPMC films are more highly hydrophilic and dissolve readily into the agar and surrounding regions, actively reducing growth around the film sample.

Similar to the results reported by Imran et al. (2010), the addition of nisin in MC-HPMC reduced the film transparency (Fig. 8.1). An opaque film is acceptable in the case of in-between-slice inserts, as being able to see product features is dependent on outer packaging.

In Fig. 8.2 the inhibition of L. innocua growth produced by nisin diffusion from the MC-HPMC coating is apparent. After 24 h incubation at 37 °C, a zone of clearing measuring ~30 mm from the edge of the active packaging was present. This is consistent with findings by Grower et al. (2004) who observed similar clearing on growth media at the highest concentration of nisin (10,000 IU/mL) incorporated in their coatings on LDPE. The nisin concentration used in the present study, (i.e. 7,500 and 10,000 IU/mL) was determined based on maximum allowable concentration of nisin permissible for pasteurized processed cheese spread in the USA (Jones, Salin, & Williams, 2005).
Figure 8.1: Change in transparency for PLA films coated by MC-MPMC coatings with 5% (w/v) nisin addition after drying at ambient conditions for 30 min.
Figure 8.2: 0 h vs. 24 h incubation at 37 °C for MC-HPMC + nisin coated PLA films on nutrient agar with ‘soft’ agar lawn. Column a = 0 h photos; b = 24 h photos; row 1 = MC-HPMC films with nisin; 2 = uncoated PLA film; 3 = blank plates with bacterial lawn.
8.3.4 Conclusion

Coating PLA film with coating of MC-HPMC and nisin at 10,000 IU/mL effectively produced a zone of clearing on bacterial lawns created by dispersing bacteria in a thin soft agar after 24 H incubation. Films were opaque in appearance, and coating was able with to withstand curling of film without fracturing. Further testing should be scaled to food systems to understand any effect of spoilage or pathogen growth on food material. MC-HPMC is a versatile, low-cost, and available in varying grades, allowing packaging converters the ability to tailor the barrier/release properties and warrants further exploration as a coating material for active packaging films.

8.4 Exploratory work conducted on PLA using the OpenAir™ plasma system

Briefly, in conducting exploratory work using the OpenAir™ plasma system (Figs. 8.3 and 8.4), it was found that at slower speeds and at lower z distances (lower distance from film surface), PLA film is at increased risk to damage from thermal degradation. This effect being due to the heat given off by the plasma source. Surface energy of plasma- treated PLA increased by over 70% from 38 dyn for un-treated PLA, to just over 65 dyn for treated films. Considering these findings, it is apparent that a variety of research avenues could be investigated using this technology.
Figure 8.3: Surface tension (Dyne) of OpenAir™ plasma treated PLA film in response to nozzle head speed
Figure 8.4: Surface tension (Dyne) of OpenAir™ plasma treated PLA film in response to change in nozzle distance from PLA (z Distance)
9.0 References


Coutinho de Oliveira, T. L., Malfitano de Carvalho, S., de Araújo Soares, R., Andrade, M. A., Cardoso, M. D. G., Ramos, E. M., & Piccoli, R. H. (2012). Antioxidant effects of Satureja montana L. essential oil on TBARS and color of mortadella-type sausages formulated with different levels of sodium nitrite. LWT - Food Science and Technology, 45(2), 204–212.


contamination and pre-slaughter control methods. Animal Health Research Reviews, 12(2), 197–211.


