ANTIMICROBIAL AND BIODEGRADABLE FOOD PACKAGING FILMS WITH CHITOSAN-BASED N-HALAMINE STRUCTURES TO PREVENT CONTAMINATION BY DRUG SUSCEPTIBLE AND RESISTANT STRAINS OF *SALMONELLA* TYPHIMURIUM

By

Vinni Thekkudan Novi

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biosystems Engineering – Master of Science

2019

ABSTRACT

ANTIMICROBIAL AND BIODEGRADABLE FOOD PACKAGING FILMS WITH CHITOSAN-BASED N-HALAMINE STRUCTURES TO PREVENT CONTAMINATION BY DRUG SUSCEPTIBLE AND RESISTANT STRAINS OF *SALMONELLA* TYPHIMURIUM

By

Vinni Thekkudan Novi

Contamination of food samples with antibiotic resistant *Salmonella* Typhimurium has become a cause for concern due to difficulty in treating infections caused by this pathogen. In one approach, chitosan/PVA-based N-halamine (CPN) film was developed and tested for its efficacy in inactivating drug susceptible and ampicillin resistant *Salmonella* Typhimurium strains. The CPN film significantly (100%) inactivated the growth of both strains during the antimicrobial sandwich assay when tested for five days since film preparation, while the CH/PVA films showed around one log reduction ($p<0.05$). CPN films reduced the drug resistant strain's growth on cheddar cheese slices by 5-6 logs at 25°C and 3-4 logs at 4°C when packaged and stored over a period of five days unlike CH/PVA films that did not show significant reduction. The second approach involves the synthesis of a stronger chitosan N-halamine-based coating on plasma treated polycaprolactone film (CH-NX/PCL film). The FTIR peaks obtained for chitosan coated PCL film (CH/PCL) showed characteristic peaks of both PCL and chitosan, specifically at 1720 cm^{-1} and 3354 cm^{-1}, respectively. The tensile strength of the PCL was higher, while the Young's modulus value was higher for CH/PCL. CH/PCL film showed better barrier against water and oxygen compared to PCL. The antimicrobial efficacy of the CH-NX/PCL film was 100% against both strains of *Salmonella* Typhimurium when compared to PCL and CH/PCL, indicating that this fabricated film has promising applications in food safety.

ACKNOWLEDGEMENTS

The research was funded by The Axia Institute – Michigan State University. This funding source was not involved in the actual research or writing of this report.

Dr. Shan Shan is acknowledged for the original fabrication of the chitosan/PVA-N-halamine films and determination of optimal conditions for the antimicrobial sandwich assay. Raymond Lesiyon is acknowledged as a valuable undergraduate contributor to this study. Ms. Xinyi Wang is acknowledged for providing consultation on plasma treatment and coating technique for film fabrication.

The author would like to acknowledge Mr. Michael Rich and the Composite Materials and Structures Center for training and offering advice on characterization techniques such as thermogravimetric analyzer, water vapor transmission tester and oxygen transmission tester. Dr. Per Askeland is acknowledged for his assistance in Plasma Reactor use and FTIR. Mr. Brian Rook is acknowledged for offering tensile strength testing services.

Additionally, the author would like to thank Dr. Shannon Manning and the Manning Laboratory for providing the bacterial cultures and advice on microbiological methods and Dr. Jade Mitchell for providing advice on experimental design. Dr. Maria Rubino and Dr. Rafael Auras are acknowledged for offering consultation on fabrication of packaging films.

The author would also like to acknowledge Dr. Evangeline Alocilja for providing guidance throughout the study and the NanoBiosensors Laboratory, specifically, Saad Sharief and Dr. Leann Lerie Matta for their help and support.

Finally, the author would like to express gratitude to family and friends for being supportive and encouraging throughout the period of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

CDC Centers for Disease Control and Prevention

PCA Peanut Corporation of America

ZnO Zinc oxide

TiO_2 Titanium dioxide

PVA Polyvinyl alcohol

PCL Polycaprolactone

ASTM American Society for Testing and Materials

CH Chitosan

PHB Polyhydroxybutyrate

PHA Polyhydroxyalkanoate

PHBV Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

PLA Polylactic acid

FDA Food and Drug Administration

EO Essential Oil

GRAS Generally Recognized as Safe

ZEO *Zataria* Essential Oil

CEO Cinnamon Essential Oil

CD Cyclodextrin

EGO *Eucalyptus globulus* Essential Oil

EEO Eucalyptus Essential Oil

TP Tea Polyphenols

MgO Magnesium Oxide

CaO Calcium Oxide

UV Ultraviolet

SEM Scanning Electron Microscopy

EDTA Ethylenediaminetetraacetic acid

BEO 4-(Bromo-acetic acid methylester)-4-ethyl-2- oxazolidinone

MIC Minimum Inhibitory Concentration

MBC Minimum Bactericidal Concentration

CFU Colony Forming Unit

LAE Lauric arginate

NaL Sodium Lactate

SA Sorbic acid

AA Acetic Acid

CA Citric Acid

LA Lactic Acid

LevA Levulinic Acid

OEO Oregano Essential Oil

TSB Tryptic Soy Broth

TSA Tryptic Soy Agar

BPLS Brilliant Green Phenol Red Lactose Sucrose Agar

PBS Phosphate Buffer Saline

NaOH Sodium Hydroxide

KI Potassium Iodide

CPN Chitosan/Polyvinyl Alcohol N-halamine

CH/PVA Chitosan/Polyvinyl Alcohol

$Na_2S_2O_3$ Sodium Thiosulphate

AATCC American Association of Textile Chemists and Colorists

ANOVA Analysis of Variance

SS Sum of Squares

DF Degrees of Freedom

MS Mean of Squares

CH-NX/PCL Chitosan-based N-halamine coated Polycaprolactone

FTIR Fourier Transform Infrared

TGA Thermogravimetric Analysis

UTS United Testing Systems

WVTR Water Vapor Transmission Rate

RH Relative Humidity

WVP Water Vapor Permeation

OTR Oxygen Transmission Rate

CH/PCL Chitosan/Polycaprolactone

OP Oxygen Permeation

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) has reported several foodborne outbreaks in the U.S. over the past few years of which most of the contaminations are due to the bacteria *Salmonella* [1]. Every year, there are about 1.2 million illnesses caused by this bacterium, with 23,000 cases of hospitalizations and about 450 deaths just in the U.S. [1]. Almost a million of these illnesses have happened due to contamination in the food consumed [1]. There are different strains of *Salmonella* involved in each of these outbreaks and most recently there have also been reports of multi-drug resistant *Salmonella* strains causing infections in individuals through contaminated food [1]. Other pathogens involved in such outbreaks are norovirus, *Campylobacter* spp., *Shigella* spp., *Escherichia coli* [2] and *Listeria monocytogenes* [3]. There have been 16 outbreaks reported in 2018 in a wide range of food products including raw meat, sprouts, dry cereals and coconut, fresh vegetables and chicken [1]. There have also been outbreaks in dairy products such as cheese in the past [4]. Most such issues occur during the food processing phase, where the food gets exposed to pathogens that lead to such large-scale outbreaks. There have been hospitalizations and death reported in extreme cases, and economic losses of affected consumers. In 2009, the estimated annual cost of illness caused by non-typhoidal *Salmonella* spp. is $3,309.3 million [5]. Apart from these, huge monetary losses for the food companies involved have also been reported, primarily due to food recalls and settlements made in lawsuits against them. Some companies have also gone bankrupt due to this. For example, Peanut Corporation of America (PCA) was a relatively small company that went down due to one of the largest food recalls that happened in recent years, a result of failing to act against *Salmonella* contamination in their peanut products [6]. There are larger companies like Kellogg's that lost more than $65 million out of their profits due to recalls and loss in sales [6].

These critical issues make it necessary to find methods to prevent such foodborne contaminations in future.

Food packaging is generally done to improve the shelf-life, quality and safety of food. Specifically, antimicrobial food packaging is designed to prevent spoilage through exposure to contaminating pathogens in the environment [7]. However, most of the contaminations occur before the food is packaged and usually through its surface [8]. Therefore, an ideal way to tackle this issue would be to develop food packaging materials that can eliminate the microorganism that invaded the food surface and prevent further contamination. Currently used commercial packaging materials are petroleum-based products and mostly synthetic in nature such as polystyrene [9]. Some of these films have been modified to incorporate antimicrobial property and tested on food samples [9]. The modification is usually in terms of antimicrobial coating applied on their surface [9]. However, there is now an increased trend towards identifying bio-based films due to their eco-friendly nature and flexibility in integrating antimicrobial agents. There are two main methods through which this can be done – by embedding the antimicrobial agent within the film and by coating the agent on the film surface. Several biodegradable polymers have been studied for this purpose including chitosan, starch, polylactic acid, etc. [10].

A wide range of antimicrobial agents have been explored for food safety, many of them being essential oils from plant sources. These essential oils are rich in active components such as terpenoids and phenolic particles, which can affect bacterial growth through disruption of the cytoplasmic membrane, disruption of the transport system in their cell wall and inhibition of protein synthesis [11]. Silver with inherent antimicrobial properties have been incorporated as antimicrobial agents in food packaging in the form of nanoparticles [11]. Inorganic nanoparticles such as zinc oxide (ZnO) and titanium dioxide (TiO_2) were studied for their ability to inactivate

microbial growth [11]. TiO_2 is photoreactive and generates hydroxyl radicals along with reactive oxygen species that can oxidize the phospholipids present in the bacterial cell membrane. Other antimicrobial agents include chitosan, enzymes such as lysozymes and bacteriocins from lactic acid bacteria [11]. Some films have also included chemical agents that can preserve food and prevent contamination, especially quaternary ammonium salts [12] while some films also contain chitosan-based N-halamine structures [13].

HYPOTHESES AND OBJECTIVES

This study focusses on the use of the natural polymer, chitosan, to prepare a biodegradable packaging film, primarily because it has the added advantage of being antimicrobial in nature [13]. The objective is to enhance the antimicrobial activity of fabricated chitosan-based food packaging films using simpler methods and cost-effective raw materials.

The novelty of the study is the simple fabrication of a chitosan-based N-halamine functionalized film, that does not require additional processing steps unlike most studied in the literature. The antimicrobial activity of such films against drug resistant pathogens, especially *Salmonella* Typhimurium have not been studied in the past and this work aims to tackle this problem.

Generally, biodegradable polymers are observed to have low mechanical strength compared to the synthetic polymers currently used in the packaging industry. However, blending such polymers with another biodegradable polymer can improve their mechanical and barrier properties [14], which are essential for any food packaging material. In this study, previously synthesized chitosan and polyvinyl alcohol (PVA) blended film, with simple generation of N-halamine structures on their surface, was tested as a potential packaging material for cheddar cheese slices. The film was specifically tested for antimicrobial activity against the common foodborne pathogen *Salmonella* Typhimurium, both drug-susceptible and drug-resistant strains. Another way to incorporate the enhanced antimicrobial chitosan activity in food packaging material is to coat them on a stronger film. There is a limited availability of biodegradable polymers bearing ideal physical properties for packaging applications. Polycaprolactone (PCL) is one such polymer obtained synthetically and having aliphatic ester linkages in its main chain. This polymer meets the American Society for Testing and Materials (ASTM) standard for biodegradability and has a melting point of 60°C. PCL with molecular weight higher than 40,000

g/mol form films that are water resistant and ideal for packaging applications [15]. This study also focusses on synthesizing ideal packaging films using PCL incorporated with the antimicrobial property of N-halamine through chitosan coating, followed by characterization of its physical properties. Antimicrobial activity of the synthesized film against drug susceptible and ampicillin resistant *Salmonella* Typhimurium was also tested.

The following are the hypotheses tested:

1. N-halamine-based chitosan films can inactivate growth of both drug susceptible and drug resistant strains of the foodborne pathogen *Salmonella* Typhimurium.
2. The fabricated chitosan/PVA based N-halamine films can inactivate growth of both strains of bacteria in contaminated cheddar cheese matrix.
3. The fabricated chitosan-N-halamine (CH-N-halamine) coated PCL film can inactivate growth of both strains of bacteria apart from having better mechanical properties for packaging applications.

CHAPTER 1

LITERATURE REVIEW

1.1. Biodegradable polymers used for packaging

1.1.1. Polysaccharide-based polymers

It is important to consider the water barrier properties of polymers while being considered for food packaging applications. In case of fresh produce, it is necessary to avoid dehydration while in case of dry food such as chips, it is important to prevent the food from turning soggy. Polysaccharide materials generally have good CO_2 and O_2 barriers. However, they are hydrophilic in nature and have poor water barrier properties. There are several polysaccharides found in nature that have been studied for food packaging application which includes starch, chitosan, cellulose, alginate and carrageenan [16].

Starch

This is the most naturally abundant biodegradable polymer that is of low cost and easily available. It has good film forming properties due to the presence of amylose, which is its linear component and the other being amylopectin, which is highly branched. Genetically modified corn is a good source of starch with high amylose content and is generally used to make biodegradable films. However, starch with high amylose content has the disadvantage of being highly crystalline in nature which would need to be gelatinized at high temperatures (above 100°C and atmospheric pressure) [17]. This polysaccharide is considered to be a good matrix to incorporate antimicrobial materials for packaging applications [18].

Cellulose

Cellulose is the most abundantly available polymer in nature that has film forming properties, making it an ideal candidate for studies as a food packaging material. It can be isolated from several plant-based sources such as hemp, wood and cotton. It has the advantage of being non-toxic and less expensive. Most often cellulose is used in the form of derivatives with improved properties. Hydroxypropyl cellulose, methylcellulose and cellophane are examples of such derivatives. Though they have good gas barrier properties, they are not resistant enough to water vapor [16]. Cellulose acetate is formed by the acetylation of cellulose and has advantages of being non-toxic, stable and odorless. It is water vapor permeable [19], which can be an advantage or a disadvantage depending on the type of food being packaged.

Alginate

Alginate is extracted from an algal source which is the brown seaweed, *Phaeophyceae*. It is a linear copolymer made of 1-4β-d-mannuronic acid (M) and α-l-guluronic acid (G) and are salts of alginic acid. They have the ability to interact with cations, which enables them to form films that can be used in packaging application. More commonly they interact with calcium to form calcium alginate. The cations serve as a gelling agent for the alginate material [16].

Pectin

Pectin consists of linear and branched regions of poly α-1,4-galacturonic acids and has a complex hetero-polysaccharide structure. This polymer, however, do not have good physical properties that can be applied as ideal food packaging material and may require modifications before being used for that purpose [20].

Chitosan

Chitosan is a derivative of the naturally occurring polymer, chitin. This is generally obtained from shells of crustaceans and insect cuticles. The removal of N-acetyl groups on chitin through alkali treatment at high temperatures produce chitosan [21]. Both chitin and chitosan polymers exhibit antimicrobial properties which makes them desirable for several applications that involve prevention of contamination. Chitosan is linear in structure and consists of (1,4)-linked 2-amino-deoxy-β-d-glucan, is non-toxic and biocompatible [16].

1.1.2. Protein-based polymers

Protein-based polymers are made of amino acid monomer units. Whey, soy and corn zein proteins have been commonly studied for the fabrication of ideal food packaging materials [18].

Whey protein

Whey protein isolate is a by-product of cheese or casein manufacture and is abundantly available in milk. It has polymeric properties and forms films with good gas barrier properties. However, these films have low tensile strength and poor water barrier properties, which necessitates its modification through blending or coating with other polymers and plasticizers [22].

Soy protein

A by-product from soy oil production is used to extract soy protein isolate that has shown film-forming properties. These films, like most other naturally occurring polymers, have excellent oxygen barrier properties and high water vapor permeability due to their hydrophilic nature [23].

Corn zein

Zein is a protein found in corn and maize that is hydrophobic in nature. This protein can form films that have been explored for food packaging applications. Though they have excellent gas barrier properties, they do not have desirable physical properties that limit their use as films for long term food storage [24]. Unlike films obtained from other protein sources, zein shows good thermoplastic behavior [25] that is useful for packaging applications, if modified to have better mechanical characteristics.

1.1.3. Microbiological polymers

Polyhydroxybutyrate (PHB)

This is a synthetically produced biodegradable polymer that has been studied in food packaging application. It belongs to the polyhydroxyalkanoates (PHAs) family and is produced using microorganisms such as *Ralstonia eutrophus*, *Bacillus megaterium*, etc. through the process of fermentation. This polymer has poor mechanical properties compared to most other biodegradable polymers, is brittle and have low gas barrier properties which may not be ideal for food storage [26].

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)

To improve the properties of PHB, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was created with three units of PHB and one unit of hydroxyvalerate. This polymer shows lower melting temperature and crystallinity with respect to PHB, making it more suitable for commercial application. However, this modification has lowered the mechanical properties along with making it thermally less stable [27].

1.1.4. Synthetically produced biopolymers

Polycaprolactone (PCL)

Polycaprolactone is a synthetic polymer fabricated by the ring-opening polymerization of the cyclic monomer ε-caprolactone [19]. This material shows good thermoplastic behavior, is biocompatible, and has good mechanical properties. Its biocompatibility makes it an ideal polymer for use in biomedical applications [28].

Polylactic acid (PLA)

Polylactic acid has been derived from natural sources and is one of the most promising biodegradable polymers for food packaging applications. It is usually obtained by the fermentation of renewable starch-rich products like corn, wheat, sugar beet, etc. this polymer can be synthesized either through condensation or ring-opening polymerization process of lactic acid. The advantages of this polymer include good mechanical properties, high barrier and low toxicity [19].

Polyvinyl alcohol (PVA)

Polyvinyl alcohol is another synthetic polymer with biodegradable properties. It has a linear structure and has good film forming properties, is non-toxic and compatible with other materials and has good adhesive properties as well. This polymer has good mechanical properties, with chemical resistance and low moisture absorption properties compared to most other hydrophilic polymers. This material has been approved by the Food and Drug Administration (FDA) to be used as food packaging material because of additional advantages such as barrier to oxygen and aromatic compounds [17].

1.2. Antimicrobial agents used in biodegradable packaging materials

1.2.1. Plant-based antimicrobial agents

Essential oils (EO)

Essential oils are generally produced by plants to protect themselves from microbial and insect attacks. They are colored liquids with volatile compounds that have antimicrobial activity. These compounds are commonly plant secondary metabolites with aromatic functional groups that give out strong odor. EOs are known to have larvicidal, antifungal, antioxidant, antitumor and anti-inflammatory activities. Due to the emergence of multidrug resistant bacterial pathogens, more research is being carried out with essential oils to identify ideal antimicrobial doses that can replace ineffective antibiotics against bacterial infections or contaminations [29].

EOs can be extracted from different parts of a plant including leaves, seeds, herbs, wood, fruits, roots and flowers. *Zataria multiflora* Boiss consists of EOs with antimicrobial activity such as carvacrol and thymol. Both these phenolic compounds have been approved by the FDA as Generally Recognized as Safe (GRAS) and so, the antimicrobial efficacy of the *Zataria* EO (ZEO) was studied against pathogens such as *L. monocytogenes, E. coliO157:H7, S. aureus* and *S.* Typhimurium, and some kinds of fungi in the past. Specifically, it has been incorporated in zein based films and tested for antimicrobial efficacy against *L. monocytogenes* and *E. coli.* Two concentrations of ZEO were tested and it was found that 5% of the EO caused around one log reduction, whereas, 10% of EO applied showed around two log reduction of both bacterial strains [25].

Cinnamon essential oil (CEO) is one of the most studied EOs for antimicrobial food packaging applications. It is a natural EO that possesses antifungal and antimicrobial activity against other

pathogens. However, CEO easily decomposes at temperatures higher than 60°C and forms benzaldehyde, losing its desirable antimicrobial properties in the process [30]. A previous study tested the activity of CEO against *Escherichia coli* and *Staphylococcus aureus* by its incorporation into a PVA based film. This antimicrobial film was fabricated using electrospinning method and consists of β-cyclodextrin (β-CD) to encapsulate CEO. The encapsulation is done to improve its stability and mask its undesirable flavor in the film. The PVA/CEO/β-CD nanofibrous film was able to inhibit both bacteria but the addition of CEO and β-CD increased the hydrophilicity of the film [31], which could potentially reduce its water barrier properties. Yet another study incorporated CEO into PVA films with the aid of Pickering emulsion to stabilize this essential oil. Due to the low thermal stability, susceptibility to oxidation and light [32], the CEO requires systems that could stabilize them while being applied as an antimicrobial agent in a potential packaging film.

Eucalyptus globulus essential oil (EGO) is another compound studied for its antimicrobial activity in food packaging films. One study used this EO in a chitosan film and tested for its antimicrobial efficacy against Gram-negative bacteria - *Salmonella enteritidis* and *Escherichia coli*; Gram-positive bacteria - *Bacillus cereus* and *Staphylococcus aureus*. In all the cases the films were able to show around 4 log reductions compared to the controls when EGO was applied in the liquid form. In its vapor form, the reduction was lesser, around one log. The active films exhibited morphological changes compared to the control, which included reduction in its tensile strength due to increase in porosity of the chitosan film with the incorporation of EGO [33]. Another study describes the fabrication of chitosan films incorporated with cumin (CEO) and eucalyptus essential oils (EEO), which was tested for increasing the refrigerated storage life of fresh chicken meat. The inhibition of *Listeria monocytogenes*, *Salmonella typhi*,

Streptococcus pyogenes and *Shigella dysenteriae* by chitosan films with cumin and eucalyptus EO were tested. The results indicated potential application of these films for enhancing the storage life of packaged food [34].

Though EO based films exhibit good antimicrobial activity, there are certain drawbacks such as high cost, low water solubility, low stability and they contain volatile compounds that produce off-odors. These properties limit their practical application as a food packaging material [35].

Plant polyphenols

Polyphenols obtained from plants show potential antimicrobial activity against pathogenic microorganisms apart from being antioxidant. They also contribute to nutritional value apart from color and taste to the packaged food [35].

Gallic acid (3,4,5-trihydroxybenzoic acid) [36] has shown potential antimicrobial activities in past studies against bacteria such as *Salmonella typhi* and *Staphylococcus aureus.* This phenolic compound was extracted from *Caesalpinia mimosoides* Lamk (Leguminosae). There are other sources of gallic acid such as the flower of *Rosa chinensis* Jacq. That has shown potential antimicrobial activity against *Vibrios* species. This compound has also shown to modify the physical properties of films such as chitosan, where its incorporation improved the film's elasticity and reduced its brittleness. One study specifically tested the incorporation of various concentrations of gallic acid and the film's activity against *Escherichia coli, Salmonella* Typhimurium, *Listeria innocua* and *Bacillus subtilis*. The results indicated that in addition to enhanced antimicrobial activity, water and oxygen barrier properties of the film also increased with the addition of this phenolic compound [37].

In one study, apple peel was used as a source of polyphenols and was incorporated into chitosan film matrix. Apple peel consists of bioactive compounds such as phenolic acids, flavonols, flavon-3-ols, anthocyanins, dihydrochalcones, procyanidins, (+)-catechin, (−)-epicatechin, chlorogenic acid, phloridzin and quercetin conjugates. The results for this study indicated that the antimicrobial activity of chitosan film was enhanced with the addition of apple peel extracts containing polyphenols. The mechanical properties of the film were also modified, where its thickness and density increased along with decrease in the water vapor permeability. However, the tensile strength and elongation at break also reduced from that of regular chitosan films. The antimicrobial activity of these films depended on the concentration of the extract incorporated into the chitosan film [38].

Tea is a source of a class of phenols called tea polyphenols (TP). They are known to have excellent antimicrobial and antioxidant properties and their antimicrobial mechanism is to prevent microbial attachment through direct disruption of cells. Specifically, TPs can inhibit Gram-negative and Gram-positive bacteria along with fungal growth. In one study four polylactic acid/tea polyphenol composite nanofibers were prepared using electrospinning and studied for its application in food packaging. It was found that the incorporation of TP into the nanofiber reduced its tensile strength and elongation at break. Its antimicrobial activities against *Escherichia coli* and *Staphylococcus aureus* showed over 90% growth inhibition, showing that the films could potentially be used to improve the shelf life of food [39].

In most cases involving the incorporation of plant-based secondary metabolites into biodegradable polymer matrix, the mechanical properties of the film are being compromised. Though their antimicrobial efficacy is promising, it is equally important to produce packaging films that have physical properties that make them ideal for long-term storage. Therefore, further

studies are required to produce films with excellent antimicrobial properties that do not adversely affect the film's physical properties.

1.2.2. Metal ion-based antimicrobial agents

Metal ion-based antimicrobials are generally termed inorganic agents and mostly possess biocidal activity in their oxidized nanoparticle forms. The reason for this activity is due to their resistance to harsh treatment conditions under which they are prepared. Some of the metal oxides that were studied for food packaging applications include TiO_2, ZnO, magnesium oxide (MgO) and calcium oxide (CaO) [40].

TiO_2 nanoparticles

TiO_2 can absorb ultraviolet (UV) light from the sun or artificial light sources that leads to a photochemical reaction involving oxidation-reduction, making the metal ion more biocidal against microorganisms due to the production of free radicals. Antimicrobial PVA films based on the incorporation of TiO_2 nanoparticles were fabricated and their mechanical and antimicrobial properties were tested for packaging *Macrobrachium rosenbergii.* The results indicated that the tensile strength and oxygen barrier property of the PVA film increased with the incorporation of the nanoparticle. The fabricated films were thermally stable compared to pure PVA films and exhibited good antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [41]. Another study reported the fabrication of chitosan films incorporated with TiO_2 nano-powder and its antimicrobial activity against *Escherichia coli, Staphylococcus aureus,* and the fungal strains, *Candida albicans*, and *Aspergillus niger*. The metal ion in the film enhanced the antimicrobial efficacy and showed 100% sterilization in 12 h [42]. Though the antimicrobial and mechanical properties exhibited by films incorporated with this metal ion are excellent, due to

their potential toxicity to human cells [40], the application of TiO_2 in food packaging systems is not yet fully practical.

Silver nanoparticles

Silver nanoparticles have been incorporated into chitosan and starch-based films for enhanced antimicrobial properties. These films showed improved tensile strength and oxygen barrier properties. However, the water barrier properties were diminished. Their antimicrobial activity against *E. coli*, *S. aureus* and *B. cereus* was tested, and the results indicated that the fabricated film can potentially be applied for food packaging. The silver nanoparticles produced in this study used γ-ray irradiation unlike the conventional method and did not require additional steps in its production.

Silver nanoparticles are conventionally produced by the reduction of silver salts by a reducing agent in the presence of a stabilizer. This means that the production of these nanoparticles would also require removal of these additional agents at the end of the process making it costly and time consuming. Additionally, the residues could be toxic [43].

ZnO nanoparticles

ZnO nanoparticles have exhibited antimicrobial activity against bacteria, including some resistant strains and is approved by the FDA as GRAS. These nanoparticles were mixed with sodium carboxymethyl cellulose to form antimicrobial packaging films and their efficacy to inhibit *Staphylococcus aureus* was tested. Specifically, the study involved testing the film as a package for pork meat for 14 days in cold storage. The scanning electron microscopy (SEM) showed that the ZnO nanoparticles were able to rupture the bacterial cells in cold storage [44]. Another study used ZnO nanoparticles in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) films.

The thermal stability of the films improved with its addition and the films showed promise in inhibiting the growth of the foodborne pathogen *Listeria monocytogenes.*

Generally metal based materials are rarely recognized as safe [45] and are usually toxic for food-based applications. Therefore, there is a need to look for sources and doses of such materials that reduces toxicity and enhances antimicrobial activity against just the pathogenic microorganism cells.

1.2.3. Other antimicrobial agents

Lysozyme

Lysozyme is a naturally occurring enzyme that has demonstrated its use as a bio-preservative for food packaging studies. One study used lysozyme obtained from hen egg white as an antimicrobial agent and was blended into cellulose acetate films. The study investigated the morphology of the film with respect to the method of lysozyme incorporation and it was found the immobilization of lysozyme improved antimicrobial activities and tensile strength of the film [46]. The antimicrobial mechanism of this lytic enzyme starts with its attack on the β-1-4-glycosidic linkage between the N-acetylmuramic acid and the N-acetylglucosamine groups of the peptidoglycan layer in the bacterial cell wall. For this reason, Gram positive bacteria are highly susceptible to the effects of lysozyme than Gram negative bacteria [47]. There have been studies incorporating lysozyme in zein films along with the inclusion of EDTA to enhance its antimicrobial activity against Gram negative bacteria [48].

Nisin

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis* and is thermally stable. This antimicrobial agent is effective in inhibiting the growth of Gram-positive bacterial species

such as *Clostridium*, *Bacillus*, *Staphylococcus* and *Listeria.* One study explains the fabrication of nisin embedded PLA films for antimicrobial food packaging. The fabricated film was tested against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Enteritidis and it was found that film had reduced the growth of *L. monocytogenes* by around 4 logs in both culture medium and liquid egg white. In the case of the *E. coli* strain, the antimicrobial effect was more prominent in orange juice samples than in the culture medium. Growth of *Salmonella* Enteritidis reduced by two logs, much lower than that seen for the other two strains. However, at lower temperature, the nisin incorporated film could reduce around 3 logs of this bacteria after 21 days in liquid egg white compared to the control [49]. Another study use chitosan/PVA films with nisin to test its activity against *Staphylococcus aureus* [50].

N-halamine-based antimicrobial agents

N-halamine consists of nitrogen-halogen covalent bonds with potential antimicrobial activity against pathogens and have been exploited in past studies for their use as an antimicrobial agent in packaging materials [51]. There are several compounds that have the N-halamine structure and they usually involve complex preparation processes with various chemicals. One of the compounds prepared for this purpose is 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinon, which was used in absorbent pads for beef [51] and chicken [52]. The preparation of N-halamine structures with the food packaging polymer usually involves the chlorination of available or synthesized N-halamine precursors and these include polymethacrylamide [53], 4-(Bromo-acetic acid methylester)-4-ethyl-2- oxazolidinone (BEO) [54], *m*-aramid [55], etc.

Studies involving N-halamine based antimicrobial films have shown excellent antimicrobial efficacy against pathogenic microorganisms. However, their complex preparation process may be time consuming and expensive and this necessitates a need to look for simpler methods to

prepare such films with minimal damage to the mechanical properties of the biodegradable film used.

1.3. Antimicrobial packaging systems against *Salmonella* contamination

The following table gives a list of antimicrobial packaging systems that have been specifically tested against the foodborne pathogen, *Salmonella.*

Table 1: Examples of studies on antimicrobial packaging systems against *Salmonella* species

***Salmonella* strain**	**Biodegradable polymer**	**Antimicrobial agent**	**Film fabrication method**	**Additional materials for fabrication**	**Antimicrobial efficacy (MIC, MBC, log/CFU reductions)**	**References**
Salmonella spp.	PLA	Silver-based nanoclay	Solvent casting method with homogeneous dispersion of silver nanoclay	Montmorillonite for silver immobilization	99.99% CFU reduction	[56]
Salmonella enterica Serovar Typhimurium (*S.* Typhimurium)	PLA	Lauric arginate (LAE)	PLA film surface activated with corona discharge and coated with LAE	Silicone used as surfactant	2 to 3 log CFU/tested film with 0.07% of the agent	[57]

Table 1: (cont'd)

Salmonella Typhimurium	PLA	Edible chitosan-acid solutions incorporating lauric arginate ester (LAE), sodium lactate (NaL), and sorbic acid (SA) alone or in combination	Commercial PLA films with antimicrobial coating	2 % of acetic acid (AA), citric acid (CA), lactic acid (LA), and levulinic acid (LevA) for chitosan coating preparation	<0.69 log CFU/ml with 1.94 mg/cm^2 of chitosan and 1.94 μg/cm^2 of LAE in PLA film	[7]
Salmonella enterica	PVA	Oregano essential oil (OEO)	PVA solution casted with OEO in emulsion	Glycerol (0.5 wt%) and Tween 20 (0.5 wt%) for emulsion	Around 4.7 log CFU/tomato on first day	[58]
Salmonella Typhimurium	Chitosan; polyamide 6/66 chitosan-based blend; chitosan-coated polyamide 6/66	Chitosan	Solvent evaporation technique; incorporation of chitosan powder in extruded polyamide 6/66; regular coating on polyamide 6/66	-	Contact inhibition	[59]

As can be seen, limited studies have focused on this pathogen, though it is one of the most common pathogens seen in foodborne outbreaks. Past literature has concentrated on inhibiting the growth of drug susceptible bacterial pathogens and have not explored the activities of antimicrobial packaging systems for drug resistant bacterial strains. Antimicrobial resistance in bacteria is usually acquired through mutations in genes that are responsible for preventing antibiotics from entering the cell transport system and inactivating the pathogen [60]. Strains that are already resistant to a biocidal agent have the potential to develop resistance to other antimicrobial agents upon continuous exposure to them through a phenomenon called selection pressure. Therefore, studying the efficacy of antimicrobial packaging material in inactivating drug resistant pathogen warrants special attention and the present study aims to achieve that.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Chitosan (CH, low molecular weight, deacetylated chitin), polyvinyl alcohol (PVA, Mw 85,000-124,000, 99+% hydrolyzed), polycaprolactone (PCL, average Mn 80,000), sodium hypochlorite, tryptic soy broth (TSB), tryptic soy agar (TSA), brilliant green phenol red lactose sucrose agar (BPLS), sodium thiosulfate penta-hydrate (≥99.5%) and phosphate buffer saline (PBS) pouches were purchased from Sigma Aldrich (St. Louis, MO). Acetic acid was purchased from EMD Millipore (Burlington, MA) and sodium hydroxide (NaOH) pellets were purchased from J.T. Baker (Phillipsburg, NJ). Potassium iodide (KI) (ACS grade, granular) was purchased from Columbus Chemical Industries (CCI, Columbus, WI). Sliced sharp cheddar cheese was purchased from a local grocery store. Bacterial cultures of *Salmonella* Typhimurium, and ampicillin resistant *Salmonella* Typhimurium (TW16633) were maintained in TSB.

2.2. Methods

2.2.1. Preparation of chitosan/PVA N-halamine-based (CPN) films

The preparation of the film is a modified procedure following previous literatures [61, 62]. Briefly, chitosan solution (2 %) was prepared by dissolving in 2 % acetic acid at 65°C for 60 minutes and PVA solution (2 %) was prepared by dissolving in deionized water at 275°C for 30 minutes. The two solutions were then mixed together to form a homogeneous blend of 50 ml in the ratio of 2 CH:3 PVA for another 30 minutes. This solution was centrifuged at 8000 rpm for 10 minutes to separate impurities and air bubbles before being casted on glass containers of uniform size (11 x 16 cm^2). The solution was dried at room temperature overnight and the dried film was treated with 1 N NaOH solution to neutralize the acidic pH caused by acetic acid. The

films were washed repeatedly with deionized water, dried and treated with 0.65 % sodium hypochlorite solution to form N-halamine structures on the film surface. They were further dried after washing repeatedly with deionized water and stored in a desiccator before they were used for further experiments.

2.2.2. Determination of active chlorine content

The active chlorine content of the film is proportional to the N-halamine structures present on the surface of the CPN films. Therefore, detecting the presence of chlorine and quantifying it is important to confirm the presence of N-halamine, which is the bioactive agent. Iodometric titration method previously used in the literature was used to quantify the chlorine content [61]. In this study, 50 mg of the CPN films were suspended in 40 ml of 2% acetic acid solution. To this, 1 g of potassium iodide was added, and the mixture was stirred vigorously. This was then titrated against sodium thiosulphate solution until the blue-black color of the film turns colorless. Unchlorinated CH/PVA films were used as controls for this experiment. The following equation is used to calculate the chlorine content of the CPN film,

$$\text{Cl}\ \% = \frac{35.45}{2}\ \text{X}\ \frac{V_{Cl}-V_0}{W_{Cl}}\ \text{X}\ 0.01\ \text{X}\ 100 \qquad (1)$$

Where V_{Cl} and V_0 are the volumes of $Na_2S_2O_3$ used up during the titration of CPN and unchlorinated CH/PVA films, respectively and W_{Cl} is the weight of the film sample used for titration, which is 50 mg in this case. CPN films 0, 1, 2, 3, 4 and 5 days old after preparation were tested to detect changes in their chlorine content over time.

2.2.3. Testing antimicrobial activity of CPN film against bacteria

The antimicrobial test against *Salmonella* Typhimurium and ampicillin resistant *Salmonella* Typhimurium strains were conducted to compare the activity of the fabricated films in eliminating both drug susceptible and drug resistant strains of food contaminants. This test is based on modified American Association of Textile Chemists and Colorists (AATCC) Test Method 100 [61]. The bacterial strains were cultured in TSB for 24 hours at 37°C. They were cultured again from this broth for 4 hours in TSB at 37°C to attain log phase. The bacterial cells were harvested by centrifugation at 8000 rpm for 10 minutes in 4°C, washed and resuspended in PBS. The density of the harvested culture was between 10^8-10^9 colony forming units per milliliter (CFU/ml). 10 µl of this was sandwiched between 1 X 1 cm^2 pieces of the CPN films for 30 minutes. This was then transferred to 5 ml of $Na_2S_2O_3$ solution and ultrasonicated for 10 minutes. The samples were then serially diluted (10^{-3}) and surface-plated on TSA plates and incubated for 24 hours at 37°C. The number of colonies were counted to check the reduction in bacterial growth. Samples without treatment with the film were used as negative control and samples treated with regular plastic film were used as positive control. CH/PVA films without treatment with sodium hypochlorite were also tested for their antimicrobial activity and compared with the activity of CPN films. CPN and CH/PVA films that were 0, 1, 2, 3, 4 and 5 days old were tested to check for any changes in their antimicrobial activity over time.

The antimicrobial activity of CPN films against the studied bacterial strains in liquid culture were also tested based on a method previously described in the literature [7]. CPN films cut into 1 x 3 cm^2 pieces were introduced into 10 ml TSB culture tubes. These tubes were immediately inoculated with *S.* Typhimurium and ampicillin resistant *S.* Typhimurium strains which were previously prepared to have a final concentration of 10^3-10^4 CFU/ml. These tubes were

incubated with shaking at 100 rpm for 0 and 24 hours. The samples were surface plated on TSA plates after serial dilutions and incubated for 24 hours at 37°C to determine the bacterial growth after treatment with the film. Bacterial culture tubes without the films were used as controls. The effect of CH/PVA film against the growth of bacteria was also tested the same way for comparison. Samples without bacterial inoculation were used to check the chlorine content of the films at 0 and 24 hours after interaction with TSB.

2.2.4. Application of CPN films on packaging cheese

Cheese slices purchased from the store were aseptically cut into four equal pieces and weighed before starting the experiment. Each cut piece of slice was transferred to a petri dish and inoculated with ampicillin resistant *S.* Typhimurium strain prepared to a final concentration of 10^8-10^9 CFU/ml. The bacterial culture was harvested following the procedure in the antimicrobial test before being used for these experiments. The cheese slices were covered with CPN and CH/PVA films and the petri dishes were sealed with paraffin films to make sure that air is kept out. Samples without inoculation with bacteria were also maintained to quantify the presence of previously existing *S.* Typhimurium growth in the store-bought cheese slices. Ampicillin resistant *S.* Typhimurium inoculated cheese pieces without treatment with any film were used as controls to quantify bacterial growth without the effect of films. Triplicates were maintained for this experiment at two different temperatures, 25°C and 4°C, respectively, to study the temperature effect on the activity of films in preventing bacterial contamination. The effect of storage time was also quantified by maintaining packaged cheese slices under the same conditions for up to five days.

The bacterial growth in cheese samples was quantified by transferring the cheese to sterile stomacher bags containing 5 ml of 0.1 % peptone water and homogenized using a stomacher for

2 minutes. The juice from the sample was serially diluted and plated on BPLS agar and incubated for 24 hours at 37°C. The color of the plate remains red in the presence of *S.* Typhimurium strains and the number of colonies counted will help quantify the bacterial growth on the cheese samples.

2.2.5. Statistical Analysis

All experiments were conducted with samples maintained as triplicates. The CFU/ml units obtained from the plate counting were converted to log CFU/ml to get the results, which were then analyzed using analysis of variance (ANOVA). The significance level used was $\alpha=0.05$.

2.2.6. Preparation of chitosan-based N-halamine coated polycaprolactone films (CH-NX/PCL)

Preparation of PCL films

Homogeneous solution of PCL was prepared by dissolving 10% PCL pellets in chloroform solution and stirring for 4-5 hours in room temperature. This solution was casted on glass containers of uniform size (11 x 16 cm^2) and heated on a hot plate at 70°C for an hour before continuing to dry the films in a fume hood for another 15-20 minutes. This is done to obtain smooth PCL films of uniform thickness. The overall film preparation process takes 5-6 hours.

Preparation of chitosan coating solution

Chitosan solution (2 %) was prepared by dissolving in 2 % acetic acid at 65°C for 60 minutes. This solution was centrifuged at 8000 rpm for 10 minutes to separate impurities and air bubbles before being poured into glass containers.

Coating chitosan on PCL

PCL being hydrophobic in nature, has low surface adhesivity and wettability [63] to natural materials such as chitosan that is hydrophilic. It is, therefore, necesary to modify the surface of PCL to improve its wettability and adhesion to the chitosan coat by increasing its hydrophilicity. Plasma treatment has been used in the past on hydrophobic materials to improve their adhesion characteristics and give them desirable properties for various applications [64, 65]. In this case, the PCL films are subjected to plasma treatment in the presence of oxygen gas for a process time of one minute. The radio frequency level was set 50% of the total, that is 300 W power. The plasma modified PCL films were then immediately dip-coated with the previously prepared chitosan solution and left to dry at room temperature overnight.

Modification of chitosan coating to give N-halamine structures

The dried chitosan coated film was treated with 1 N NaOH solution to neutralize the acidic pH caused by the acetic acid used in preparing the coating. The films were washed repeatedly with deionized water, dried and treated with 0.65 % sodium hypochlorite solution to form N-halamine structures on the chitosan coated film surface. They were dried again after copiously washing with deionized water and stored before being used for further experiments. The treatment of chitosan in the above-mentioned steps is a modification of the procedure followed in previous literature [61].

2.2.7. Characterization of CH-NX/PCL films

Fourier Transform Infrared (FTIR) analysis

FTIR analysis for PCL, chitosan, CH/PCL and CH-NX/PCL was carried out using FTIR spectrometer (FT/IR-4600typeA, JASCO, Maryland, USA). The spectra were obtained at room temperature for the range of 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Thermogravimetric analysis (TGA)

The thermogravimetric analysis of PCL (9.4 mg), CH/PCL (14 mg) and CH-NX/PCL (20 mg) was carried out using TGA 500 (TA instruments, DE, USA) to check the thermal degradation rate of these films at high temperatures. The materials were heated in aluminium pans from 25 to 500°C and the weight loss of samples as a function of temperature was recorded in oxygen atmosphere.

Mechanical properties

The tensile strength of PCL and CH/PCL films were tested using United Testing Systems (UTS) model SFM-20 load frame by following the ASTM D882-12 method [66]. Samples were cut into six-inch strips of one-inch width for the tests. The thickness of the samples was noted before the tests began. Triplicates for each film was maintained and the tests were conducted at room temperature. The tensile strength and young's modulus were calculated for every sample to determine the mechanical properties of PCL films before and after coating with chitosan. The following equations were used for the calculations:

$$Tensile\ strength\ (Mpa) = \frac{Maximum\ Force}{Film\ thickness\ X\ Width} \quad (2)$$

$$Young's\ modulus = \frac{Slope}{Film\ thickness\ X\ Width} \quad (3)$$

Where, the slope is obtained from the linear portion of the plot of force versus extension.

Barrier properties

Water vapor transmission rate (WVTR):

The moisture barrier properties of PCL films before and after coating with chitosan was tested using MOCON PERMATRAN-W® 3/33 (Modern Controls Inc., USA). The films were sandwiched between aluminium foils with an uncovered area of 5 cm^2 for the mositure to pass through. The relative humidity (RH) was maintained at 100% and the temperature at 23°C. In the case CH/PCL film, the coated chitosan surface was placed facing the source of moisture in the cell. The film thickness was noted, and the films were conditioned for one hour before the measurements began. The system generated WVTR values for the films over hours and this was used to calculate the permeation of moisture in each film. Equation 4 was used to calculate the water vapor permeation (WVP):

$$WVP = \frac{WVTR\ X\ l}{\Delta p} \quad (4)$$

Where, l is the thickness of the film and Δp is the vapor pressure calculated from the saturated vapor pressure at the given temperature and RH.

Oxygen transmission rate (OTR):

Oxygen transmission rates through PCL films before and after chitosan coating was quantified using MOCON OX-TRAN® 2/21. Conditioning was done for one hour before the measurements began. The films were sandwiched between aluminium foils placed inside the cells with 5 cm^2 open area through which the oxygen gas (100%) was passed. The film thickness was noted before the samples were placed in the cells. The surface of chitosan coating in the CH/PCL film

was placed facing the source of oxygen gas. A mixture of nitrogen and hydrogen gases were passed through the other side. The experiments were carried out at 0% RH and 23°C. The oxygen permeability values were calculated from the OTR values obtained from the system. Equation 5 was used to do the calculations:

$$Oxygen\ permeability = \frac{OTR\ X\ l}{\Delta P} \quad (5)$$

Where, l is the thickness of the film and Δp = 1 atm.

2.2.8. Determination of active chlorine content

N-halamine structures on the CH-NX/PCL film surface can be quantified by quantifying the chlorine ions attached to the amino groups of the chitosan coating. Since the active antimicrobial agent of the film is N-halamine, determining the active chlorine content of the film is crucial to understanding the efficacy of the film in preventing bacterial contamination. Previously in the literature, an iodometric titration method was applied to quantify chlorine content in treated chitosan films [61]. This study follows the same procedure for the CH-NX/PCL films. Around 50 mg of the CH-NX/PCL film was suspended in 40 ml of 2% acetic acid solution. To this, 1 g of potassium iodide powder was added, and the mixture was stirred vigorously. This solution turns yellow and the film turns blue black in the presence of chlorine. It was then titrated against sodium thiosulphate solution until the film and solution turns colorless. Unchlorinated CH/PCL and chlorinated PCL films without the chitosan coating were used as controls for this experiment and equation 1 was used to calculate the chlorine content of the CH-NX/PCL film,

The chlorinated PCL films were subjected to copious washing using deionized water to remove excess chlorine on its surface, the same way CH-NX/PCL films were treated.

2.2.9. Testing antimicrobial activity of CH-NX/PCL film against bacteria

The antimicrobial activity of the fabricated CH-NX/PCL film was tested against drug susceptible and ampicillin resistant strains of *Salmonella* Typhimurium to compare its efficacy in eliminating both drug susceptible and drug resistant strains of this foodborne pathogen. A modified version of the American Association of Textile Chemists and Colorists (AATCC) Test Method 100 [61] was used for the antimicrobial activity tests. TSB was used to culture the bacterial strains for 24 hours at 37°C. They were re-cultured from this broth for 4 hours at 37°C to attain log phase. The cells were then harvested by centrifugation at 8000 rpm for 10 minutes in 4°C, washed using PBS and resuspended in the same. This was done to achieve a culture density of 10^8-10^9 colony forming units per milliliter (CFU/ml). Out of this culture, 10 µl was sandwiched between 1 X 1 cm^2 pieces of the CH-NX/PCL films (sterilized before use) for a period of 30 minutes. The sandwich was later transferred to 5 ml of $Na_2S_2O_3$ solution and ultrasonicated for 10 minutes. Following this the samples were serially diluted using PBS and surface-plated on TSA plates. These plates were incubated for 24 hours at 37°C. Bacterial growth reduction was quantified by counting the number of colonies grown on the plates. Negative and positive controls were samples without treatment with the film and samples treated with plain PCL film. CH/PCL films without sodium hypochlorite treatment were also tested for their antimicrobial efficacy and compared.

2.2.10. Statistical analysis

The tensile strength tests and barrier tests were done with replicates for PCL and CH/PCL films. The antimicrobial efficacy tests were done for PCL, CH/PCL and CH-NX/PCL films with triplicates. In all the cases, the calculated properties were compared for each of the films and significant difference was analyzed using ANOVA with significance level α=0.05.

CHAPTER 3

TESTING ANTIBACTERIAL ACTIVITY OF CHITOSAN/PVA-BASED N-HALAMINE FILM AGAINST DRUG SUSCEPTIBLE AND AMPICILLIN RESISTANT *SALMONELLA* TYPHIMURIUM AND ITS APPLICATIONS IN PACKAGING CHEDDAR CHEESE SLICES

3.1. Introduction

This study focusses on the use of the natural polymer, chitosan, to prepare a biodegradable packaging film, primarily because it has the added advantage of being antimicrobial in nature [13]. Chitosan is composed of β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [21] and the positive charges of its amine group is considered the reason for the antimicrobial activity. This positive charge reacts with the negative charge of the bacterial cell wall that leads to cell lysis [67]. The charge on chitosan is induced by the protonation of the amino groups in the presence of an acidic environment or through modifications introduced in their structure [68]. However, the killing efficiency of this material is highly dependent on the pH of the system and is seen to increase with acidic conditions [11]. This may not be ideal for most food packaging conditions as they do not necessarily have to be acidic in nature. Methods to improve its antimicrobial activity in non-acidic conditions have also been investigated. One way to do this is to exploit the presence of amine groups in chitosan, where the amine group, upon reaction with a chlorine-based compound can form N-halamine structures that enhances the antibacterial activity at most pH conditions [61].

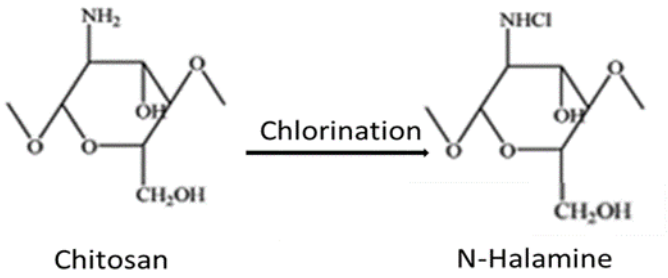

Figure 1: Chlorination of chitosan forms N-halamine structures that enhances the antimicrobial property of the film [69]

Generally, biodegradable polymers are observed to have low mechanical strength compared to the synthetic polymers currently used in the packaging industry [14]. However, blending such polymers with another biodegradable polymer can improve their mechanical and barrier properties [14], which are essential for any food packaging material. In this study, previously synthesized chitosan and polyvinyl alcohol (PVA) blended film, with simple generation of N-halamine structures on their surface, was tested as a potential packaging material for cheddar cheese slices. The film was specifically tested for antimicrobial activity against the common foodborne pathogen *Salmonella* Typhimurium, both drug-susceptible and drug-resistant strains.

Salmonella contamination of cheese products have been reported in the past, which is often accompanied with food recalls. Flat Creek Farms recalled three lots of cheese due to potential *Salmonella* contamination. Two of the lots involved cheddar cheese [70]. Another company, Miss Bonnie's Gourmet cheese also had to recall their cheddar cheese spread due to potential contaminations by *Salmonella* [71]. It is, therefore, necessary to check the application of the fabricated antimicrobial film in preventing bacterial contamination in cheddar cheese. This study specifically uses cheddar cheese slices to test the application of the film as a packaging material. Testing the antimicrobial activity of the fabricated film, particularly in packaging cheese slices

contaminated with drug resistant strain of *Salmonella* Typhimurium can give information on the possibility of using the film for commercial purposes.

3.2. Methods

Chitosan (CH)/PVA-based N-halamine film was prepared by blending chitosan solution prepared in acetic acid with PVA solution prepared in deionized water. The blended solution were casted glass plates and left to dry overnight. The dried films were treated with NaOH solution to neutralize the acidic pH from the acetic acid, was dried and chlorinated using sodium hypochlorite solution to form N-halamine structures.

The chlorine content of the film was tested using iodometric titration method and compared with the control, which was unchlorinated CH/PVA films. The antimicrobial activity of these films was tested using a modified version of the American Association of Textile Chemists and Colorists (AATCC) Test Method 100 against drug susceptible and ampicillin resistant strains of *Salmonella* Typhimurium. Their biocidal activity against these pathogens were also tested in liquid culture. The efficiency of the fabricated film as an antimicrobial food packaging material was also tested by packing cheddar cheese slices and storing over a period of five days at 25°C and 4°C.

3.3. Results and Discussion

3.3.1. Determination of active chlorine content

The chlorination of CH/PVA films involves an interaction between the amino groups of chitosan and chlorine molecules of sodium hypochlorite solution, which leads to the formation of N-halamine structures on the film surface. The hydrophilic nature of chitosan possibly caused the film to swell when introduced into the diluted sodium hypochlorite solution that lead to the

exposure of its amino groups to the chlorine molecules for interaction [61]. Copious washing of the film after chlorination removes excess unbound chlorine from the film's surface. This means that the chlorine remaining on the film are bound to the amino groups of the chitosan and quantifying them would give a measure of the N-halamine structures on the film. In this study, the change in active chlorine content of the film during its storage over five days after preparation have been quantified. Figure 2 shows that the chlorine content decreases linearly over time as the shelf life increases. This is significant in determining how long the film can remain active against specific bacterial contaminants when used for packaging. The loss of chlorine molecules from the film could be due to the hydrophilic nature of chitosan, which causes it to interact with the moisture in the surrounding environment that promotes dissociation of chlorine molecules [61].

The chlorine content was found using equation 1 and expressed as a percentage. The initial chlorine content was calculated to be 1.81%. On the fifth day of the film's storage from preparation, the chlorine content was found to be 1.44% and the reduction from initial content was 0.37%, which may not cause significant changes in the N-halamine content on the CPN films.

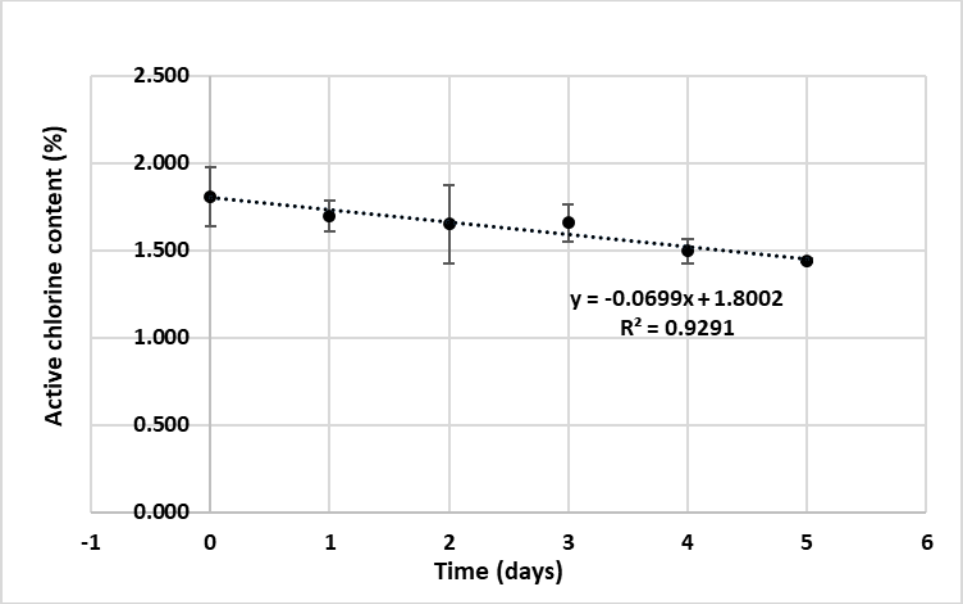

Figure 2: The change in active chlorine content of CPN films over five days

3.3.2. Antimicrobial activity of CPN films

The antimicrobial activities of CPN and CH/PVA films were tested against ampicillin resistant and drug susceptible strains of *S.* Typhimurium. Figures 3a and 3b compares the initial bacterial concentration in broth culture to bacterial growth obtained after the antimicrobial sandwich assay. The data shows the effect of the CPN and CH/PVA films against the two strains of bacteria as their storage period increases, along with the control, which were not treated with any films but were subjected to similar conditions of the assay. As seen in the figures, the antimicrobial activity of CPN films is 100 % and there is no bacterial growth for both the drug resistant and drug susceptible strains, irrespective of the age of the film ($p<0.05$). Tables 3 and 5 show *p* values, 2.85E-50 for *S.* Typhimurium and 9.13E-65 for ampicillin resistant *S.* Typhimurium, which are less than 0.05, showing that there is a significant reduction in bacterial growth when treated with CPN film compared to the original bacterial concentration. This means that the loss of chlorine over a period of five days, as seen in Figure 2, has not significantly

affected the antimicrobial efficacy of the CPN film against both drug susceptible and ampicillin resistant strains. The original bacterial concentration used for the assay each day for both strains of bacteria was 9.5±0.2 log CFU/ml. From the figures it is evident that bacterial growth for samples treated with CH/PVA films and the control is not reduced to a greater extent when compared to those treated with CPN film. The little to no bacteria reduction in the antimicrobial assay control samples when compared to the original concentration gives evidence that the ultrasonication treatment with sodium thiosulphate solution in the assay do not influence the bacterial growth. However, almost one log reduction in bacterial growth was noted for samples that were treated with CH/PVA films compared to the original samples on almost all days ($p<0.05$), which is evidenced by the statistical analysis shown in Tables 2 and 4, where the p values are 1.71E-11 for *S*. Typhimurium and 2.3E-09 for ampicillin resistant *S.* Typhimurium. This could be due to the presence of chitosan in the film's structure, which has potential inherent antimicrobial activity [13].

The initial concentration of bacteria used in the test was between 10^8-10^9 and this might be too high for the given amount of nutrients in TSB. This could mean that the bacterial cells could have already reached steady state and not growing enough due to lack of nutrients by this point. Therefore, the effect of the CPN film could have been enhanced leading to a 100% bacterial reduction in this condition.

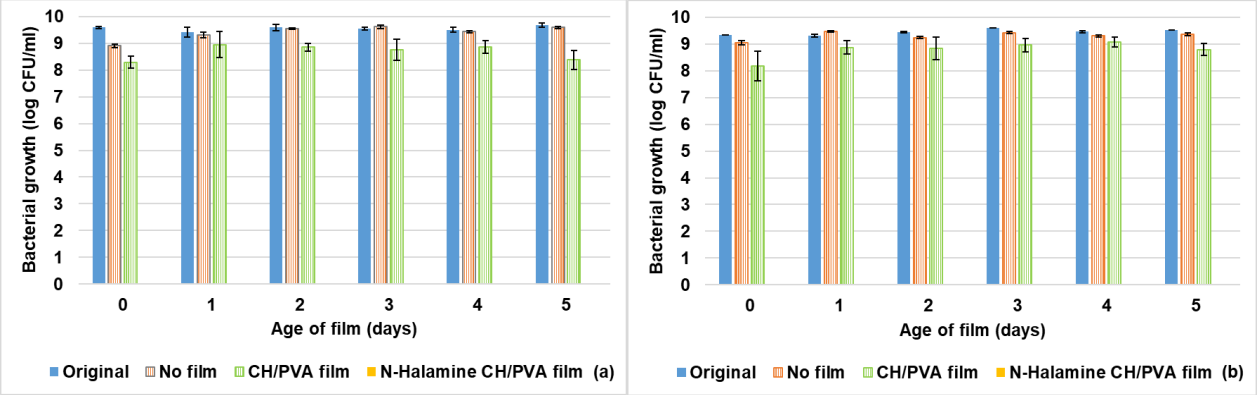

Figure 3: Comparison of bacterial growth when treated with N-halamine CH/PVA (CPN) and CH/PVA films (contact time – 30 minutes; detection limit – serial dilution of 10^{-3}); (a) effect on drug susceptible *Salmonella* Typhimurium; (b) effect on ampicillin resistant *Salmonella* Typhimurium

Table 2: ANOVA for *Salmonella* Typhimurium treated with CH/PVA film compared with control

Source of Variation	**Sum of squares (SS)**	**Degrees of freedom (df)**	**(Mean of squares) MS**	**F**	**P-value**	**F critical**
Sample (between treatments)	7.970476	1	7.970476	139.6642	1.71E-11	4.259677
Columns (between film age)	0.349923	5	0.069985	1.22632	0.327448	2.620654
Interaction (between treatments and film age)	0.850945	5	0.170189	2.98217	0.03119	2.620654
Within	1.369652	24	0.057069			
Total	10.541	35				

Table 3: ANOVA for *Salmonella* Typhimurium treated with CPN film compared with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatments)	820.8478	1	820.8478	277272.8	2.85E-50	4.259677
Columns (between film age)	0.066821	5	0.013364	4.514298	0.004839	2.620654
Interaction (between treatments and film age)	0.066821	5	0.013364	4.514298	0.004839	2.620654
Within	0.07105	24	0.00296			
Total	821.0525	35				

Table 4: ANOVA for Ampicillin resistant *Salmonella* Typhimurium treated with CH/PVA film compared with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatments)	4.915809	1	4.915809	85.18153	2.3E-09	4.259677
Columns (between film age)	1.492281	5	0.298456	5.171674	0.002328	2.620654
Interaction (between treatments and film age)	0.864321	5	0.172864	2.995404	0.030663	2.620654
Within	1.385035	24	0.05771			
Total	8.657446	35				

Table 5: ANOVA for Ampicillin resistant *Salmonella* Typhimurium treated with CPN film compared with control

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F critical**
Sample (between treatments)	803.5983	1	803.5983	4474809	9.13E-65	4.259677
Columns (between film age)	0.091207	5	0.018241	101.5762	2.42E-15	2.620654
Interaction (between treatments and film age)	0.091207	5	0.018241	101.5762	2.42E-15	2.620654
Within	0.00431	24	0.00018			
Total	803.785	35				

To ensure that the reduction in bacterial growth on the agar plates during the assay is not due to its inaccessibility while being sandwiched between the films, the assay was conducted using a regular plastic film without any antimicrobial activity of its own. The results were compared with the original growth, control and those treated with CPN and CH/PVA films. Figures 4a and 4b indicate that the treatment with regular plastic film has reduced the growth of bacteria only by 0.5±0.1 logs, which means that sandwiching the bacteria between the films did not significantly influence the bacterial concentration.

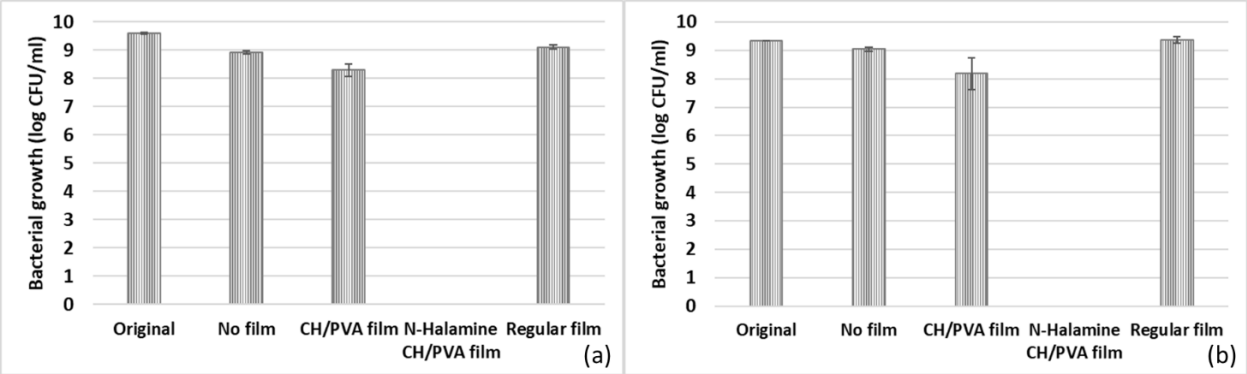

Figure 4: Comparison of bacterial growth under different conditions of the antimicrobial sandwich assay to the original bacterial concentration in the initial culture (contact time – 30 minutes; detection limit – serial dilution of 10^{-3}); (a) drug susceptible *Salmonella* Typhimurium; (b) ampicillin resistant *Salmonella* Typhimurium

N-halamines are a combination of halogens with nitrogen based functional groups that have potential antimicrobial activities. In this study, the CPN films contain N-halamines on its surface through the interaction of the amino group of chitosan with the chorine molecules from the sodium hypochlorite treatment. The advantages of N-halamine-based antimicrobial agents is that they have oxidative halogens in their structures that inherently have strong antimicrobial potential in them. Since the halogens present in these structures are bound to the amino groups, they are highly stable unlike the free halogens and are comparatively safer to use. Once the oxidative chlorine group is consumed during its activity against a microorganism, they can be recharged again by exposing the amino groups on the surface of CPN films to sodium hypochlorite solution [72]. It is noted that the antimicrobial mechanism of N-halamines depend on the transfer of its oxidative halogen group to the bacterial cell wall. The interactions between this halogen ion and the bacterial cell wall is seen to cause destruction of the cells irrespective of whether the pathogen has drug resistant genes. Therefore, using N-halamine based antimicrobial agents have an advantage over using antibiotics in combating drug resistant strains of bacteria as well [73]. In this study, the results obtained for the sandwich assay evidently suggests that the

growth of both the non-drug resistant and ampicillin resistant *S.* Typhimurium strains have been significantly reduced when treated with CPN films containing N-halamine structures on their surface. It has also been noted that there is no significant variation in the effect of CPN films over the two bacterial strains.

The antimicrobial mechanism of N-halamine has shown to require direct contact with bacterial cells for their effective elimination. However, it is hypothesized that the antimicrobial action of N-halamines can also be initiated with the release of their halogen molecules into an aqueous environment that can be exposed to the pathogens [72]. The sandwich assay conducted in this study gives evidence of the biocidal activity of N-halamine upon direct contact with the bacterial cells. The oxidative chlorine ions (Cl^+) present in the N-halamine structure interacts with the charges on the bacterial cell wall and penetrates the cell through its transport system. This interaction is followed by the generation of reactive oxidation species that can interrupt regular cellular functions, thereby, causing cell necrosis [69, 72, 74].

From Figure 3, it can be inferred that there is no significant difference in the activity of the CPN film on eliminating bacteria when the film's storage period increases. The CPN film was able to eliminate 100 % of bacterial cells when treated on both the non-drug resistant and ampicillin resistant *S.* Typhimurium strains. From the chlorine titration experiments conducted previously, it can be said that the decrease in the chlorine content of CPN films each day has not significantly affected its biocidal activity. As noted in the previous section, the reduction in the chlorine content is too small to cause serious changes to the film's N-halamine content. This means that there could potentially be more N-halamine structures on the film's surface that are not yet saturated by interactions with bacteria and can still potentially be used after longer storage periods. The effect of CH/PVA films on bacterial growth also have not shown significant

difference with respect to its storage period and remains similar to the results obtained on the first day of test.

As mentioned previously, N-halamine could potentially fight bacteria by first releasing their halogens into an aqueous environment. To check whether this would be applicable in the case of the CPN film, its antimicrobial activity was also tested against bacterial broth cultures of both non-drug resistant and ampicillin resistant *S.* Typhimurium. The tests were conducted for two days since the preparation of the CH/PVA and the CPN films and the results are shown in Figure 5. Bacterial growth in broth samples treated with CH/PVA and CPN films were compared with the control, which was not exposed to any film. From the results it can be noted that there is no apparent significant antimicrobial effect of CH/PVA or CPN films on the bacterial growth of either of the strains used in this study. The initial bacterial growth was found to be around 9 logs for both strains, which was the same for the cultures treated with the films as well. After 24 hours, the growth was higher, reaching around 12 logs in all the cases indicating that there is no reduction of growth over time.

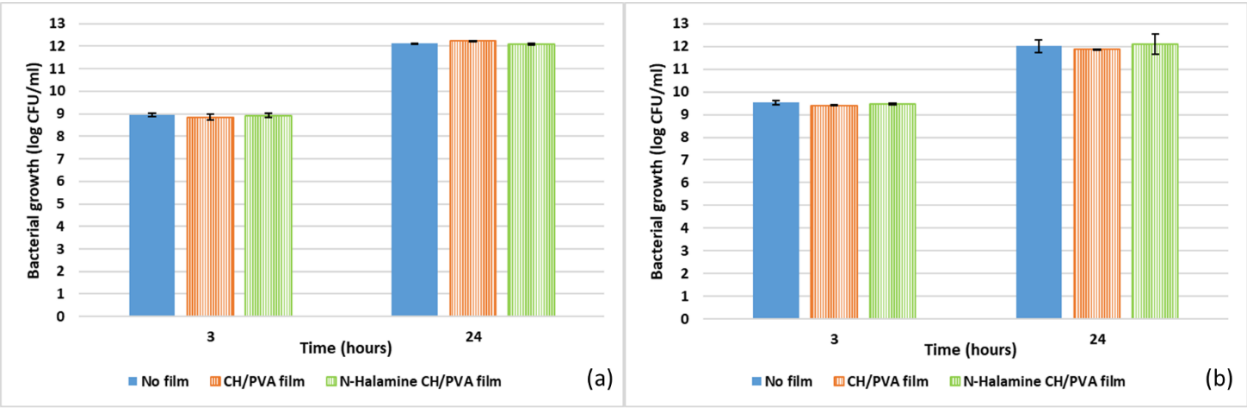

Figure 5: Comparison of bacterial growth in broth culture treated with CH/PVA and CPN films over time; (a) drug susceptible *Salmonella* Typhimurium; (b) ampicillin resistant *Salmonella* Typhimurium

Table 6: Comparison of growth on day 0 between both strains when treated with CPN film along with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatment condition)	0.006537	1	0.006537	1.318368	0.284058	5.317655
Columns (between the two strains)	0.947679	1	0.947679	191.1332	7.24E-07	5.317655
Interaction (between treatment condition and strains)	0.000985	1	0.000985	0.198744	0.667555	5.317655
Within	0.039666	8	0.004958			
Total	0.994867	11				

There is a significant difference between the growth of both the strains at 3 hours (*p* value = 7.24E-07), where the drug resistant strain shows almost one log higher growth than the drug susceptible strain. This indicates that both strains show differences in growth and tolerance to the antimicrobial agent, which is the N-halamine structures on the CPN film. The *p* value for sample and interaction exceeds 0.05, suggesting that there is no significant difference between the bacterial growth observed for control samples and those treated with CPN film. The two strains seem to behave similarly when under the two different treatment conditions, suggesting that the CPN film has little effect in reducing bacterial growth in the broth maintained in this experiment irrespective of the type of strain. After 24 hours, the growth of both strains has increased considerably and have possibly reached steady state condition due to which a significant difference between bacterial growth is not evident in both the strains. Table 7 shows *p* values exceeding 0.05, which means that there is no significant difference between the bacterial growth in both strains when treated with the CPN film in broth and compared to the control.

Table 7: Comparison of growth on day 1 between both strains when treated with CPN film along with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatment condition)	0.001393	1	0.001393	0.019253	0.893074	5.317655
Columns (between the two strains)	0.083855	1	0.083855	1.159018	0.31306	5.317655
Interaction (between treatment condition and strains)	0.002587	1	0.002587	0.035755	0.854731	5.317655
Within	0.578804	8	0.07235			
Total	0.666639	11				

The antimicrobial efficacy of CPN films could have been insignificant in this case due to the saturation of chlorine ions present on the 1 x 3 cm^2 strips of films by the bacterial cells that encounter the film. Even if the chorine ions were released into the broth, they may not have been enough to attack the bulk of the bacterial cells that remained and continued to replicate in the broth over time. The broth was also shaken at 100 rpm, causing an aerobic environment which was ideal to improve the growth rate of the bacteria. Tests could be done without shaking to test the effect of the film in eliminating the pathogen.

Another reason could be its exposure to TSB, which largely contains water molecules that can interact with the film's surface and alter its properties. The interaction of chlorine ions on the film with the surrounding aqueous environment could cause a reverse reaction where the chlorine gets released into the broth. As mentioned previously, this could happen due to the hydrophilic nature of chitosan that interacts with the surrounding moisture [61]. The reduction in

the chlorine percentage on CPN films means that the number of N-halamine groups on its surface has reduced as well. This in turn leads to its decreased antimicrobial activity when interacting with bacteria in broth culture. Figure 6 shows the residual chlorine content on CPN films of size 1 x 3 cm^2 when placed in tubes containing TSB for 3 and 24 hours. The results seem to confirm the loss of chlorine ions from the film's surface during this interaction. The initial chlorine content of the film, as found previously, was 1.81%, and about 78% of this was lost from the film within the first three hours. At the end of the 24 hours, the final chlorine content on the film treated with TSB was around 0.16%, which is close to 91% reduction from the initial chlorine content. This indicates that chlorine ions have been released into the broth.

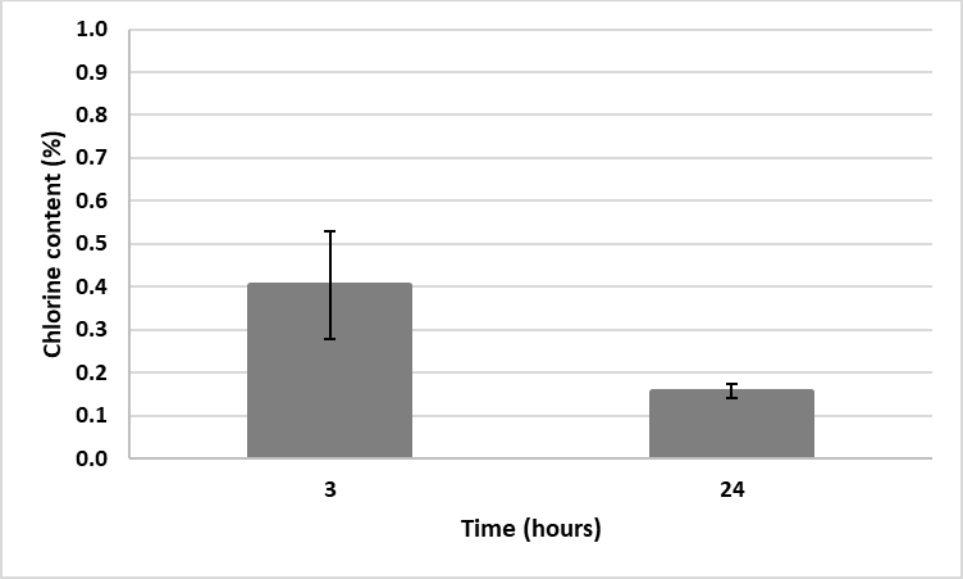

Figure 6: Remaining chlorine content (%) in CPN films after incubation in TSB against time (almost 60% reduction after 24 hours)

3.3.3. Application of CPN films on packaging cheese

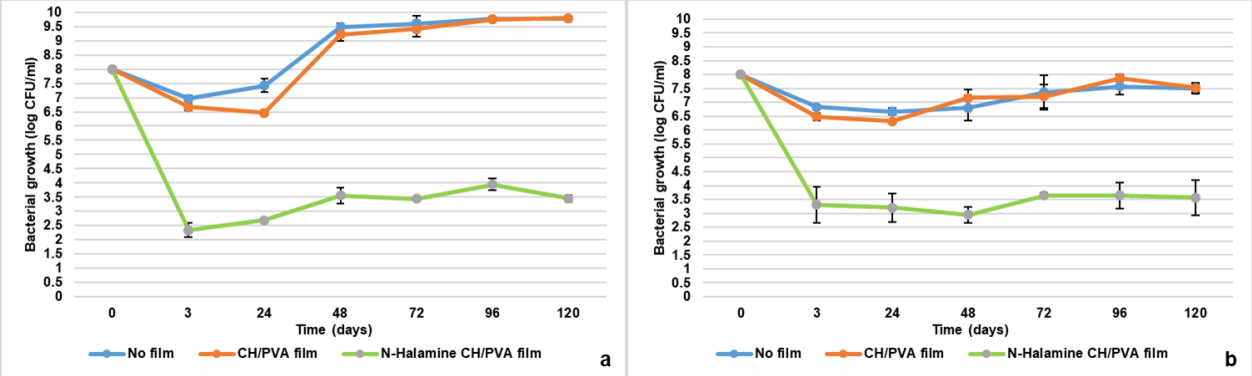

Figure 7: Comparison of bacterial growth (log CFU/ml of peptone water used) in cheese slices packaged with CH/PVA and CPN films over storage time (a) at 25°C; (b) at 4°C

The effect of temperature on the antimicrobial activity of the CPN films as packages for cheddar cheese slices was tested at 25°C and 4°C to compare the quality of the packaged food in room temperature and when stored in refrigerators. The samples were plated on BPLS agar, which is a selective medium for *Salmonella* species. The colonies grow in red because they do not ferment the lactose or sucrose present in the agar. *Escherichia coli* also grows on this agar. However, it turns the medium yellow due to the fermentation of the sugars present in the agar. This medium was used in these experiments to ensure that only the inoculated ampicillin resistant *S.* Typhimurium strain is quantified. A negative control was used in these experiments where, cheese samples were treated the same way but without bacterial inoculation. This will be useful to quantify any existing *Salmonella* contamination in the cheese.

From Figure 7, it can be observed that the bacterial growth on days 0 and 1 after packaging are lower than that of days 2, 3, 4 and 5. This is more prominent in cheese samples maintained at 25°C than at 4°C. The statistical analyses shown in Tables 8 and 10 give *p* values equal to 3.22E-23 and 1.16E-06, respectively, which are less than 0.05. This proves that there is a significant

difference in bacterial growth over the days when stored at these two different temperatures. It is also evident that cheese slices packaged with CH/PVA has not shown significant reduction in bacterial growth from day 2 onwards when compared to the controls, which were samples not packaged with either films when stored at 4°C (p value = 0.83). However, the statistical analysis in Table 8 shows significant difference (p value = 3.14E-05) between the control and the samples stored using CH/PVA film at 25°C, though it is not as effective as CPN film in eliminating the pathogen based on the log reductions seen in Figure 7. This means that CH/PVA films had little effect on reducing bacterial contamination on cheese. On the other hand, CPN films showed around 3-6 log reductions in bacterial growth compared to the controls (p<0.05) irrespective of the temperatures at which the samples were stored as evidenced by the p values for interaction in Tables 9 and 11. At 4°C, the samples stored in CPN film showed similar growth pattern as the control over the days (p value = 0.0108), though there is a significant bacterial reduction in CPN treated samples compared to the control (p value = 7.91E-20), which is evident from the p value of interaction in Table 11 being greater than 0.05, which is 0.397. At both temperatures, the bacterial log reduction on days 0 and 1 is lower than that observed for the remaining storage period. At 25°C, around 5 logs and at 4°C, around 3 logs of bacterial growth reduction were observed, while the remaining period shows around 6 and 4 log reductions at 25°C and 4°C, respectively. Every cheese sample was inoculated with the same concentration of ampicillin resistant *S.* Typhimurium and its growth is seen to increase until day 2 in samples stored at 25°C followed by a stable bacterial concentration until day 5. However, this trend is not prominent at 4°C, possibly due to the inhibition of *S.* Typhimurium strains at lower temperatures. At refrigerated temperatures *Salmonella* strains slows growth and continues to stay dormant. The results obtained in this case are consistent with previous studies on *Salmonella* growth in food

samples [75, 76]. Studies conducted on cooked ham resulted in *Salmonella* population remaining the same when stored at lower temperatures such as 5°C, while there was an increase in growth at higher temperatures such as 25°C [75]. *S.* Typhimurium growth in inoculated buffalo mozzarella cheese was shown to reduce at 4°C and increased at 20°C when tested over a period of 12 days [76].

From the results above, it is evident that the CPN films can reduce the growth of ampicillin resistant *S.* Typhimurium in cheese at temperatures 25°C and 4°C. However, a complete elimination of contamination could not be achieved, as shown by the 2-3 log bacterial growth during the entire storage period, irrespective of the temperature. This could be due to the inaccessibility of the N-halamine structures on the CPN films to the bacterial cells that could have diffused into the food matrix. While inactivation of bacterial cells in contact with the film surface is happening, the remaining bacterial cells in the food matrix may not be affected and can continue to grow. The cheese matrix provides for a nutrient rich substrate for bacteria to grow. However, the growth of ampicillin resistant *Salmonella* Typhimurium has not increased from day 3 to day 5 and this could possibly be due to the inhibition of bacterial growth accompanied with the inactivation effect of the chitosan-based packaging film, causing the surviving bacteria to grow at a slower pace. Also, the concentration of bacteria used to inoculate the cheese samples is around 10^8, which could be too high compared to actual bacterial concentration contaminating food products. This could possibly be the reason for bacteria remaining in the food matrix and further studies at lower concentrations can give insight into the efficacy of the proposed antimicrobial film in eliminating bacteria in practical situation.

Different food samples have varying texture and physical properties that make them more susceptible to contamination than others. In this study, the food sample selected for antimicrobial

efficacy test, was porous in nature and could have allowed the diffusion of bacterial culture into the cheese matrix more easily. For less porous food materials, this process may have happened slower, in which case the CPN film could have closely interacted with more bacterial cells on the surface and eliminated those as well. Therefore, the texture of the food sample tested may also play a role in affecting the capability of the proposed packaging film in reducing contamination.

As discussed in the previous section, direct contact of films is required for effective elimination of all bacterial cells. Therefore, the film was able to eliminate only those bacterial cells that remained on the cheese surface, while those that diffused into the cheese matrix survived.

Table 8: ANOVA – Cheese packed with CH/PVA film at 25°C compared with control

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F critical**
Sample (between treatments)	0.656662	1	0.656662	26.11706	3.14E-05	4.259677
Columns (between days)	60.27707	5	12.05541	479.4734	3.22E-23	2.620654
Interaction (between treatments and days)	0.906137	5	0.181227	7.207861	0.000303	2.620654
Within	0.603433	24	0.025143			
Total	62.4433	35				

Table 9: ANOVA – Cheese packed with CPN film at 25°C compared with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatments)	274.4269	1	274.4269	9503.589	1.05E-32	4.259677
Columns (between days)	26.27412	5	5.254824	181.9781	2.9E-18	2.620654
Interaction (between treatments and days)	4.008775	5	0.801755	27.76532	3.06E-09	2.620654
Within	0.693027	24	0.028876			
Total	305.4028	35				

Table 10: ANOVA – Cheese packed with CH/PVA film at 4°C compared with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatments)	0.004158	1	0.004158	0.0461	0.831809	4.259677
Columns (between days)	6.665219	5	1.333044	14.77923	1.16E-06	2.620654
Interaction (between treatments and days)	0.797638	5	0.159528	1.768656	0.157556	2.620654
Within	2.16473	24	0.090197			
Total	9.631745	35				

Table 11: ANOVA – Cheese packed with CPN film at 4°C compared with control

Source of Variation	SS	df	MS	F	P-value	F critical
Sample (between treatments)	129.0651	1	129.0651	782.2672	7.91E-20	4.259677
Columns (between days)	3.158917	5	0.631783	3.829257	0.010825	2.620654
Interaction (between treatments and days)	0.88893	5	0.177786	1.077565	0.397571	2.620654
Within	3.959724	24	0.164989			
Total	137.0727	35				

CHAPTER 4

SYNTHESIS OF CHITOSAN-N-HALAMINE-COATED POLYCAPROLACTONE FILM AGAINST DRUG SUSCEPTIBLE AND AMPICILLIN RESISTANT *SALMONELLA* TYPHIMURIUM FOR FOOD PACKAGING APPLICATIONS

4.1. Introduction

Chitosan is a natural polymer, a derivative of the compound chitin, that is commonly found on the shells of crustaceans. It is formed from the alkaline treatment of chitin and is reported to have antimicrobial properties [77], apart from being biodegradable. However, chitosan derivatives have been proven to show much higher efficacy against pathogens than chitosan itself. This is especially proven for the N-halamine derivative of this natural polymer [61]. Chitosan is composed of β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [21]. The amine group of this polymer can interact with halogens to form N-halamine structures. The halogen ions of this derivative, then, upon interaction with any pathogen, gets transferred into the cells and disrupt their metabolic activity [69].

The use of chitosan as a biodegradable food packaging material has been investigated in the past. However, due to its poor physical properties, that often do not meet the requirements of an ideal food packaging system, it is combined with other physically stronger polymers [78]. This chapter discusses the fabrication of plasma treated PCL films coated with chitosan and chlorinated to form N-halamine structures. The physical properties of the film were tested and its antimicrobial efficacy against drug susceptible and ampicillin resistant *Salmonella* Typhimurium strains were quantified.

4.2. Methods

Chitosan-N-halamine-coated PCL films were fabricated beginning with the preparation of smooth PCL films in chloroform. The dried films are plasma treated in oxygen environment and immediately coated with chitosan solution prepared in acetic acid. The coated PCL film was left to dry overnight before being treated with NaOH solution to neutralize the acidic pH of the acetic acid. This was dried and treated with sodium hypochlorite solution to form N-halamine structures on the chitosan coated surface of PCL films.

The chlorine content of the fabricated antimicrobial film was quantified using iodometric titration with chlorinated PCL and unchlorinated chitosan-coated PCL films as controls. Characterization of the films were done using FTIR and TGA. The mechanical properties such as tensile strength and Young's modulus were also measured for plain and chitosan-coated PCL films. The barrier properties of the film against moisture and oxygen were also quantified. The antimicrobial efficacy of the fabricated films against drug susceptible and ampicillin resistant *Salmonella* Typhimurium was tested using the modified version of American Association of Textile Chemists and Colorists (AATCC) Test Method 100.

4.3. Results and Discussion

4.3.1. Characterization of CH-NX/PCL films

Fourier Transform Infrared (FTIR) analysis

Figure 8 shows the FTIR spectra for pure PCL, CH/PCL and pure chitosan films. As can be noted from the figure, the spectra for CH/PCL films include characteristic peaks of both PCL and chitosan. The characteristic peaks of PCL are visible in the spectra, which include peaks at 1720.19 cm^{-1} attributing to the carbonyl group (-C=O), ~2941 cm^{-1} and ~2863 cm^{-1} attributing to

the asymmetric and symmetric CH_2 stretching, respectively [79]. Similar peaks can be noted in the spectra for CH/PCL at 2932.23, 2862.81 and 1719.23 cm^{-1} showing evidence that the modified film retains the chemical structure of PCL. The spectra for chitosan show its characteristic peaks at ~1571 cm^{-1} associated with the N-H band of the primary amines or amide II [80], 3354 cm^{-1} for the O-H stretch that overlap with the N-H stretch, 2914.88 cm^{-1} and 2869.55 cm^{-1} for the C-H stretch, 1641.12 cm^{-1} C-O stretch of the acetyl group and the amide II band, ~1571 cm^{-1} for N-H stretch, 1374.99 cm^{-1} for the asymmetric C-H stretch bending of CH_2 group and 1063.54 cm^{-1} for the skeletal vibration with the bridge C-O stretch of the glucosamine residue [81]. The CH/PCL films also show same peaks at 3354 cm^{-1} and 1374.99 cm^{-1} and similar peaks for the other functional groups at ~1654, ~1560, 1169.69 and 1064.51 cm^{-1}. This is evidence of CH/PCL film retaining the chemical structure of chitosan, when it is coated on the plasma treated PCL films.

Considering there are no overlapping peaks or new peaks in the CH/PCL film's FTIR spectra other than those observed for pure PCL and pure chitosan, it can be concluded that there are no covalent interactions or chemical reactions between the PCL film and the chitosan coating [82]. This means that the plasma treatment assisted in adhesion of the chitosan coating on PCL film, but did not significantly alter its chemical structure. FTIR spectrum for CH-NX/PCL film was found to be similar to the spectrum for CH/PCL film with no significant changes.

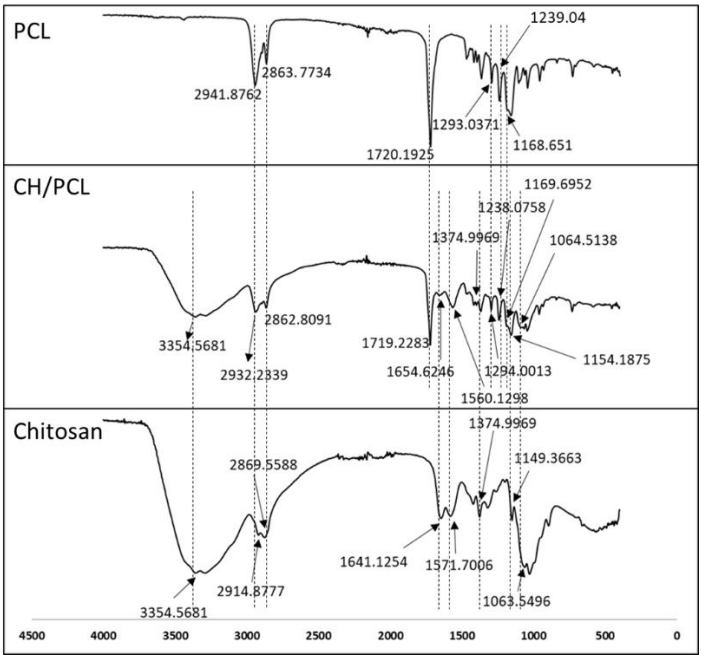

Figure 8: Comparison of the FTIR spectra of pure PCL, chitosan coated PCL (CH/PCL) and chitosan

Thermogravimetric analysis (TGA)

The TGA curves for PCL, CH/PCL and CH-NX/PCL films were obtained in oxygen atmosphere to account for their degradation properties in the presence of air at high temperatures. The TGA curve in Figure 9 (A) for PCL shows degradation beginning at 283.68°C. The thermal degradation of this polymer was accelerated during its reaction with the oxygen gas present in the system, which is the reason for the lower degradation temperature compared to the usual ~400°C as seen in nitrogen environment. The decrease in weight happens in three steps (283.68°C, 361.18°C and 392.13°C) which is in accordance with past literature [83]. The experiment was carried out until 500°C and at 474.29°C, only 6.73% of the original weight of PCL remains.

In the case of CH/PCL, the degradation begins slowly beyond 100°C and rapidly occurs around 275°C. The curve is not as sharp as was observed in the case of PCL. However, this is still in accordance with the degradation that would be observed for PCL and chitosan. Chitosan begins degradation around 300°C in oxygen atmosphere just as in the case of PCL [84]. This could be the reason why there is little difference in the initial degradation temperatures of both PCL and CH/PCL films. This also means that the plasma treatment and chitosan coating of the PCL film did not significantly affect its thermal properties.

The TGA curve for CH-NX/PCL indicates that degradation began at 302.36°C and continues to decrease beyond 500°C. The weight loss occurs in steps just as in the case of PCL. The CH-NX/PCL film's TGA curve looks similar to the one for chitosan as well [84], due to the presence of the chitosan coating. The degradation around 300°C in the cases of CH/PCL and CH-NX/PCL goes well with the degradation of PCL and the potential polysaccharide degradation of the chitosan coating [83]. The TGA curves for CH/PCL and CH-NX/PCL films vary to some extent, and this could be the effect of the chlorination of the latter.

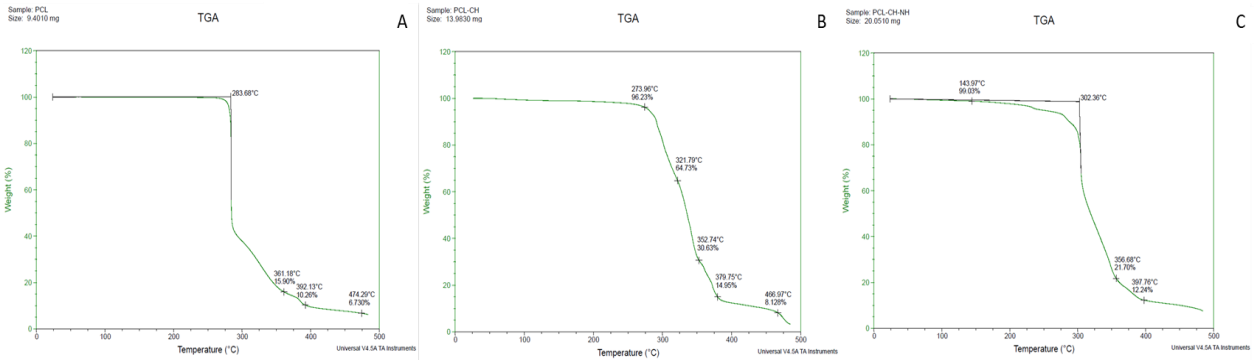

Figure 9: TGA curves for (A) PCL; (B) CH/PCL; (C) CH-NX/PCL

Mechanical properties

The tensile strength and young's modulus of PCL and CH/PCL films were calculated from the force and extension data obtained and is displayed in Figure 10.

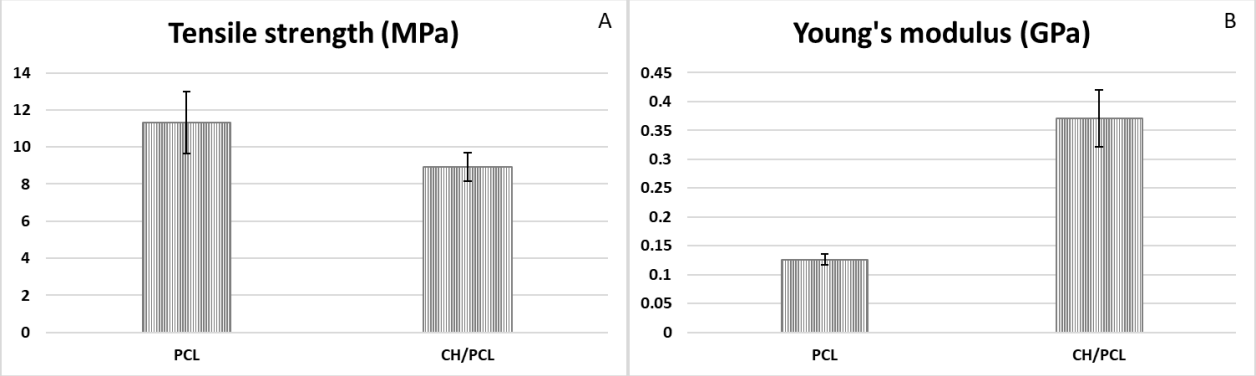

Figure 10: Mechanical properties of PCL and CH/PCL films (with statistically significant differences between each film type (p<0.05)) (A) Tensile strength (MPa); (B) Young's modulus (GPa)

The tensile strength of a material is a measure of the maximum strain that it can withstand before breaking. The tensile strength of CH/PCL was found to be around 8.907 MPa, while that of PCL film was found to be 11.302 Mpa, showing that the PCL film can withstand higher stress compared to its modified counterpart (p<0.05). This could be due to the changes induced in the physical properties of the PCL film while it was subjected to plasma treatment and the subsequent coating with chitosan, known to have undesirable mechanical properties [78]. The Young's modulus value indicates the resistance of any material to deformation under load. This value is higher for materials that are stiff and less flexible. They do not show any difference in their shape at the time of breaking. In this study, the CH/PCL film have higher Young's modulus (~0.37 GPa) compared to PCL (~0.13 GPa), indicating that former is stiffer compared to the

latter (p<0.05). This was also visible in the way the two films broke, which is shown in Figure 11.

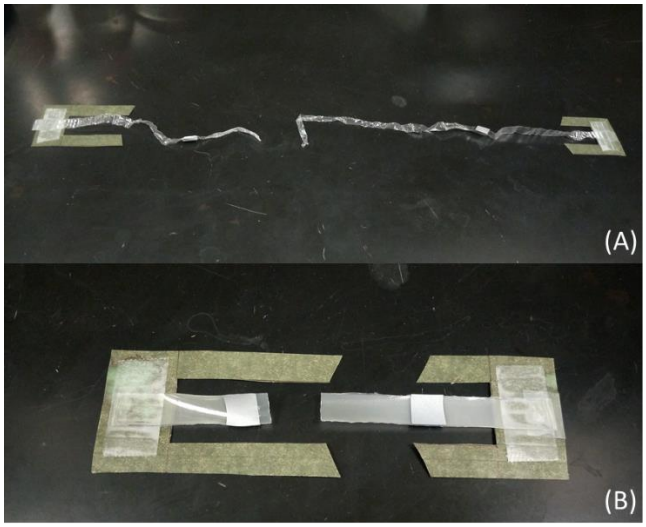

Figure 11: Strips of films after mechanical testing (A) PCL; (B) CH/PCL

Barrier properties

Water vapor transmission rate (WVTR):

The water vapor transmission rates of pure PCL and CH/PCL films were quantified and used to calculate the water vapor permeability. The thicknesses of the films play an important role in the barrier properties of the film and was used for these calculations. The vapor pressure at 23°C and 100% RH is 2810 Pa. From Table 12, it can be noted that water vapor permeability of PCL film is higher than that of CH/PCL (p<0.05). The thicknesses of both the films are similar (~0.2 mm), indicating that the variations in the permeability values for both films depend on the modifications made on the PCL film. The lower permeability of water for CH/PCL film indicates that the plasma treated PCL film coated with a layer of chitosan has increased its barrier against moisture. This may be desirable in food packaging applications that require the packaging

material to protect the food from moisture and subsequent contaminations from microbial sources.

Oxygen transmission rate (OTR):

The oxygen transmission rates were obtained for PCL and CH/PCL films at 23°C, 0% RH and 1 atm. Based on the noted thicknesses for these films (~0.2 mm), the oxygen permeability (OP) values were calculated. Table 12 shows the values OP values and the thicknesses of both the films. It is observed that the OP value for CH/PCL film is lower than that of PCL (p<0.05). This is possibly due to the chitosan coating which has high oxygen barrier properties [85]. Generally, synthetic polymers like PCL have high OTR values. This is undesirable for food packaging since oxygen can easily pass through such films and oxidize the food [86]. The presence of oxygen can also promote the growth of biological contaminants and reduce the shelf-life of the food. The presence of chitosan has increased the oxygen barrier properties of the PCL film, making it desirable for food packaging applications. Here the thicknesses of both the films are similar and so, any variation the barrier properties depend on the modification of PCL.

Table 12: Water vapor and oxygen permeability of pure and chitosan coated PCL

	WVP (g m/m^2 s Pa)	**OP (cc cm/cm^2 s Pa)**
PCL	12.82E-10	9.16E-14
CH/PCL	0.63E-10	3.54E-14

4.3.2. Determination of active chlorine content

To understand the antimicrobial efficacy of the CH-NX/PCL films against the selected strains of the pathogen, the active chlorine content of the film was quantified using an iodometric titration method. The amount of chlorine attached to the surface of the film was found to be 1.5%, when

tested on the same day it was synthesized. Chlorinated PCL film without chitosan coating and unchlorinated CH/PCL film were used as controls and it was found that neither of the films had chlorine on them. The absence of chlorine on the chlorinated PCL film is because of the absence of chitosan coating on the film. There were no amino groups present for the chlorine ions to attach to and so, N-halamine structures could not be formed. This also indicates that the presence of chlorine on the CH-NX/PCL films can directly be correlated to the presence of the antimicrobial N-halamine structures on the film. The absence of chlorine ions on the CH/PCL films indicates that there are no chlorine ions previously associated with the chitosan coating and all the chlorine ions come from the sodium hypochlorite treatment of the coated PCL films. Figure 12 shows the titration solutions for chlorinated and uncoated PCL, unchlorinated CH/PCL and CH-NX/PCL films. It can be observed that only the CH-NX/PCL turns blue black in the presence of potassium iodide in the solution, indicating the presence of chlorine ions on its surface.

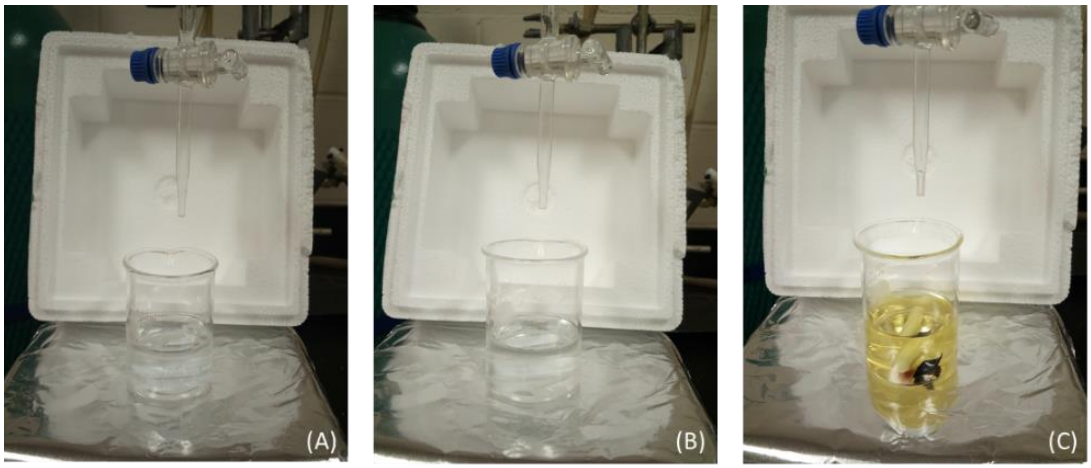

Figure 12: Iodometric titration solutions with (A) chlorinated and uncoated PCL film; (B) CH/PCL film; (C) CH-NX/PCL film

4.3.3. Testing antimicrobial activity of CH-NX/PCL film against bacteria

The antimicrobial activity of CH-NX/PCL film was tested against the drug susceptible and ampicillin resistant strains of *Salmonella* Typhimurium to check its efficacy against the pathogens irrespective of whether they are resistant to antibiotics. The efficacy of CH-PCL in eliminating these pathogens was also studied and compared with that of CH-NX/PCL film. Figure 13 shows the bacterial growth in log CFU/ml for both the strains when treated with these films. From the graph, it is clearly observed that 100% of each strain was eliminated in the antimicrobial assay, when treated with the CH-NX/PCL film ($p<0.05$). Table 14 shows p value to be 2.29E-11 for ampicillin resistant *S.* Typhimurium strain, while the p value could not be determined in Table 16 for *S.* Typhimurium since there was absolutely no difference in growth for within the control group and treatment group. Though not significant enough for eliminating contamination, the CH/PCL films also show bacterial growth reduction of around one log for ampicillin resistant *Salmonella* Typhimurium, which is 8.5 logs, and around two logs for drug susceptible *Salmonella* Typhimurium, which is 8 logs, as opposed to the original culture growth of 9.5 and 10 logs obtained without any treatment for each strain, respectively ($p<0.05$). Tables 13 and 15 show p values to be 0.0047 and 1.3E-04 for the drug resistant and drug susceptible strains, respectively. This is possibly due to the inherent antimicrobial activity of the chitosan coating on the film. However as seen from the results chitosan's efficacy is not high enough to eliminate contamination completely. While drug susceptible *Salmonella* Typhimurium was reduced by two logs, ampicillin resistant *Salmonella* Typhimurium was reduced by one log. The difference in the activity of CH/PCL on both the strains could be due to the difficulty of chitosan molecules to interact with the drug resistant strain causing lower bacterial reduction in this case.

PCL film did not show significant growth reduction of either strains when compared to the original bacterial concentration for ampicillin resistant and drug susceptible *Salmonella* Typhimurium, respectively. This indicates the absence of any antimicrobial activity associated with the film. Samples without treatment with films also did not show significant growth reduction when compared to the original. This indicates that the treatment steps followed in the assay do not significantly play a role in reducing bacterial growth on their own and any bacterial elimination occurs due to the antimicrobial film that is being tested. The absence of bacterial growth for samples treated with CH-NX/PCL film indicate that the N-halamine structures formed on the film's surface play a crucial role in eliminating the pathogens. The results from the iodometric titration also confirms the presence of chlorine ions on this film, that interacts with the amino groups of the chitosan coating to form the antimicrobial N-halamine structures.

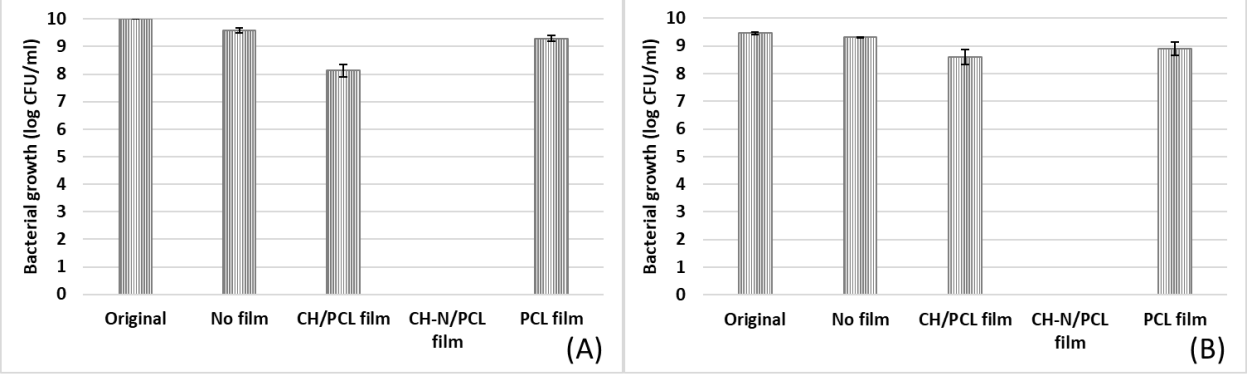

Figure 13: Bacterial growth reduction for (A) ampicillin resistant *Salmonella* Typhimurium; (B) drug susceptible *Salmonella* Typhimurium (contact time – 30 minutes; detection limit – serial dilution of 10^{-3})

Table 13: ANOVA for Ampicillin resistant *Salmonella* Typhimurium treated with CH/PCL film and control group

Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	1.276671	1	1.276671	32.38964	0.004708	7.708647
Within Groups	0.157664	4	0.039416			
Total	1.434335	5				

Table 14: ANOVA for Ampicillin resistant *Salmonella* Typhimurium treated with CH-NX/PCL film and control group

Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	134.0797	1	134.0797	512186.9	2.29E-11	7.708647
Within Groups	0.001047	4	0.000262			
Total	134.0808	5				

Table 15: ANOVA for *Salmonella* Typhimurium treated with CH/PCL film and control group

Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	5.450014	1	5.450014	212.5153	0.000129	7.708647
Within Groups	0.102581	4	0.025645			
Total	5.552595	5				

Table 16: ANOVA for *Salmonella* Typhimurium treated with CH-NX/PCL film and control group

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Control	3	30	10	0		
CH-NX/PCL	3	0	0	0		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F critical**
Between Groups	150	1	150	65535	_	7.708647
Within Groups	0	4	0			
Total	150	5				

The chlorine ions on CH-NX/PCL are stable due to their attachment to the amino groups of the chitosan coating. This makes them much safer compared to free chlorine ions, which is essential when considering food-based applications [72]. The chlorine ions in the N-halamine structure are oxidative in nature and upon contact with bacterial cells, get transferred to the cell wall. This interaction causes cell destruction due to the interference of the ions in the metabolic functions of the cell. Since this process happens irrespective of whether the bacterial strain has drug resistant strains, the application of N-halamine for eliminating drug resistant pathogen contaminations is promising [73]. The results of the sandwich assay confirm this activity, wherein, the CH-NX/PCL film was efficient in eliminating both the drug susceptible and ampicillin resistant *Salmonella* Typhimurium. The chlorine ions consumed in the elimination of such pathogens can be replenished by sodium hypochlorite treatment of the used film and reapplied for food packaging purposes [72].

CONCLUSION

The antimicrobial activity of CPN films against both drug susceptible and ampicillin resistant *S.* Typhimurium strains was evident in the sandwich assay and in ampicillin resistant bacteria inoculated cheese samples. The correlation of chlorine percentage of the N-halamine structures on the CPN films with its antibacterial activity was specifically evident from the fact that the CH/PVA films without these ions did not show significant bacterial inactivation. However, all tests were conducted for five days. Further testing beyond those five days could possibly give an insight into how long the CPN films could be used effectively to eliminate pathogens. This can also help in identifying the critical chlorine content at which its biocidal efficacy is intact. This will help in designing films with lower chlorine content that meets the required standards and still be able to retain its biocidal activity. Although total bacterial elimination was not possible in cheese samples using the CPN films, there was a significant bacterial reduction when compared to the samples that were not treated with these films, showing that CPN films are effective in eliminating ampicillin resistant *S.* Typhimurium contamination in food samples upon direct contact. Further tests would need to be conducted to create a more effective film design that targets the bacteria that are not in contact with the film.

The fabrication of N-halamine based antimicrobial film using plasma treated and chitosan coated polycaprolactone polymer was done to obtain an ideal packaging material with strong physical properties. This film along with pure PCL and chitosan coated PCL were characterized using FTIR and TGA and the fabricated films showed properties similar to PCL and chitosan. Their physical properties in terms of mechanical strength, water vapor and oxygen barrier were also analyzed, and it was found that the modified PCL films showed promise as food packaging materials with higher strength and barrier compared to pure PCL film. The chlorine content of

the CH-NX/PCL film was found to be 1.5% and correlates to its antimicrobial activity. The sandwich assay tests showed that the fabricated CH-NX/PCL film can eliminate both drug susceptible and ampicillin resistant *Salmonella* Typhimurium strains with 100% efficacy, unlike the one to two log reductions obtained by treatment with CH/PCL film. The physical properties of the film, together with its biodegradability and antimicrobial activity makes the CH-NX/PCL film a promising food packaging material.

Though the antimicrobial efficacy of CPN and CH-NX/PCL films depended on the chitosan-based N-halamine structures, their mechanical properties depended on the polymers used for fabrication. Both films showed excellent antimicrobial property against the two strains of bacteria. However, PCL based films were prepared keeping in mind the necessity to apply the films in practical packaging systems. The PVA based films, though improved the mechanical properties of chitosan, did not perform well under moist environment due to absorption of water, making them unfit for practical applications. The PCL based films on the other hand were able to overcome this problem due to their high barrier to water and resemblance to synthetic polymers currently used in food packaging.

Future studies:

The fabrication of chitosan-based N-halamine films proposed in this study requires the use of diluted sodium hypochlorite for the chlorination process. Though the preparation process uses diluted quantities of this chemical, there is a set standard for allowable chlorine that can come in contact with food products consumed by humans, according to FDA regulations. For this reason, further studies must be done to determine the minimum chlorine percentage that remain within FDA limit and can form N-halamine structures to effectively eliminate pathogens. Toxicity tests

on human cells may also be required to ensure safety in using these films for food-based applications.

This study specifically used drug susceptible and ampicillin resistant strains of *S.* Typhimurium. However, there has been outbreaks due to multidrug resistant strains of this bacteria associated with meat products [1]. Further studies will be required to study the antimicrobial efficacy of CPN and CH-NX/PCL films against other strains of *Salmonella*. There are other foodborne pathogens that top the list in causing outbreaks as mentioned previously and studies to test the efficacy of these films against Gram positive, other Gram negative and fungal contaminants must be done to check the applicability of these films in preventing contamination in food matrix in a broad sense.

Cheddar cheese slices were used for testing the application of CPN films in packaging and eliminating contamination. Based on the results it is evident that the properties of the food also play a role in influencing the antimicrobial activity of the packaging film. Therefore, the film's application in a wide range of food products such as meat, vegetables, dry food such as cereals, etc. will have to be tested to check if the antimicrobial activity is retained while packaging a broad range of food products. Also, the physical properties of the fabricated film will differ based on the type of food being packaged for shelf-life considerations and there is scope for incorporating other biodegradable polymers as the foundation for the chitosan/N-halamine-based antimicrobial activity.

REFERENCES

[1] Salmonella Homepage | CDC [Internet]. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention; [cited 2019May29]. Available from: https://www.cdc.gov/salmonella/

[2] Franz CM, den Besten HM, Böhnlein C, Gareis M, Zwietering MH, Fusco V. Reprint of: Microbial food safety in the 21st century: Emerging challenges and foodborne pathogenic bacteria. Trends in Food Science and Technology. 2019;84:34-7.

[3] Johnson JR. Foodborne Illness Acquired in the United States. Emerging Infectious Diseases. 2011;17(7):1338–9.

[4] Ratnam S, March SB. Laboratory studies on salmonella-contaminated cheese involved in a major outbreak of gastroenteritis. Journal of Applied Bacteriology. 1986 Jul;61(1):51-6.

[5] Hoffmann S, Batz MB, Morris Jr JG. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. Journal of food protection. 2012 Jul;75(7):1292-302.

[6] Leighton P. Mass salmonella poisoning by the peanut corporation of America: State corporate crime involving food safety. Critical Criminology. 2016 Mar 1;24(1):75-91.

[7] Guo M, Jin TZ, Yang R. Antimicrobial polylactic acid packaging films against Listeria and Salmonella in culture medium and on ready-to-eat meat. Food and bioprocess technology. 2014 Nov 1;7(11):3293-307.

[8] Proulx J, Hsu LC, Miller BM, Sullivan G, Paradis K, Moraru CI. Pulsed-light inactivation of pathogenic and spoilage bacteria on cheese surface. Journal of dairy science. 2015 Sep 1;98(9):5890-8.

[9] Suganya A, Shanmugvelayutham G, Hidalgo-Carrillo J. Plasma Surface Modified Polystyrene and Grafted with Chitosan Coating for Improving the Shelf Lifetime of Postharvest Grapes. Plasma Chemistry and Plasma Processing. 2018 Sep 1;38(5):1151-68.

[10] Khaneghah AM, Hashemi SM, Limbo S. Antimicrobial agents and packaging systems in antimicrobial active food packaging: An overview of approaches and interactions. Food and bioproducts processing. 2018 Sep 1;111:1-9.

[11] Sung SY, Sin LT, Tee TT, Bee ST, Rahmat AR, Rahman WA, Tan AC, Vikhraman M. Antimicrobial agents for food packaging applications. Trends in Food Science & Technology. 2013 Oct 1;33(2):110-23.

[12] Pellerito A, Ameen SM, Micali M, Caruso G. Antimicrobial Substances for Food Packaging Products: The Current Situation. Journal of AOAC International. 2018 Jul 1;101(4):942-7.
[13] Li R, Hu P, Ren X, Worley SD, Huang TS. Antimicrobial N-halamine modified chitosan films. Carbohydrate polymers. 2013 Jan 30;92(1):534-9.

[14] Zhang R, Xu W, Jiang F. Fabrication and characterization of dense chitosan/polyvinyl alcohol/poly-lactic-acid blend membranes. Fibers and Polymers. 2012 May 1;13(5):571-5.

[15] Folegatti MI, Antunes AJ, Marcondes JA. Mechanical and permeability properties of milk protein films. Brazilian Archives of Biology and Technology. 1998;41(3):0-.

[16] Cazón P, Velazquez G, Ramírez JA, Vázquez M. Polysaccharide-based films and coatings for food packaging: A review. Food Hydrocolloids. 2017 Jul 1;68:136-48.

[17] Aguirre-Loredo RY, Velazquez G, Gutierrez MC, Castro-Rosas J, Rangel-Vargas E, Gómez-Aldapa CA. Effect of airflow presence during the manufacturing of biodegradable films from polymers with different structural conformation. Food packaging and shelf life. 2018 Sep 1;17:162-70.

[18] Kuorwel KK, Cran MJ, Sonneveld K, Miltz J, Bigger SW. Antimicrobial activity of biodegradable polysaccharide and protein-based films containing active agents. Journal of Food Science. 2011 Apr;76(3):R90-102.

[19] Xie J, Hung YC. UV-A activated TiO2 embedded biodegradable polymer film for antimicrobial food packaging application. LWT. 2018 Oct 1;96:307-14.

[20] Biddeci G, Cavallaro G, Di Blasi F, Lazzara G, Massaro M, Milioto S, Parisi F, Riela S, Spinelli G. Halloysite nanotubes loaded with peppermint essential oil as filler for functional biopolymer film. Carbohydrate polymers. 2016 Nov 5;152:548-57.

[21] Aranaz I, Mengíbar M, Harris R, Paños I, Miralles B, Acosta N, Galed G, Heras Á. Functional characterization of chitin and chitosan. Current chemical biology. 2009 May 1;3(2):203-30.

[22] Oymaci P, Altinkaya SA. Improvement of barrier and mechanical properties of whey protein isolate based food packaging films by incorporation of zein nanoparticles as a novel bionanocomposite. Food Hydrocolloids. 2016 Mar 1;54:1-9.

[23] Wang H, Wang L. Developing a bio-based packaging film from soya by-products incorporated with valonea tannin. Journal of cleaner production. 2017 Feb 1;143:624-33.

[24] Arcan I, Yemenicioğlu A. Incorporating phenolic compounds opens a new perspective to use zein films as flexible bioactive packaging materials. Food Research International. 2011 Mar 1;44(2):550-6.

[25] Kashiri M, Cerisuelo JP, Domínguez I, López-Carballo G, Muriel-Gallet V, Gavara R, Hernández-Muñoz P. Zein films and coatings as carriers and release systems of Zataria multiflora Boiss. essential oil for antimicrobial food packaging. Food Hydrocolloids. 2017 Sep 1;70:260-8.

[26] Dhar P, Bhardwaj U, Kumar A, Katiyar V. Poly (3--hydroxybutyrate)/cellulose nanocrystal films for food packaging applications: Barrier and migration studies. Polymer Engineering & Science. 2015 Oct;55(10):2388-95.

[27] Yu H, Yan C, Yao J. Fully biodegradable food packaging materials based on functionalized cellulose nanocrystals/poly (3-hydroxybutyrate-co-3-hydroxyvalerate) nanocomposites. Rsc Advances. 2014;4(104):59792-802.

[28] Xu FJ, Wang ZH, Yang WT. Surface functionalization of polycaprolactone films via surface-initiated atom transfer radical polymerization for covalently coupling cell-adhesive biomolecules. Biomaterials. 2010 Apr 1;31(12):3139-47.

[29] Murbach Teles Andrade BF, Nunes Barbosa L, Da Silva Probst I, Fernandes Júnior A. Antimicrobial activity of essential oils. Journal of Essential Oil Research. 2014;26(1):34–40.

[30] Niu B, Yan Z, Shao P, Kang J, Chen H. Encapsulation of Cinnamon Essential Oil for Active Food Packaging Film with Synergistic Antimicrobial Activity. Nanomaterials. 2018 Aug;8(8):598.

[31] Wen P, Zhu DH, Wu H, Zong MH, Jing YR, Han SY. Encapsulation of cinnamon essential oil in electrospun nanofibrous film for active food packaging. Food Control. 2016 Jan 1;59:366 76.

[32] Fasihi H, Noshirvani N, Hashemi M, Fazilati M, Salavati H, Coma V. Antioxidant and antimicrobial properties of carbohydrate-based films enriched with cinnamon essential oil by Pickering emulsion method. Food Packaging and Shelf Life. 2019 Mar 1;19:147-54.

[33] Azadbakht E, Maghsoudlou Y, Khomiri M, Kashiri M. Development and structural characterization of chitosan films containing Eucalyptus globulus essential oil: Potential as an antimicrobial carrier for packaging of sliced sausage. Food packaging and shelf life. 2018 Sep 30;17:65-72.

[34] Sharafati Chaleshtori F, Taghizadeh M, Rafieian-kopaei M, Sharafati-chaleshtori R. Effect of chitosan incorporated with cumin and eucalyptus essential oils as antimicrobial agents on fresh chicken meat. Journal of food processing and preservation. 2016 Jun;40(3):396-404.

[35] Bouarab Chibane L, Degraeve P, Ferhout H, Bouajila J, Oulahal N. Plant antimicrobial polyphenols as potential natural food preservatives. Journal of the Science of Food and Agriculture. 2019 Mar 15;99(4):1457-74.

[36] Rui L, Xie M, Hu B, Zhou L, Yin D, Zeng X. A comparative study on chitosan/gelatin composite films with conjugated or incorporated gallic acid. Carbohydrate polymers. 2017 Oct 1;173:473-81.

[37] Sun X, Wang Z, Kadouh H, Zhou K. The antimicrobial, mechanical, physical and structural properties of chitosan–gallic acid films. LWT-Food Science and Technology. 2014 Jun 1;57(1):83-9.

[38] Riaz A, Lei S, Akhtar HM, Wan P, Chen D, Jabbar S, Abid M, Hashim MM, Zeng X. Preparation and characterization of chitosan-based antimicrobial active food packaging film incorporated with apple peel polyphenols. International journal of biological macromolecules. 2018 Jul 15;114:547-55.

[39] Liu Y, Liang X, Wang S, Qin W, Zhang Q. Electrospun Antimicrobial Polylactic Acid/Tea Polyphenol Nanofibers for Food-Packaging Applications. Polymers. 2018 May;10(5):561.

[40] Hoseinnejad M, Jafari SM, Katouzian I. Inorganic and metal nanoparticles and their antimicrobial activity in food packaging applications. Critical reviews in microbiology. 2018 Mar 4;44(2):161-81.

[41] Tang ZP, Chen CW, Xie J. Development of antimicrobial active films based on poly (vinyl alcohol) containing nano-TiO2 and its application in macrobrachium rosenbergii packaging. Journal of Food Processing and Preservation. 2018 Aug;42(8):e13702.

[42] Zhang X, Xiao G, Wang Y, Zhao Y, Su H, Tan T. Preparation of chitosan-TiO2 composite film with efficient antimicrobial activities under visible light for food packaging applications. Carbohydrate polymers. 2017 Aug 1;169:101-7.

[43] Yoksan R, Chirachanchai S. Silver nanoparticle-loaded chitosan–starch based films: Fabrication and evaluation of tensile, barrier and antimicrobial properties. Materials Science and Engineering: C. 2010 Jul 20;30(6):891-7.

[44] Suo B, Li H, Wang Y, Li Z, Pan Z, Ai Z. Effects of ZnO nanoparticle-coated packaging film on pork meat quality during cold storage. Journal of the Science of Food and Agriculture. 2017 May;97(7):2023-9.

[45] Castro-Mayorga JL, Fabra MJ, Pourrahimi AM, Olsson RT, Lagaron JM. The impact of zinc oxide particle morphology as an antimicrobial and when incorporated in poly (3-hydroxybutyrate-co-3-hydroxyvalerate) films for food packaging and food contact surfaces applications. Food and Bioproducts Processing. 2017 Jan 1;101:32-44.

[46] Gemili S, Yemenicioğlu A, Altınkaya SA. Development of cellulose acetate based antimicrobial food packaging materials for controlled release of lysozyme. Journal of Food Engineering. 2009 Feb 1;90(4):453-62.

[47] Duan J, Park SI, Daeschel MA, Zhao Y. Antimicrobial chitosan-lysozyme (CL) films and coatings for enhancing microbial safety of mozzarella cheese. Journal of Food Science. 2007 Nov;72(9):M355-62.

[48] Mecitoğlu Ç, Yemenicioğlu A, Arslanoğlu A, Elmacı ZS, Korel F, Çetin AE. Incorporation of partially purified hen egg white lysozyme into zein films for antimicrobial food packaging. Food Research International. 2006 Jan 1;39(1):12-21.

[49] Jin T, Zhang H. Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. Journal of Food Science. 2008 Apr;73(3):M127-34.

[50] Wang H, Zhang R, Zhang H, Jiang S, Liu H, Sun M, Jiang S. Kinetics and functional effectiveness of nisin loaded antimicrobial packaging film based on chitosan/poly (vinyl alcohol). Carbohydrate polymers. 2015 Aug 20;127:64-71.

[51] Ren T, Qiao M, Huang TS, Weese J, Ren X. Efficacy of N-halamine compound on reduction of microorganisms in absorbent food pads of raw beef. Food control. 2018 Feb 1;84:255-62.

[52] Ren T, Hayden M, Qiao M, Huang TS, Ren X, Weese J. Absorbent Pads Containing N-Halamine Compound for Potential Antimicrobial Use for Chicken Breast and Ground Chicken. Journal of agricultural and food chemistry. 2018 Feb 12;66(8):1941-8.

[53] Liu Y, Li L, Pan N, Wang Y, Ren X, Xie Z, Buschle-Diller G, Huang TS. Antibacterial cellulose acetate films incorporated with N-halamine-modified nano-crystalline cellulose particles. Polymers for Advanced Technologies. 2017 Apr;28(4):463-9.

[54] Chen X, Liu Z, Cao W, Yong C, Xing X. Preparation, characterization, and antibacterial activities of quaternarized N-halamine-grafted cellulose fibers. Journal of Applied Polymer Science. 2015 Nov 15;132(43).

[55] Lee J, Whang HS. Poly (vinyl alcohol) blend film with m-aramid as an N-halamine precursor for antimicrobial activity. Journal of Applied Polymer Science. 2011 Nov 15;122(4):2345-50.

[56] Busolo MA, Fernandez P, Ocio MJ, Lagaron JM. Novel silver-based nanoclay as an antimicrobial in polylactic acid food packaging coatings. Food Additives and Contaminants. 2010 Nov 1;27(11):1617-26.

[57] Theinsathid P, Visessanguan W, Kruenate J, Kingcha Y, Keeratipibul S. Antimicrobial activity of lauric arginate-coated polylactic acid films against Listeria monocytogenes and Salmonella typhimurium on cooked sliced ham. Journal of food science. 2012 Feb;77(2):M142-9.

[58] Kwon SJ, Chang Y, Han J. Oregano essential oil-based natural antimicrobial packaging film to inactivate Salmonella enterica and yeasts/molds in the atmosphere surrounding cherry tomatoes. Food microbiology. 2017 Aug 1;65:114-21.

[59] Rodríguez-Núñez JR, López-Cervantes J, Sánchez-Machado DI, Ramírez-Wong B, Torres-Chavez P, Cortez-Rocha MO. Antimicrobial activity of chitosan-based films against Salmonella typhimurium and Staphylococcus aureus. International journal of food science & technology. 2012 Oct;47(10):2127-33.

[60] Tsai TM, Chang HH, Chang KC, Liu YL, Tseng CC. A comparative study of the bactericidal effect of photocatalytic oxidation by TiO2 on antibiotic-resistant and antibiotic sensitive bacteria. Journal of Chemical Technology & Biotechnology. 2010 Dec;85(12):1642-53.

[61] Cao Z, Sun Y. N--halamine-based chitosan: Preparation, characterization, and antimicrobial function. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2008 Apr;85(1):99-107.

[62] Yu Z, Li B, Chu J, Zhang P. Silica in situ enhanced PVA/chitosan biodegradable films for food packages. Carbohydrate polymers. 2018 Mar 15;184:214-20.

[63] Suntornnond R, An J, Chua. CK. Effect of gas plasma on polycaprolactone (PCL) membrane wettability and collagen type I immobilized for enhancing cell proliferation. Materials

Letters. 2016 May 15;171 :293-6.

[64] Lin WC, Mohd Razali NA. Temporary Wettability Tuning of PCL/PDMS Micro Pattern Using the Plasma Treatments. Materials. 2019 Jan;12(4):644.

[65] Chaiwong C, Rachtanapun P, Wongchaiya P, Auras R, Boonyawan D. Effect of plasma treatment on hydrophobicity and barrier property of polylactic acid. Surface and Coatings Technology. 2010 Jun 25;204(18-19):2933-9.

[66] ASTM International. ASTM D882-12, Standard Test Method for Tensile Properties of Thin Plastic Sheeting. ASTM International; 2012.

[67] Goy RC, Britto DD, Assis OB. A review of the antimicrobial activity of chitosan. Polímeros. 2009 ;19 (3):241-7.

[68] Goy RC, Morais ST, Assis OB. Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on E. coli and S. aureus growth. evista Brasileira de Farmacognosia. 2016 Feb;26(1):122-7.

[69] Demir B, Broughton R, Qiao M, Huang TS, Worley S. N-halamine biocidal materials with superior antimicrobial efficacies for wound dressings. Molecules. 2017;22(10):1582.

[70] Flat Creek Farm Recalls Cheeses for Potential Salmonella Contamination. [Internet]. Food Safety News. 2018 [cited 2019Jul3]. Available from: https://www.foodsafetynews.com/2014/12/flat-creek-farm-recalls-cheeses-for-potential salmonella-contamination/

[71] Marler O. Salmonella Concern Prompts Cheese Spread Recall. [Internet]. Food Safety News. 2011 [cited 2019Jul3]. Available from: https://www.foodsafetynews.com/2011/08/salmonella-concerns-prompts-cheese-spread recall/#more-17142

[72] Dong A, Wang YJ, Gao Y, Gao T, Gao G. Chemical insights into antibacterial N-halamines. Chemical reviews. 2017 Mar 2;117(6):4806-62.

[73] Sun X, Cao Z, Porteous N, Sun Y. An N-halamine-based rechargeable antimicrobial and biofilm controlling polyurethane. Acta biomaterialia. 2012 Apr 1;8(4):1498-506.

[74] Natan M, Gutman O, Lavi R, Margel S, Banin E. Killing mechanism of stable N-halamine cross-linked polymethacrylamide nanoparticles that selectively target bacteria. ACS nano. 2015 Jan 26;9(2):1175 -88.

[75] Szczawińska ME, Szczawiński J, Łobacz A. Effect of temperature on the growth kinetics of Salmonella Enteritidis in cooked ham. Bulletin of the Veterinary Institute in Pulawy. 2014 Mar 1;58(1):47-56.

[76] Serraino A, Finazzi G, Marchetti G, Daminelli P, Riu R, Giacometti F, Losio MN, Rosmini R. Behaviour of Salmonella Typhimurium during production and storage of artisan water buffalo mozzarella cheese. Italian Journal of Animal Science. 2012 Jan 1;11(3):e53.

[77] Khattak S, Wahid F, Liu LP, Jia SR, Chu LQ, Xie YY, Li ZX, Zhong C. Applications of cellulose and chitin/chitosan derivatives and composites as antibacterial materials: current state and perspectives. Applied microbiology and biotechnology. 2019 Mar 1;103(5):1989-2006.

[78] Zhang ZH, Han Z, Zeng XA, Xiong XY, Liu YJ. Enhancing mechanical properties of chitosan films via modification with vanillin. International Journal of biological macromolecules. 2015 Nov 1;81 :638-43.

[79] Dávila JL, Freitas MS, Inforçatti Neto P, Silveira ZD, Silva JV, d'Ávila MA. Fabrication of PCL/β-TCP scaffolds by 3D mini-screw extrusion printing. Journal of Applied Polymer Science. 2016 Apr 15;133(15).

[80] Zhong X, Ji C, Chan AK, Kazarian SG, Ruys A, Dehghani F. Fabrication of chitosan/poly (ε-caprolactone) composite hydrogels for tissue engineering applications. Journal of Materials Science: Materials in Medicine. 2011 Feb 1;22(2):279-88.

[81] Kumar S, Koh J, Kim H, Gupta MK, Dutta PK. A new chitosan–thymine conjugate: Synthesis, characterization and biological activity. International journal of biological macromolecules. 2012 Apr 1;50(3):493-502.

[82] Cooper A, Bhattarai N, Zhang M. Fabrication and cellular compatibility of aligned chitosan–PCL fibers for nerve tissue regeneration. Carbohydrate Polymers. 2011 Apr 22;85(1):149-56.

[83] Vogel C, Siesler HW. Thermal Degradation of Poly (ε--caprolactone), Poly (L--lactic acid) and their Blends with Poly (3--hydroxy--butyrate) Studied by TGA/FT-IR Spectroscopy. InMacromolecular symposia 2008 May (Vol. 265, No. 1, pp. 183-194). Weinheim: WILEY VCH Verlag.

[84] Cesano F, Fenoglio G, Carlos L, Nisticò R. One-step synthesis of magnetic chitosan polymer composite films. Applied Surface Science. 2015 Aug 1;345:175-81.

[85] Park SH, Lee HS, Choi JH, Jeong CM, Sung MH, Park HJ. Improvements in barrier properties of poly (lactic acid) films coated with chitosan or chitosan/clay nanocomposite. Journal of Applied Polymer Science. 2012 Jul 25;125(S1):E675-80.

[86] Khan RA, Dussault D, Salmieri S, Safrany A, Lacroix M. Mechanical and barrier properties of carbon nanotube reinforced PCL--based composite films: Effect of gamma radiation. Journal of Applied Polymer Science. 2013 Mar 5;127(5):3962-9.