Incorporation of selected plant extracts into edible chitosan films and the effect on the antiviral, antibacterial and mechanical properties of the material

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Doctor of Philosophy in the Graduate School of The Ohio State University

By

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Graduate program in Food Science and Technology

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2013

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ABSTRACT

Edible films and coatings are increasingly being used as carriers of functional additives including antimicrobial agents. Consumer interest in naturally-derived antimicrobials has also increased. Plant extracts such as grape seed and green tea extracts are known to have antiviral as well as antibacterial activities. Even though there are several researches that have investigated the use of edible films and coatings as carriers of antimicrobial agents against foodborne bacterial pathogens, unfortunately data is lacking on the use of these same technologies on foodborne viruses. Therefore, this study seeks to develop edible films and coatings that can control foodborne viruses. The film’s antibacterial and mechanical properties were also tested.

Chapter 3 of this study investigated the virucidal activity of green tea extract (GTE) dissolved in deionized water and also incorporated into chitosan film forming solutions (FFS) and into chitosan films. For comparison, the antibacterial activity of the films was also investigated against Listeria innocua and Escherichia coli K12. The viral infectivity after treatments was measured by plaque assays. The 1, 1.5 and 2.5% aqueous GTE solutions significantly (p<0.05) reduced murine norovirus (MNV-1) plaques by 1.69, 2.47, and 3.26 log after 3 h exposure, respectively. Similarly, the FFS containing 2.5 and 5.0% GTE reduced MNV-1 counts by 2.45 and 3.97 log_{10} PFU/ml, respectively
after 3 h exposure. Additionally, the edible films enriched with the GTE (5, 10 and 15%) were also effective against MNV-1. After a 24 h incubation period, the 5 and 10% GTE films significantly (p<0.05) resulted in MNV-1 titer reductions of 1.60 and 4.50 log_{10} PFU/ml, respectively. The film containing 15% GTE reduced norovirus plaques to undetectable levels in 24 h. Finally, all the GTE films (5, 10 and 15%) reduced L. innocua and E. coli K12 populations to undetectable levels in tryptic soy broth after 24 h exposure.

Chapter 4 investigated the effect of grape seed extract in solution, and in edible coatings and films against MNV-1. Also, the films were tested against L. innocua and E. coli K12, surrogates for L. monocytogenes and E. coli O157:H7, respectively. Grape seed extract concentrations of 1, 1.5 and 2.5% dissolved in deionized water resulted in MNV-1 plaque reductions (p<0.05) of 1.75, 2.60 and 3.58 log_{10} PFU/ml, respectively, after 3 h. The chitosan solutions incorporated with the GSE (2.5 and 5%) also reduced MNV-1 titers significantly (p<0.05) by 2.68 and 4.00 log_{10} PFU/ml, respectively, after 3 h. Additionally, incorporation of the GSE into the chitosan films enhanced its efficacy against MNV-1, L. innocua and E. coli K12. The chitosan films containing 5, 10, and 15% GSE showed reductions of 0.92, 1.89 and 2.27 log_{10} PFU/ml, respectively after 4 h of incubation. Also after 24 h, the 5 and 10% GSE films reduced MNV-1 titers by 1.90 and 3.26 log_{10} PFU/ml, respectively, while the 15% GSE film reduced MNV-1 to undetectable levels. For E. coli K12, there were reductions of 2.28, 5.18 and 7.14 log_{10} CFU/ml after 24 h exposure by the 5, 10, and 15% GSE films, respectively. Also, L.
inocua counts were reduced by 3.06, 6.15 and 6.91 log$_{10}$ CFU/ml by the 5, 10 and 15% GSE films, respectively.

The final part of this dissertation (chapter 5) investigated the effect of glycerol, GTE and GSE on the mechanical and moisture barrier properties of chitosan film. To optimize the quantities of glycerol, GTE and GSE in the chitosan film samples, the tensile strength (TS), percent elongation at break (%E), thickness, moisture content, water vapor permeability and solubility were determined for each treatment combination. The results showed that, TS significantly (p<0.05) decreased after the incorporation of glycerol. The tensile strength of 2% chitosan (used as the control) was 48.09 ± 5.17 MPa. The films incorporated with GTE or GSE and glycerol were significantly (p<0.05) lower in TS compared to the control. Values of 11.07 ± 2.50, 6.42 ± 0.68 and 6.55 ± 0.80 MPa were recorded for the 5, 10, and 15% GSE films, respectively. For the 5, 10 and 15% GTE films, TS values of 12.48 ± 2.52, 5.43 ± 0.59 and 5.07 ± 1.27 MPa were obtained, respectively. Also, the %E obtained for the 2% chitosan control film was 4.98 ± 0.94. The 5, 10 and 15% GSE films produced %E values of 16.75 ± 2.02, 30.95 ± 6.92 and 30.22 ± 4.98%, respectively. The values obtained for the 5, 10, and 15% GTE films were 23.77 ± 3.30, 39.12 ± 4.27 and 43.49 ± 1.06%, respectively. The WVP of chitosan film without the extract was 0.349 ± 0.039 g.mm/m$^2$.h. kPa. The 5, 10, and 15% GTE films produced WVP values of 0.417 ± 0.007, 0.457 ± 0.005 and 0.569 ± 0.025 g.mm/m$^2$.h. kPa, respectively. Also, the 5, 10 and 15%GSE films recorded WVP values of 0.422 ± 0.085, 0.496 ± 0.083 and 0.524± 0.029 g.mm/m$^2$.h. kPa, respectively
DEDICATION

To my children Daisy and David Amankwaah
ACKNOWLEDGMENTS

To God be all the Glory. My deepest gratitude goes to the Almighty God under whose Mighty hands I have receive protection and the gift of life to see this day.

I very grateful for the support and direction I received from my advisor Dr. Melvin Pascall for all these years. I want to also thank my committee members Dr. Jianrong Li, Dr. John Litchfield and Dr. Jiyoung Lee for their help.

The virus studies were conducted in the lab of Dr. Jianrong Li and I am very appreciative for this opportunity he granted me. Also, I am thankful to the members of the virology lab: Dr. Yuanmei Ma, Erin Di Caprio, Dr. Yongwei Wei, Fangfei Lou, Elbashir Araud and Yu Zhang for creating a friendly atmosphere in the lab. I am also thankful to Drs. Gabe Sanglay and Lizanel Feliciano for their willingness to always assist me. Additionally, I want to express my gratitude to my food packaging lab mates: Ruey Bruce, Kyra Jones, Breanna Wingate and Dr. Jaesung Lee for all their help and support.

Finally, I want to thank my family for their love, sacrifices as well as their prayers. I am especially grateful to my wife Akua Frempomah Amankwaah for her love, support and sacrifices. May the good Lord richly bless you Akua!
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CHAPTER 1

INTRODUCTION

1.1 Rationale and Significance

Microbial contamination affecting the safety and quality of foods can occur at any point during production, processing, transport, retail trade, domestic storage, or meal preparation. Foodborne illnesses associated with pathogenic micro-organisms present a major public health concern throughout the world. In the United States, the CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, of which 128,000 are hospitalized, and 3,000 die of foodborne diseases (CDC 2011a).

Noroviruses (formerly named Norwalk-like viruses (NVLs) or small round structured viruses (SRSVs)) are commonly associated with most foodborne viral outbreaks. The Center for Disease Control and Prevention (CDC) estimates that more than 21 million cases of acute gastroenteritis occur each year due to Noroviruses infection, and 58% of all foodborne disease outbreaks can be attributed to Noroviruses (CDC 2011a). This makes Noroviruses the leading cause of gastroenteritis in the United States. Increased incidence of foodborne illnesses from Listeria monocytogenes, and Escherichia coli O157:H7 have also spurred greater interest in finding innovative means to control microbial growth in foods while maintaining quality, freshness, and safety (Jin and Zhang 2008).
Antimicrobial food packaging can serve as an innovative technique to control infectious agents in foods. As an additional hurdle to other inactivation techniques, antimicrobial packaging would not only reduce the risk of pathogen contamination, it would also help to extend the quality and shelf life of minimally processed foods. Antimicrobial food packaging has several advantages over the common practice of directly adding antimicrobial agents to foods (Vermeiren and others 2002). Limitations of the direct application include rapid loss of activity due to interactions with food components, and dilution caused by the food mass. Since micro-organisms can grow on the surface of solid and semi-solid foods, applying and maintaining an effective concentration of a selected preservative on the food is important (Ouattara and other, 2000; Cagri and others 2004). Also, direct addition, because of the depletion or neutralization of the antimicrobial agents, will not prevent the recovery of injured cells or re-growth of surviving cells (Zhang and others 2004).

Incorporating active agents in packaging films is one technique that will help to minimize the problems associated with direct addition of antimicrobials to foods. This can provide stability to the agents. The antimicrobial packaging material can be fabricated by either incorporating active agents in the polymer matrix or they can be coated onto the surface of the polymer. Furthermore, slow migration of the antimicrobial agents from the packaging material to the surface of the food would help maintain a steady concentration of the active agents throughout the shelf life of the product (Gennadios and others 1997).
Antimicrobial packaging has worked well in past applications as an intervention technique to minimize bacterial growth in selected ready-to-eat foods, as have been reported by several researchers (Zhang and others 2004; Shin and others 2010; Suppakul and others 2003). This same approach is also feasible for ready-to-eat (RTE) foods that are susceptible to contamination with foodborne viruses such as human norovirus. Since the food package serves as the last barrier before consumption, especially for minimally processed and RTE foods, bestowing an antimicrobial ability to the package can help to curtail outbreaks associated with pathogenic bacterial and viral infectious agents.

Current consumer demands are for foods that are free from or contain low levels of synthetic preservatives. As a result, interest has also risen in the use of naturally-derived antimicrobial agents in active packages. Additionally, the quest for antimicrobial agents with a broad spectrum of activity is increasing. Naturally-derived plant extracts from green tea and grape seeds have been shown to have a broad spectrum of activities such as antibacterial, antifungal and antiviral (Cushnie and Lamb 2005). Moreover, because these plants derived extracts contain multiple active agents, it is possible that the development of antimicrobial resistance is less likely to occur (Palaniappan and Holley 2010).

In food packaging, edible films and coatings have received considerable attention in recent years. These films could contribute to the reduction of environmental pollution because they are biodegradable, plus they can serve as carrier for various additives.
1.2 Specific Objectives

The long term goal of this research project is to develop polymeric films with both antiviral/virucidal and bactericidal efficacies. The specific objectives in our quest to achieve the long term goal are as follows:

1. Development of a chitosan-based active film incorporated with green tea and grape seed extracts.

2. Test the antibacterial and virucidal efficacies of the developed films against 3 surrogates: Murine norovirus, *Escherichia coli* K12 and *Listeria innocua*.

3. Test the moisture barrier, and mechanical properties of the developed films.
CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne pathogens and illness

The presence and growth of microorganisms in food may cause spoilage and result in a reduction in quality and quantity (Soliman and Badeea 2002). Foodborne illnesses associated with pathogenic micro-organisms also present a major public health concern throughout the world.

2.1.1 Selected Bacteria Pathogens

*Escherichia coli* O157:H7 can be isolated from the feces of many animals. The organism is gram-negative, enteric bacteria that has been associated with foodborne diseases such as bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Griffin and Tauxe 1991). The majority of *E. coli* O157:H7 outbreaks are associated with bovine products; however, other food products such as leafy greens have been linked to its outbreaks (Feng 1995). Two important constituents of the extracellular membrane matrix of *E. coli* are the curli fimbriae and polysaccharide cellulose. These constituents help in attaching the organism to abiotic surfaces and animal cells such as the epithelium of the intestine (Saldana and others 2009). These components also contribute to cell to cell aggregation and biofilms formation and make the bacteria resistant to chemical and
other physical methods designed to remove them from contaminated surfaces (Ryu and others 2005). These organisms also produce shiga toxins that are responsible for causing diseases. *Listeria monocytogenes* is a ubiquitous microorganism found in the environment and can be isolated from soil, vegetation, and animals. It is gram positive, facultative anaerobe, psychrotrophic, and a facultative intracellular bacterium. *Listeria* causes listeriosis a potentially lethal disease characterized by bacteremia and meningoencephalitis, especially in immune compromised individuals, the elderly, pregnant women and infants but can also cause self-limiting, febrile gastroenteritis in healthy individuals (Schuppler and Loessner 2010). The mechanism of how *Listeria* causes diarrhea has been associated with its direct invasion of the intestinal mucosa epithelial (Ooi and Lorber 2005). *Listeria* has been associated with the consumption of contaminated foods that are minimally processed. These include foods such as soft cheeses, frankfurters and milk (Swaminathan and Gerner-Smidt 2007, Dass and others 2011). *L. monocytogenes* is able to grow at refrigerated temperatures, making them important in outbreaks associated with foods that are store at this temperature. A multistate *Listeria* outbreak involving tainted cantaloupe occurred in 2011 and resulted in 30 deaths and 1 miscarriage out of 146 infected people (CDC 2011b).

Foodborne outbreaks due to *Salmonella* also pose a significant threat to public health. They are rod-shaped, gram-negative, non spore formers belonging to the *Enterobacteriaceae* family. Although the majority of outbreaks in which Salmonella spp. is the etiological agent are linked with ingestion of contaminated foods of animal origin (Greig and Ravel 2009), the association with vegetables has been increasing in the last
few years (Lynch and others 2009). *Salmonella* serovars are prevalent in nature and can be found in the intestinal tract of both wild and domesticated animals (Allerberger and others 2002). Symptoms of infection with these organisms include diarrhea, abdominal pain, and vomiting within 12-72 hours of consuming a contaminated food.

Overall, the increased consumption of minimally processed ready-to-eat foods is the driving force behind the high levels of foodborne illnesses. This is so because ready-to-eat foods can easily become recontaminated or cross contaminated with foodborne pathogens by food handlers and dirty equipment surfaces during processing or packaging.

2.1.2 Foodborne Viruses

Viruses have been implicated in a number of foodborne illnesses and have become a great threat to public health. Viruses are very small micro-organisms, ranging in size from 20 to 300 nm, and comprise of a core of either RNA or DNA with a protein coat. They are intracellular obligate and unlike bacteria cannot grow outside a host. They are therefore unable to multiply or grow in foods but are able to contaminate and survive in food and cause illness. Foodborne viruses can be grouped into three diseased categories: (1) viruses that cause gastroenteritis e.g. astrovirus, rotavirus, adenovirus, human norovirus and sapovirus; (2) hepatitis viruses e.g. hepatitis A and hepatitis E; and (3) those that cause other illnesses such as enteroviruses e.g. coxsackie, polio, echo and aichi viruses (Koopmans and others 2006).

Gastroenteritis viruses cause diarrhea in humans by destroying the mature enterocyte of the intestinal villi (Carter 2005). The destruction of these cells by these viruses can affect the reabsorption of water from the gut, resulting in diarrhea.
Additionally, the surface area available for absorption is reduced because of the retraction of the damaged intestinal villi. Hepatitis viruses attack the hepatocytes of the liver and impair its functions including the breaking down of hemoglobin. Viruses that are important in foodborne outbreaks are the enteric viruses that infect via the gastrointestinal tract. They then propagate in the digestive tract and subsequently attack the cells there or enter other organs (e.g. liver) to cause diseases (Vasickova and others 2005). Among foodborne viruses, the human norovirus is the leading agent responsible for foodborne outbreaks worldwide. Table 2.1 lists the properties of various foodborne viruses.

2.1.2.1 Human Noroviruses

Norovirus belongs to the family Caliciviridae, and it’s a nonenveloped, single-stranded, and positive sense RNA virus. Its size ranges from 27 to 38nm and appears under an electron microscope as small blurry structures (Figure 2.1). Its etiological significance is due to its high resistance to heat and low pH and hence it is able to survive technologies designed to control foodborne bacteria. It also has a low infection dose and only a few virions are required to cause disease. As low as 10 virus particles can cause disease (Koopmans and Duizer 2004). Human norovirus (HuNoV) infection is characterized by symptoms such as vomiting, diarrhea, nausea, cramps, chills, dehydration, and headache with an incubation period of 1-3 days (Koopmans 2008). Transmission can occur through person-to-person contact, or through the ingestion of contaminated food or water. Seafoods, fresh produce, and ready-to-eat foods are examples of high-risk foods associated with HuNoV outbreaks. The virus is transmitted
Table 2.1 Properties of other enteric foodborne viruses (Carter 2005)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus family (Genus)</th>
<th>Foodborne</th>
<th>Size (Genome)</th>
<th>Features</th>
<th>Associated illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aichivirus</td>
<td>Picornaviridae (Kobuvirus)</td>
<td>Yes, Shellfish</td>
<td>28nm (ssRNA)</td>
<td>Knob-like projections, cultivable Vero Cells</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Picornaviridae (hepatovirus)</td>
<td>Yes</td>
<td>28nm (ssRNA)</td>
<td>Little surface detail, Non enveloped, Fhrk-1 cells</td>
<td>Hepatitis, mild in the young</td>
</tr>
<tr>
<td>Hepatitis E</td>
<td>Unclassified</td>
<td>Mainly water</td>
<td>34nm (ssRNA)</td>
<td>Calicivirus-like structure Not Cultivable</td>
<td>Hepatitis, severe in pregnancy</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>Not reported</td>
<td>100nm (dsDNA)</td>
<td>Icoshedral Cultivable Graham 293 cells</td>
<td>Mild diarrhea, shedding may be prolonged, mainly affect children</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Reoviridae</td>
<td>Rare often water</td>
<td>70nm (dsRNA)</td>
<td>Multilayered Segmented genome Cultivable Ma104 cells</td>
<td>Diarrhea-common in the young, incidence increasing with age but increases in the elderly</td>
</tr>
<tr>
<td>Human Sapovirus</td>
<td>Caliciviridae (sapovirus)</td>
<td>Yes (rare), shellfish</td>
<td>34nm (ssRNA)</td>
<td>Cup-like depressions on surface, not cultivable</td>
<td>Explosive projectile vomiting in older children/young adults</td>
</tr>
</tbody>
</table>
via the fecal-oral route but transmission through vomitus is also possible either by means of aerosols or direct contact with infectious vomitus. Outbreaks typically occur in a closed setting such as cruise ships, restaurants, hospitals, prisons, nursing homes, schools and daycare centers as a result of environmental contamination. Although outbreaks occur all year round, in temperate climates it is common in the winter months (Seymour and Appleton 2001).
2.1.2.2 Surrogates for Human Noroviruses

The challenge associated with HuNoV studies is that, in spite of the huge public health impact, the virus cannot be grown in cell culture. Additionally, there are no small animal models; hence the HuNoV pathogenesis is poorly understood. Studies on its persistence, inactivation, and transmission have therefore been limited to cultivable virus surrogates including Murine norovirus (MNV-1), Feline calicivurs, Porcine Sapovirus and Tulane virus (TV). These surrogates are used owing to either their similarity to HuNoV in one or more of the following areas: stability, size, genetical and biochemical features. The MNV-1 infects mice and has been used as a HuNoV surrogate since discovered by Wobus and others (2006). The factors that make MNV-1 a good surrogate are: 1. HuNoV and MNV-1 have similar size, and shape; 2. the two share similar biochemical features, 3. they are genetically related because they both belong to the genus *Norovirus* with similar genome size and gene organization; and 4. they are also acid and heat resistant and can persist in the environment (Cannon and others 2006, Wobus and others 2006). However, there are differences in the virus receptors, pathogenesis and immunity. Murine noroviruses are not enteric (i.e. they don’t cause gastroenteritis), they use sialic acid as a receptor, whereas HuNoV uses the histo-blood group antigens (HBGAs) as receptors for viral binding (Wobus and others 2006, Tan and Jiang 2005).

Feline calicivirus also belongs to the same *Caliciviridae* family and has been used as a surrogate. However, it is not enteric and is unstable in acidic conditions (Cannon and others 2006). Tulane virus and Porcine Sapovirus are relatively new surrogates. Both also
belong to the *Caliciviridae* family and share similar resistance to pH and heat when compared with HuNoV. Porcine Sapovirus causes diarrhea in pigs whilst Tulane virus causes diarrhea in monkeys. Tulane virus also uses the same receptors as the HuNoV (Farkas and others 2010) and could serve as a better surrogate for HuNoV (Li and others 2012b).

### 2.2 Intervention technologies

Due to the high economic costs and health effects associated with these foodborne outbreaks, both thermal and nonthermal food processing methods are used to ensure the safety of the food supply. Thermal treatment (60-100°C) is typically used as a preservation technology for the reduction of microbial contamination of foods (Tiwari and others 2009). Sterilization temperature of 121°C is used for heat resistant spores of *Clostridium botulinum*. However, the high temperatures used can result in undesirable quality changes in many foods including nutrient loss, texture, flavor, and color. Other intervention strategies include the use of antimicrobial chemicals during washing of the products, e.g. fresh produce, with the aim of sanitizing the food products. Unfortunately, consumers are concern with the safety of these chemicals in their foods and therefore demand fresh products with no added chemicals.

Several nonthermal technologies are being used as alternatives to heat to extend product’s shelf life and reduce the levels of pathogenic organisms. The potential of nonthermal technologies to ensure food safety and at the same time meet the demands for the retention of nutrition and quality attributes, has resulted in much interest in the use these preservation techniques for inactivating microorganisms and enzymes in foods.
Examples of nonthermal processes include high hydrostatic pressure, electrolyzed water, irradiation, ozone treatment and antimicrobial packaging.

2.3 Antimicrobial packaging

2.3.1. Antimicrobial Packaging Systems and Types

Antimicrobial food-packaging films can be divided into two main categories: (1) migrating systems that allow the antimicrobial agent to migrate into the food; and 2) non-migrating systems that do not release the antimicrobial substances (Suppakul and others 2003). In the migrating system, a preservative agent is either coated to the surface or incorporated within the matrix of the polymer. Release of this substance is triggered when the food comes in contact with the film. Figure (2.2a-c) shows a schematic representation of the first system in which (A) represents a packaging material with the agent incorporated into the matrix and its migration towards the food product. (B) Represents the same concept but a multilayer material is illustrated where the inner layer contains the agent. The inner layer can be useful in controlling the release of the antimicrobial compound. (C) Represents a coating layer of antimicrobial formulation on the surface of a food-packaging material. Figure 2.2d represents a non migrating system. The efficacy of this system is possible only when the microorganisms get in contact with the surface of the packaging material (Quintavalla and Vicini 2002).

Non migrating/immobilization systems use ionic and covalent immobilization of antimicrobial agents and they capitalize on the presence of certain functional groups on both the polymer and the antimicrobial agent (Appendini and Hotchkiss 2002).
Alternatively, surface modifications have been used to bestow antimicrobial activity to polymeric films by irradiation or other chemical methods (Ozdemir and others 1999; Cohen and others 1995; Shearer and others 2000). For example, Cho and others (2000) were able to synthesize an antimicrobial biopolymer containing a chito-oligosaccharide (COS) side chain.

Figure 2.2. Food Packaging systems and migration phenomenon (Bastarrachea 2011)
These authors introduced the COS to polyvinylacetate (PVA) by cross-linking with N-methylolacrylamide (NMA). Subsequent antibacterial testing revealed that the film inhibited the growth of *Staphylococcus aureus*. It is important to note that both the migrating and non-migrating systems require a direct contact with the food except when volatile antimicrobial agents are used.

Han (2000) grouped packaging systems into two groups based on the mode of migration. These were 1) Package/food system; and 2) Package/headspace/food system. A package/food system is where the package contacts the food without headspace, for example, individually wrapped cheese. Active agents that have been incorporated into the package will therefore migrate by diffusion and partitioning. In package/headspace/food systems, the migration processes include evaporation or equilibrated distribution of substances between the headspace, packaging material, and/or the food.

### 2.3.2 Factors affecting the design of antimicrobial packaging

A number of factors pose challenges to the development and use of antimicrobial food packaging. Inhibitory activity can be lost when the antimicrobial compound is combined with the polymeric materials. This can occur if there is interaction with the polymer molecules or due to the processing heat when the package is being formed. Han (2000) reviewed the factors that should be considered in developing an antimicrobial package. These are explained in the next sections (2.3.2.1 to 2.3.2.5).
2.3.2.1 Processing conditions and the effect on residual antimicrobial activity

An importance parameter to consider during the design of antimicrobial packaging is the chemical and thermal stability of the agent. When thermal processing such as extrusion is used for film formation, the high temperature, shearing forces and pressure can either inactivate the antimicrobial agent or cause its evaporation from the film (Han 2000; Han and Floros 1999). The residual amount of the agent in the polymer after processing is important since this will be the effective amount for microbial inactivation. To minimize the effect of temperature, Han (2000) recommended the use of master batches of the antimicrobial agent in the resin for preparation of packages. He also mentioned the quantification of parameters such as temperature, dwell time, and pressure. Alternatively, binders such as ethyl vinyl acetate (EVA), and polyethylene glycol (PEG) could be used to retain the antimicrobial agents. Microencapsulation of the antimicrobial agents can also be done to minimize their evaporation or deterioration during processing. Additionally, the use of copolymerization or lamination to get a multilayer film can be a viable option. Using a multilayer film could allow a sub-inner layer containing the antimicrobial agent to be developed at a low temperature. This sub-layer could then be laminated or coated onto outer layers which can act to protect the antimicrobial agent or limit its rate of transfer from the multilayer film.

2.3.2.2 Characteristics of antimicrobial agents and packaged foods

The nature of the food substance to which the antimicrobial film is applied can also be a factor that can limit the antimicrobial activity of the film. This could either
affect the release rate, solubility or activity of the antimicrobial agent. The pH and water activity of the food for instance, would not only affect the growth rate of the microbes but can also influence the ionization potential of some active agents such as organic acids and their salts (Han 2000). As an example, films containing benzoic anhydride are more effective in inhibiting molds at low pH values than at high pH (Weng and Hotchkiss 1993). Rico-Pena and Torres (1991) found a lower diffusion rate of sorbic acid and potassium sorbate from HMPC-palmitic acid film with an increase in pH and a decrease in water activity. Also, the water activity of a packaged food could affect the chemical stability and potentially dilute and limit the efficacy of an antimicrobial agent.

The characteristics of the antimicrobial agent such as polarity, hydrophilicity or hydrophobicity, solubility and molecular weight are important considerations for specific applications. These factors could affect the polymer and antimicrobial agent compatibility, and also the rate of diffusion of the antimicrobial agent from the polymer. Non polar LDPE was reported to be more compatible with additives that have high molecular weight and low polarity (Weng and Hotchkiss 1993). When antimicrobial agents bind too tightly to the polymer, this will impede the amount and rate of the release of the agents. Wong and others (1996) investigated the effect of different ionic states of antimicrobial agents (ascorbic acids, and sodium ascorbate) on the diffusion rates in a calcium-alginate film. These authors reported that the diffusion rate was highest for sorbic than sodium sorbate because of interaction with the film matrix.
2.3.2.3 Storage temperature

The main advantage of an antimicrobial packaging compared with the direct addition of bioactive agents to the food is the controlled release of the antimicrobial agent over the course of the shelf life of the food (Gennadios and others 1997). The diffusion rate of the agent should therefore be controlled so that its concentration in the film would be sufficient to remain effective throughout the shelf life of the product (Cooksey 2000). High storage temperature will increase the migration rate of the antimicrobial agent resulting in early loss of its efficacy. The temperature during production, distribution and storage should be predicted in order to determine its effect on the residual antimicrobial activity (Perez-Perez and others 2006).

2.3.2.4 Physical properties of the packaging materials

The addition of various ingredients to antimicrobial packages/films could have a negative or positive influence on the engineering properties of the film. These include parameters such as the mass transfer of gases through the films, tensile, thermal, and morphological properties of the material (Bastarrachea 2011). The magnitude of these properties depends on factors such as the type of film material, the film preparation procedure, and the nature of the antimicrobial agent used. In designing the package material, it is therefore important to investigate how the properties of films are affected after the incorporation of ingredients. These properties play an important role in determining the application of the film and the shelf life of the packaged food product.
Section 2.9 describes the various properties of edible films and how they are affected by different factors including addition of additives such as antimicrobial agents.

2.3.2.5 Regulation

The application and use of edible active packages/films demands that all ingredients, including the biopolymer, solvents, functional and other additives must have U.S Food and Drug Administration (FDA) generally recognized as safe (GRAS) status. Since the films or coatings will come in contact with foods and, in the case of edible films, it is important that they are approved for food contact or as a food additive. Additionally, the manufacturing process for edible films or coatings must be in accordance with the requirements of FDA good manufacturing practices (GMP) (Krochta and De Mulder-Johnston 1997). When allergens such as milk, eggs, peanuts, tree nuts, soy, wheat, shellfish, and fish are part of the ingredients, the film must be labeled in conformance with the Food Allergen Labeling and Consumer Protection Act (Janjarasskul and Krochta 2009).

2.4 Edible films and coatings

Petroleum-based synthetic packaging has been used by the food industry for decades as a barrier to provide protection from environmental factors such as light, oxygen and even micro-organisms. About 40,000,000 tons of synthetic packages (e.g. LDPE, PET, nylon) are used yearly (Srinivasa and Tharanathan 2007). Unfortunately, due to the non biodegradable nature of these synthetic plastics, there are concerns over
the vast amount of environmental waste that is generated annually. This has resulted in an increased interest in the use of biodegradable plastics such as edible films and coatings. Edible films are not to totally replace synthetic plastic but they can help to minimize the negative impact of plastic packaging. Hernandez-izquierdo and Krochta (2008) defined edible film as “a thin layer of edible material formed on a food as a coating or preformed as a film that can be placed between food in multi-component products as separation layers.” Edible films can be used as a food wrap, or formed into pouches to contain foods. The purpose of edible films and coatings include inhibition of migration of moisture, oxygen, carbon dioxide, aromas, and lipids; act as carrier for food additives (e.g. antioxidants, antimicrobials, nutrients, colorant and flavors); and/or improving the mechanical integrity or handling characteristics of the food (Krochta and De Mulder-Johnston 1997; Bourtoom 2009).

2.5. Biopolymers for film formation

To make edible films or coatings, at least a biopolymer that has the ability to form a continuous and cohesive structural matrix is required. Biopolymers for making edible films and coating fall into 3 categories, namely polysaccharides, proteins and lipids. Polysaccharides and proteins together are hydrocolloids and are typically used as additives in the food industry to serve various functions such as stabilizers, gelling agents, thickeners and water binding agents. Films can be made either from individual biopolymers or their composites, which combine various material properties (Akter and others 2012).
2.5.1. Polysaccharide-based films

Polysaccharides are macromolecules formed from repeating units of mono- and disaccharides joined together by glycosidic bonds. They have excellent barrier to oxygen as a result of intermolecular hydrogen bonding in the polymer chains, which results in a highly packed and ordered structure. However, their hydrophilic nature due to the presence of polar hydroxyl groups makes them poor moisture barriers. Various techniques and additives are therefore used to improve the poor moisture barrier properties of polysaccharide films. Examples include the addition of salts, variation of solvent type, pH, modification of the hydroxyl functional groups, heat, lipids, cross linking agents and the use of nanocomposites (De Moura and others 2009).

2.5.1.1 Carrageenens-based films

Carrageenens are polysaccharides that are extracted from seaweed such as red algae. They are sulphated galactans with the polysaccharide chains mainly composed of α-D-galactopyranose and β-D-galactopyranose monomers linked by α-(1 → 3) and β-(1 → 4) bonds. Most of the sugar units have one or two sulfate groups esterified to a hydroxyl group at carbon atoms C-2 or C-6. Carrageenens are extensively used in the food, dairy, and pharmaceutical industries as gelling, emulsifying, and stabilizing agents (Roberts and Quemener 1999). They are sub grouped into 3 major classes (Figure 2.3) depending on the number of sulfate groups or the degree of negative charges they contain. These are (1) kappa(κ)-, (2) lambda (λ) and (3) iota (ι)-carrageenens. Kappa carrageenan has one negative charge obtained from the sulphate group per disaccharide
and has a tendency to form excellent films with the highest tensile strength when compared with λ- and ι-carrageenan films (Park 1996). Iota and λ-carrageenens have 2 and 3 negative charges per disaccharide, respectively.

Figure 2.3. Structures of the disaccharide repeating units of major carrageenan types.
2.5.1.2. Chitosan-based film

Chitosan has been extensively studied for its film forming ability and inherent antimicrobial activity (Wang and others 2013). Chitosan is derived from chitin, the second most abundant polysaccharide after cellulose (Akter and others 2012). The process of synthesizing chitosan involves deacetylation (removal of acetyl groups) of the chitin, which can be obtained from the exoskeleton of insects, crustaceans and fungi. Chitin represents a cheap source of this very important natural polymer because it is a waste by-product from shrimp, lobster, krills and crab processing. The deacetylation process involves treating the chitin with an aqueous 40–45% (w/v) NaOH solution at 90–120°C for 4–5 h (Dash and others 2011). The conditions used determine the final molecular weight and the degree of deacetylation (DD) of chitosan, two important parameters that affect its biological and functional properties. These properties include solubility, viscosity, crystallinity and others expounded on in Table 2.3.

Chitosan is a linear copolymer of β-(1→4) linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose (Figure 2.4). It is a polycationic polymer that has one amino group and two hydroxyl groups in the repeating glycosidic units. The carbohydrate backbone is very similar to cellulose. Chitosan is highly crystalline because of intra and inter molecular hydrogen bonding of the linear chains (Dash and others 2011). As a result, chitosan has limited solubility in water. Therefore, chitosan film forming solutions are obtained by dissolving them in acidic solutions. The presence of amide and hydroxyl groups provides reactive sites on chitosan for various
Table 2.2. Relationship between structural parameters and properties of chitosan (Dash and others 2011)

<table>
<thead>
<tr>
<th>Property</th>
<th>Structural characteristics(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>↑ DD</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>↓ DD</td>
</tr>
<tr>
<td>Biodegradability</td>
<td>↓ DD, ↓ Molecular weight</td>
</tr>
<tr>
<td>Viscosity</td>
<td>↑ DD</td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>↑ DD</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>↑ DD, ↓ Molecular weight</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>↑ DD, ↓ Molecular weight</td>
</tr>
</tbody>
</table>

\(^a\)↑ – Directly proportional to property; ↓ – inversely proportional to property.

DD: Degree of deacetylation

Figure 2.4. Structures of chitin and chitosan.
reactions and this makes it possible to alter the biological and functional properties of this biopolymer (Coma and others 2002, Pillai and others 2009) (Figure 2.5).

2.5.1.3 Cellulose derivative films

Cellulose is the most abundant polysaccharide and it consists of β (1→4) linked D-glucose units. It can be found in the plant cell wall and is responsible for the structural integrity of the cell. Owing to the linear nature of cellulose monomer, it can form tightly packed hydrogen-bonded crystalline microfibers, which are water insoluble (Zugenmaier 2006). However, the water solubility of cellulose can be modified to improve its function and use. Derivatives of cellulose such as methylcellulose (MC), hydropropyl methylcellulose (HPMC) and carboxymethyl cellulose (CMC) are available to make transparent and flexible films (Espinoza-Herrera 2011). Modification of cellulose can be accomplished by treating them with alkali to swell the fibers. Subsequently, bulky side groups such as methyl or carboxyl methyl are introduced by adding methylene chloride.
or chloroacetic acid, respectively. Substitution with these bulky groups improves the water solubility by opening up the polymer chains via the disruption of the hydrogen bonds (Janjarasskul and Krochta 2009). Consequently, the degree of substitution and type of functional group on the substitutes affects the properties of films formed from these derivatives. Espinoza-Herrera (2011) reported the tensile strengths of films made from cellulose derivatives in the order of CMC > HPMC >MC. Carboxymethyl cellulose and MC used by the author was reported to have degrees of substitution of 0.7 and 2.2, respectively. It can be said that the higher the degree of substitution the lower the tensile strength.

2.5.1.4 Starch-based films

Starch is the main storage carbohydrate in most plants and it can be found in foods such as corn, cassava, tapioca, potato, wheat and rice. Starch consists of monomers of glucose units linked together by glycosidic bonds. Both native and modified starches have been used in the food industry in sauces, soups and meat product to modify texture, viscosity, moisture retention and for edible film formation (Thomas and Atwell 1997).

Starches are made up of two main components: (1) amylose (figure 2.6) and (2) amylopectin (figure 2.7). Amylose is the linear portion linked by α (1, 4) glycosidic bonds with excellent film forming ability (Palviainen and others, 2001). Amylopectin linked by α (1, 6) and α (1, 4) bonds forms brittle films because it is highly hydroxylated, which aids extensive crosslinking. Also, because amylopectin is highly branched, it interferes with the ability of the polymer chains orienting closely together (Li and others
Hydrogen bonding in the starch molecule makes them insoluble in cold water, but when heated, disruption of the crystalline structure occurs and this allows interaction with water and increases its solubility (Hoover 2001). The process of heating starch to break down the intermolecular bonds is referred to as gelatinization. Starch gelatinization is therefore an important step that is needed for starch film formation (Carvalho 2008). Poor mechanical and water stability have limited the application of starch-based films. In a study by Reddy and Yang (2010), citric acid improved the tensile strength and decreased water solubility of starch films when used as a crosslinking agent.

Figure 2.6. Structure of Amylose chain
2.5.2 Protein-based films

Protein-based films are attractive because they have better gas barrier and mechanical properties when compared with lipid- or polysaccharide-based films (Bourtoom 2009). This is so because proteins have a number of different monomers (i.e. 20 polar and non polar amino acids) with a range of functional groups that can make up the protein molecule. Hence, protein chains can form several intermolecular bonds and interactions, including hydrogen bonds, van der Waal forces, disulfide bonds, and covalent bonds resulting in a cohesive and stabilized film matrix (Krochta and others 1994; Janjarasskul and Krochta 2010). However, just like their hydrocolloid
polysaccharide counterparts, protein-based films also have poor water resistance. Problems associated with using these biopolymers can be reduced by blending them with other substances such as lipids, which are hydrophobic. Additionally, chemical crosslinking agents such as glyceraldehyde and formaldehyde can be used to improve the films properties. Hernandez-Munoz and other (2004) reported that these crosslinking agents can improve properties such as water barrier, and material toughness. The major step required for forming films from proteins is denaturation. Denaturation is a process in which proteins lose the secondary, tertiary or quaternary structure, which is present in their native state. This process results in the unfolding of the protein chains thereby exposing the various functional groups to aid in film formation during subsequent drying. Protein denaturation can be achieved by heating, solvents or acids.

2.5.2.1 Whey protein films

Whey proteins are by-products of cheese processing and have been used in edible film formation because of their transparency, flavorlessness, flexibility and excellent nutritional and functional properties (Banerjee and Chen 1995; Fang and others 2002; McHugh and others 1994). Whey proteins are a mixture of globular proteins including β-lactoglobulin, α-lactalbumin, bovine serum albumen and immunoglobbins. β-lactoglobulin is the most abundant of the whey globular proteins and it can form films on its own. Krochta (1998) reported of a similarity in vapor and oxygen permeabilities of whey protein isolate (WPI) and β-lactoglobulin films. Heat denaturation of the globular whey proteins exposes the sulfhydryl groups thereby promoting new intermolecular
disulfide and hydrophobic bond formation (Krochta 1998; McHugh and others 1994). Therefore, to form WPI films, heating 10% whey solutions at neutral pH and 90°C for 30min has been recommended by McHugh and others (1994).

2.5.2.2 Corn zein films

Zein proteins are a group of alcohol-soluble compounds found in the endosperm of corn. Zein proteins form films via hydrophobic, hydrogen and limited disulfide bonds between their chains (Krochta 1997, 2002; Lai and Padua 1997). Zein films can be formed by both solvent casting and thermoplastic processing. To form films by solvent casting, corn zein has to be dissolved in an aqueous ethanol solution because it is insoluble in water (Gennadios and others 1993; Luecha and others 2010). Its high in non-polar amino acids including leucine, proline and alanine, hence its water insolubility. Thermoplastic processing of zein can be achieved in two major steps: (1) resin formation, followed by (2) film formation using blown film extrusion. The resin is prepared by first dissolving them in alcohol solution, together with a plasticizer such as oleic acid. The solution is then precipitated by mixing with cold water, drying and film preparation (Wang and Padua 2003).

2.5.2.3 Soy Protein Films

Soy protein isolate (SPI) films have low aroma and oxygen permeabilities, which make them very useful for oxygen sensitive products or for preserving flavors (Gennadios and others 1993; Ghorpade and others 1995; Guerrero and others 2011). Soy
protein films are smoother, clearer and more flexible when compared to films from other biopolymers (Guilbert and others 1995). Soy proteins can also form films by a two-step process that involves heat denaturation followed by dehydration. During the drying step, the unfolded proteins from the denaturation step link through intermolecular interactions, such as disulfide and hydrophobic interactions (Gennadios and Weller 1991; Cho and Rhee 2004). The presence of polar and non-polar side chains of soy proteins aid the formation of strong intra- and inter-molecular interactions, such as hydrogen bonding, dipole–dipole, charge–charge, and hydrophobic interactions (Wittaya 2012).

2.5.3 Lipid-based films

Lipids-based films are good barriers to moisture and are used to prevent moisture migration in foods. Lipid-based hydrophobic film formers include waxes, fatty acids and alcohols, cocoa-based compounds and their derivatives, and acetylated glycerides (Debeaufort and Voilley 2009). Examples of applications for lipid-based coatings or films on food include coatings on fresh fruits and vegetables to prevent desiccation, prevention of moisture migration in pizza and dough products, and prevention of flavor loss in cereal-based products (Debeaufort and Voilley 2009).

2.5.4 Composite films

Composite films are made by combining different polymers to obtain new materials that have desirable functional properties. The challenges encountered in the use of edible films include its high water vapor permeability and poor mechanical properties. Composite edible films and coatings therefore take advantage of the different beneficial
functional properties of each category of the biopolymer film formers. Hydrocolloid-based films, as discussed previously, can have good mechanical properties but are poor barriers to moisture and gases. Lipid-based films on the other hand have good barrier properties but inferior mechanical properties compared to the hydrocolloids. By combining polysaccharide, protein and/or lipid films, characteristics such as permeability and mechanical properties can consequently be improved. Thus, in an edible composite film, the hydrocolloid fraction plays a more structural role in the matrix as they have the ability to interact and form a continuous network, whereas the lipid fractions serve as the moisture barrier component. Alternatively, composites films can be made by combining a polymer with a non-polymer. Also, biopolymers can also be combined with other synthetic polymers.

Composite film formation can either be achieved by: (1) an emulsion technique, where an emulsion of lipid and hydrocolloid are blended together with the help of emulsifiers/surfactants or (2) using a multilayer system in which the lipid layer is deposited on a preformed hydrocolloid film (Wu and others 2002; Krochta and De Mulder-Johnston 1997). For composite films, the effectiveness of the various components to improve the functional properties does not only depend on the characteristics of the component but more importantly on the interactions between the components in the matrix (Gontard and others 1994). The compatibility of the individual parts in the blend is therefore very important. The use of surfactants, which are surface active agents and heating to melt the lipids, can both help to improve the compatibility of the blend. The
main advantage of a composite film is the improvement in both mechanical and barrier properties.

Even though composite films have several advantages over films made from individual components, there are still ongoing challenges with the barrier and mechanical properties because of the hydrocolloid fractions which typically are present in greater proportions (McHugh and others 2010). Composite films are therefore now been designed with the addition of nanoparticles, nanofibers and nanoemulsions for additional benefits. The nanoemulsions, because of their extremely small oil droplet size would prevent coalescence and hence improve the blend compatibility. In one study, Bilbao-Sainz and others (2010) fabricated a composite film from either microcrystalline cellulose (MCC) or lipid-coated MCC nanoparticles (LC-MCC) and HPMC. The authors, using these nanoparticles either alone or in combination with the hydrophobic material were able to form HPMC films that had reduced water permeability and an increased tensile strength. Specifically, the HPMC/MCC and HPMC/LC-MCC composites films showed up to 53% and 48% increase in tensile strength, respectively, in comparison with unfilled HPMC films. Also the addition of the unmodified MCC nanoparticles reduced the moisture permeability up to 40% and use of LC-MCC increased barrier property to 50%.

2.6 Additives to improve the functional properties of edible films and coatings

To improve the workability of edible films and coatings, various additives including plasticizers and emulsifiers are added. Also, edible films and coatings can serve as carriers for various functional groups including antioxidants, antimicrobials, nutrients
Hydrocolloids, lipids or their composites can encapsulate these functional additives and protect them from thermal degradation and interaction with food components. Sections 2.6.1 to 2.6.5 give a detailed discussion on selective additives used in the formation of edible films.

2.6.1. Plasticizers

Plasticizers are low molecular weight compounds that are added to edible films or coating solutions to improve the flexibility and mechanical properties of the dried films (Embuscado and Huber 2009). The problem with edible films is that they can be brittle and difficult to handle because of intermolecular interactions between and within polymer chains. Plasticizers can be used to reduce this brittle nature that is responsible for chipping and cracking of these films and thus make them flexible and more elastic. Plasticizers can also be used to improve the processability of polymers by lowering the glass transition temperature or $T_g$ (Vieira and others 2011). The $T_g$ is that critical temperature of a polymer at which the material changes from glassy to a rubbery state. At the glassy state the material is hard and brittle compared to the rubbery state, where the material will be elastic and flexible. Lowering the $T_g$ of a polymer makes it possible to use a lower temperature to thermally form films. Therefore, it is possible to use a thermoplastic processing method such as extrusion to make edible films without thermal degradation. Plasticizers help reduce the intermolecular forces thereby increasing the polymer chain’s mobility. Several theories exist to explain the mechanism of how plasticizers improve film’s flexibility. These include the lubricity, gel, and free volume
theories (Han 2005; Daniels 2009). The lubricity theory states that the plasticizers act as an internal lubricant, and reduces the intermolecular force between the chains of the polymer. Plasticizers therefore weaken the polymer-polymer interactions by intermingling and shielding one polymer chain from another. The gel theory postulates that plasticizers help to reduce the internal forces holding the three-dimensional network by attaching themselves loosely to the polymer chains. Such attachment therefore reduces the number of polymer-polymer interactions. Lastly, the free volume theory states that plasticizers lower the glass transition temperature by increasing the free volume.

Other effects of plasticizer addition include a reduction of viscosity and the degree of crystallinity within the polymer. Reduction in viscosity by plasticizers can be an important processing aid to help with the incorporation of fillers, lowering of the power demands and an increased in plastic flow (Veira and others 2011). The addition of plasticizers can however, increase gas and moisture transmission through the film because it causes a reduction in crystallinity. Additionally, the poor barrier properties are due to the inherent hydrophilicity of commonly used plasticizers. Examples of plasticizers are water, glycerol, sorbitol, and polyethylene glycol. To improve the barrier properties, hydrophobic (e.g. beewax, lauric acid, stearic acid, oleic acid, citrate esters) and amphipillic (e.g. glycol monostearate, acetic acid esters of monoglyceride) plasticizers are used (Andreuccetti and others 2009).

An ideal plasticizer should be able to meet some of the following requirements: (1) they should be non volatile, (2) have low molecular weight and (3) be miscible or compatible with the polymer. Volatile plasticizers could be lost during drying or thermal
processing or even during storage and render the films brittle. Plasticizers are therefore required to have low vapor pressure and low diffusion rate (Wilson 1995). Also, a lower molecular size would allow the plasticizer to align between the polymer chains without any steric hindrance. Miscibility is also important to avoid phase separation of the plasticizer, which is referred to as blooming or bleeding. Blooming is where the plasticizers migrate to the film surface as exudates and it can occur when the levels of added plasticizer exceeds its limit in the polymer matrix.

2.6.2. Antioxidant agents

Antioxidants can be added to edible films to protect oxygen sensitive foods by reducing the deteriorative action of oxidation. Moradi and others (2011) found that the antioxidant activity of chitosan film increased when incorporated with essential oil and grape seed extract. Also, a protein-based film from fish skin gelatin was successfully blended with citrus essential oil. This was shown to improve its 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity (Tongnuanchan and others 2012). Martins and others (2012) also incorporated α-tocopherol into chitosan films. Results showed that the control film without the antioxidant had a radical scavenging activity of 10.6% while chitosan with 0.1 and 0.2% α-tocopherol had 97.42 and 97.71% scavenging activities, respectively. Thus, when chitosan was incorporated with α-tocopherol, its antioxidant activity increased. Green tea extract also had a similar effect when incorporated into a chitosan film (Siripatrawan and Harte 2010; Siripatrawan and Noipha 2012).
2.6.3 Emulsifiers/Surfactants

Emulsifiers or surfactants have also been incorporated into edible films and coatings. Some common ones include lecithin, polysorbates, sodium dodecyl sulfate, glycerol monostearates and acetylated monoglycerides. These compounds are surface active agents with both polar and non polar functional groups. In coating applications, they can help the uniformity of the coating and adhesion (Krochta 2002). Emulsifiers are important additives in composite films, especially when lipids are blended with hydrocolloid. These agents help to improve miscibility. Emulsifiers also aid the incorporation of various additives including antioxidant and antimicrobial agents into edible films and coatings.

2.6.4. Colorants, Nutrients, Nutraceuticals, and Probiotics

An important advantage of edible films is the fact that they can be ingested together with the food. As a result, edible films can be used as carriers for vitamins, minerals, probiotics and nutraceuticals with potential health benefits. Protein-based films can also be a source of protein when ingested with the food. Moreover, the sensory properties of foods can be enhanced by the addition of flavoring agent to the packaging material (Martin-Belloso and others 2009).

As an example, a natural colorant was added to films to prevent photo-oxidation of polyunsaturated fatty acids (PUFA) in salmon oil (Akhtar and others 2010). In other examples, film forming hydrocolloids were used as materials for the encapsulation of flavors and aromas (Pegg and Shahidi 2007; Fabra and others 2009). This is because the hydrophilic character of hydrocolloid-based films makes them excellent barriers to non
polar aroma compounds. In yet another example, Tapia and others (2007) incorporated bifidobacteria into alginate and gellan films to obtain a functional probiotic coating that was used to coat fresh-cut fruits.

2.6.5. Antimicrobial agents

Antimicrobial agents can also be added to films to control spoilage and pathogenic microbial growth in foods. With the increasing consumer demand for minimal use of chemical additives, attention has shifted to the use of naturally-derived antimicrobial agents to control foodborne pathogens. Additionally, the quest for antimicrobial agents with broad spectrum efficacies has increased. The advantage of using a natural based antimicrobial is its safety and broad range of activity. For instance, plant extracts are known to have a broad spectrum of antimicrobial activities because they may contain different constituents of active compounds. Plant essential oils and extracts that are used as antioxidants agents in edible films can also function as antimicrobial agents (Siripatrawan and Harte 2010; Siripatrawan and Noipha 2012; Martins and others 2012). Antimicrobial agents such as organic acids (e.g. citric, acetic and propionic acids), enzymes (e.g. lysozyme), bacteriosins (e.g nisin), fungicides (e.g. benomyl and imazalil), nitrites and lactoferin have all been used in edible films and coatings. Table 2.4 gives some examples of commercial applications of antimicrobials in food packaging.
Table 2.3. Some examples of commercial application of antimicrobials in food packaging

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Trade Name (s)</th>
<th>Producer</th>
<th>Packaging Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver zeolite</td>
<td>Aglon</td>
<td>Aglon Technologies</td>
<td>Paper, milk containers</td>
</tr>
<tr>
<td></td>
<td>Novarone</td>
<td>Toagosei Co</td>
<td>Bags, coatings, labels</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Microban</td>
<td>Microban</td>
<td>Deli-wrap, reheatable containers</td>
</tr>
<tr>
<td>Allyisothiocyanate</td>
<td>WasaOuro</td>
<td>Lintec Corp.</td>
<td>Labels, sheets</td>
</tr>
<tr>
<td>Chlorine dioxide</td>
<td>Microsphere</td>
<td>Dry Company Bernand Tech Inc.</td>
<td>Sachets</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>Freshpax</td>
<td>Multisorb Tech</td>
<td>Sachets</td>
</tr>
<tr>
<td></td>
<td>Verifrais</td>
<td>Sarl Codimer</td>
<td>Sachets</td>
</tr>
<tr>
<td>Ethanol vapor</td>
<td>Oitech</td>
<td>Nippon Kayaku</td>
<td>Sachets</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Bioka</td>
<td>Bioka Ltd</td>
<td>Sachets</td>
</tr>
</tbody>
</table>

Source: Han (2000)

Apple-based edible films containing plant antimicrobials were evaluated for their activity against *Salmonella enterica*, *E. coli* O157:H7 and *Listeria monocytogenes* on meat and poultry products (Ravishankar and other 2009). Surface inoculated products were wrapped with edible films containing 3 concentrations of cinnamaldehyde or carvacrol at 0.5%, 1.5%, and 3% and incubated at either 23 or 4°C for 72 h. At 23°C on chicken breasts, films with 3% antimicrobials showed reductions of 4.3 to 6.8 log$_{10}$ CFU/g of both *S. enterica* and *E. coli* O157:H7. Films with 1.5% and 0.5% antimicrobials showed 2.4 to 4.3 and 1.6 to 2.8 log$_{10}$ CFU/g reductions, respectively. At
4°C, films with 3%, 1.5%, and 0.5% carvacrol reduced the bacterial populations by about 3, 1.6 to 3, and 0.8 to 1 log_{10} CFU/g, respectively. Films with 3% and 1.5% cinnamaldehyde induced log reductions of 1.2 to 2.8 and 1.2 to 1.3 CFU/g, respectively.

In another study, Min and others (2010) tested the effectiveness of gelatin films containing commercial antimicrobial agents. Nisaplin at 0.5% and Guardian at 1% inhibited L. monocytogenes on bologna by 4 and 3 log_{10} CFU/g reductions, respectively at 4°C for 56 days. Also, Raybaudi-Massilia and others (2008) reported that cinnamon, clove and lemongrass oils at 0.7% or their active compounds of cinnamaldehyde, eugenol and citral at 0.5% incorporated into alginate films, reduced E. coli O157:H7 population by at least 4 log_{10} CFU/g on fresh cut apples.

2.7 Plant extracts: Green tea and grape seed extracts

Plant extracts are important sources of antimicrobial and antioxidant agents (Perumalla and Hettiarachyhty 2011). Extracts from oregano, thyme, mustard, cinnamon, onion, garlic and clove have been used to control micro-organisms in foods. The major chemicals responsible for the activity of plant extracts include polyphenols, quinones, flavanoids, alkaloids, and lectins (Cowan 1999). When used with other preservation technologies such as low temperature and low pH in a hurdle system or in combination with other preservatives, plant extract are known to have synergistic effect on foodborne pathogens (Gadang and others 2008; Over and others 2009; Beuchat and others 1994). Sections 2.7.1 to 2.7.3 describe the use of two important plant extracts including grape seed and green tea extracts in food preservation.
2.7.1 Green tea and grape seed Extracts

Green tea and grape are popular beverages with diverse health benefits. These benefits are due to their antioxidant, antimicrobial and anti-inflammatory properties (Xia and others 2010). The tea beverage which is widely consumed is obtained from the tea plant, *Camellia sinensis* L. They can be grouped into three different types: non-fermented-green tea, fermented-black tea and the semi fermented-oolong tea. This classification is based on the degree of fermentation during the manufacturing process (Wei and others 2009). Grape seed (*Vitis vinifera*) extract is a by-product from grape juice and wine processing. It is a polyphenolic rich extract that is obtained from the seeds by solvents extraction, which is further dried and purified (Lau and King 2003). Both grape seed and green tea extracts are sold commercially as dietary supplements with standardized polyphenolic contents. They both have Generally Recognized as Safe (GRAS) status approval by the Food and Drug Administration (FDA).

2.7.2 Chemical composition of green tea and grape seed extracts

Catechins are the major active polyphenolic compounds that are found in green tea and grape seed extracts. These catechins can combine with gallic acid to form gallate esters (Negro and others 2003; Weber and others 2007). They can also exist in monomeric phenolic compounds, such as catechin itself, epicatechin and epicatechin-3-O-gallate, or in dimeric, trimeric and tetrameric procyanidin forms (Figures 2.8 and 2.9). Grape seed extracts are rich in oligomeric proanthocyanidins (OPC), which are the dimeric, trimeric and tetrameric procyanidin forms. Green tea extract, on the other hand is rich in monomeric catechins, epicatechin and epicatechin-3-gallate. Six primary
catechins in green tea are (-) epicatechin, (-) epicatechin gallate, (-) epigallocatechin, (+) gallocatechin (GC), (+) catechin and (-) epigallocatechin gallate (Kajiya and others, 2004). (-) Epigallocatechin gallate is the most abundant catechin found in tea extract accounting for about 65% (Zaveri 2006).

Figure 2.8. Sample structure of OPC (trimer of epigallocatechin) found in grape seed extracts
2.7.3 *Mechanism of antimicrobial activity of green tea and grape seeds extracts*

The polyphenols in green tea and grape seed extracts act on the outer cell membrane or cytoplasmic membrane of a bacterium as the major point of interaction. According to Perumalla and Hettiarachyhty (2011), the hydroxyl groups and conjugated
double bonds in the reactive groups of these extracts are involved in binding to the cell wall components to bring about inhibition. The bacterial cell is composed of a phospholipid bilayer and proteins, which can interact with the hydroxyl and conjugate double bonds presents in the phenol groups of these extracts. The damage to the membrane that results from this interaction can lead to the death of the bacterium either by; (1) physical disruption of the membrane; (2) dissipation of the proton motive force (PMF) and/or (3) inhibition of membrane-associated enzyme activity (Shimamura and others 2007; Juven and others 1994). Among the catechins, EGCG and ECG are the most potent due to the presence of the galloyl moiety (Shimamura and others 2007; Cox and others 2001). Also, the presence of gallic acid esters increases the affinity of catechins for the lipid bilayers (Hashimoto and others 1999). Another damaging effect to the bacterial cell could be alteration of the cell morphology by influencing the osmotic pressure of the cell. This will result in the disruption of the cytoplasmic membrane and leakage of cell constituents (Davidson and Naidu 2000). Table 2.5 gives examples of various application of green tea and gape seed extract in food preservation.
Table 2.4. Summary of research findings involving grape seed and green tea extracts (Modified from Perumalla and Hettiarachyhty 2011)

<table>
<thead>
<tr>
<th>Model system</th>
<th>Multiple hurdle approach</th>
<th>Summary of findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI broth culture</td>
<td>Tartaric acid (37.5 mM) + GTE (20 or 40 mg/mL) or GSE (20 or 40 mg/mL)</td>
<td>GTE alone (20 or 40 mg/mL) or in combination with tartaric acid (37.5 mM) reduced Salmonella, Listeria and <em>E. coli</em> by at least 3.5 log CFU/mL.</td>
</tr>
<tr>
<td>Tryptic soy broth with 0.6% yeast extract (TSBYE)</td>
<td>GSE (1%) alone or in combination with nisin (6400 IU) at 37°C</td>
<td>No detectable levels of <em>L. monocytogenes</em> observed by 12 h of incubation. Minimum detection limit was 10 CFU/mL.</td>
</tr>
<tr>
<td>Turkey frankfurters</td>
<td>GSE (1%) alone or in combination with nisin (6400 IU) at 37°C</td>
<td>No detectable levels of <em>L. monocytogenes</em> observed by 28 days of storage at 4 °C and 10 °C. Minimum detection limit was 100 CFU/g.</td>
</tr>
<tr>
<td>Soy protein isolate film</td>
<td>GSE (1% w/w), nisin (10,000 IU/g), and EDTA (0.16% w/w)</td>
<td><em>L. monocytogenes</em> reduced by 2.9 logs. <em>E. coli</em> O157:H7 and <em>S. typhimurium</em> were reduced by 1.8 and 0.6 log CFU/mL, respectively</td>
</tr>
<tr>
<td>Soy protein isolate coated on turkey frankfurters</td>
<td>Nisin (10,000 IU) + GSE (1%) or GTE (1%)</td>
<td>Samples containing nisin combined with either GSE/GTE reduced <em>Listeria</em> population (7.1 CFU/g) by &gt; 2 log CFU/g after 28 days at 4 and 10 °C</td>
</tr>
</tbody>
</table>

(CONTINUED)
<table>
<thead>
<tr>
<th>Material</th>
<th>Coating/Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein isolates coated on turkey franks</td>
<td>GSE (0.5%), nisin (6000 IU/g), EDTA (1.6 mg/mL), and malic acid (1%)</td>
<td>A combination of nisin, GSE, and malic acid reduced <em>L. monocytogenes</em> (5.5 log CFU/g) to 2.3 logs and <em>S. typhimurium</em> (6.0 log CFU/g) to 1 log CFU/g in the samples with nisin, malic acid, GSE, and EDTA after 28 days at 4 °C.</td>
</tr>
<tr>
<td>Spinach</td>
<td>Electrostatic spray of malic acid (2%), GSE (2%), and tartaric acid (2%)</td>
<td>Malic acid and GSE provided 2.6 and 3.3 log CFU/g reductions of Listeria and Salmonella on the days 7 and 14 respectively.</td>
</tr>
<tr>
<td>Probiotics</td>
<td>Green tea extract (TEAVIGO™) and Lactobacillus spp. or Bifidobacterium in RCM (Re-inforced Clostridial Broth)</td>
<td>Synergistic effect (reduction) of probiotics (4–5 log CFU/mL) and GTE (400 μg/mL) against <em>Staphylococcus aureus</em> (2–15 fold) and <em>Streptococcus pyogenes</em> (3–30 fold).</td>
</tr>
<tr>
<td>Edible Gelidium corneum-gelatin (GCG) films for packing for pork loins</td>
<td>Green tea extract and packing</td>
<td>Pork chops packed with GCG film containing GTE (2.80%) decreased <em>E. coli</em> O157:H7 (0.69 to 1.11 log CFU/g) and <em>L. monocytogenes</em> populations (1.05 to 1.14 CFU/g) respectively when compared to control after 4 days of storage.</td>
</tr>
</tbody>
</table>
TABLE 2.4: CONTINUED

| Beef patties | Sulphite (100 mg/kg) and GSE or GTE (300 mg/kg) | Combination of low sulphite and GTE-0.6 and 1.7 log CFU/g reductions in total viable counts and total coliform counts respectively on 9th day of storage. Combination of low sulphite and GSE- 0.8 and 2.1 log CFU/g reductions in total viable counts and total coliform counts respectively on 9th day of storage. |

2.8 Methods and Mechanism of film formation

The formation of edible films and coatings is dependent on cohesion forces such as covalent (e.g., disulfide bonds); ionic and hydrogen bonds between the polymer chains (Janjarasskul and Krochta 2010). Edible films can therefore be obtained by two main techniques: solution casting/wet method and thermoplastic/dry method. These two methods are discussed in details below:

2.8.1 Solvent casting/wet method

In the wet method also known as solvent casting, a dispersion or emulsion of the biopolymer is prepared in the presence of a solvent. The resultant solution is then dried to evaporate the solvent followed by peeling of the dried film sample. Solvents such as water and ethanol are commonly used to dissolve the ingredients. During the drying process, a continuous stable film structure is formed as a result of chemical and physical
interactions between the molecules (Cagri and others 2003), which can be peeled as a
free standing film. Additives such as plasticizers, surfactants, and/or cross-linking agents
may be added to improve the properties of the resultant film.

Chitosan, starch, whey proteins, caseinates or hydropropyl methylcellulose have
been successfully used to form films by this technique (De Moura and others 2009; Caner
and others 1998). For some proteins such as whey, casein, wheat gluten and soy protein,
the solution has to be heated to aid protein denaturation, which is required for film
formation. Denaturation of protein exposes the functional groups by unfolding the
polymeric structures and this aid intermolecular and intra molecular interaction upon
cooling. In solvent casting, a batch or continuous method can be used (Du and others,
2008). The method used can therefore affect the physico-chemical properties of the final
film. Du and others (2008) reported higher water vapor and oxygen permeabilities when
films formed by a batch method were compared to continuous casting. The authors
concluded that these findings were due to the higher temperature used in the continuous
casting method, which reduced the interstitial spaces thereby making the film thinner and
denser as a result of evaporation.

2.8.2. Thermal Processing method

A dry process also referred to as thermoplastic or thermal processing method can
also be used to make edible films. This is because polysaccharides and proteins can
exhibit thermoplastic behavior at low moisture content (Hernandez-izquierdo and
Krochta 2008). Although protein-based films are commonly used in thermoplastic
processing, the use of extrusion for polysaccharides such as starch (Li and others 2011;
Pushpadass and others 2009), and starch/chitosan blend (Pelissari and others 2009; 2011) have been reported. A thermoplastic material softens and melts when heated to a point below its temperature of decomposition.

An advantage of using the thermoplastic method is the potential for large-scale production of edible films. Additionally, for protein-based films the temperature used during the thermal process can help promote a more extensive protein denaturation and higher cross-linking (Hernandez-izquierdo and Krochta 2008). The disadvantage however, is that the high temperature used can result in polymer degradation. To prevent polymer degradation, plasticizers are added to reduce the glass transition temperature. Reducing the glass transition temperature makes it possible to use a lower processing temperature thus minimizing thermal degradation (Hernandez-izquierdo and Krochta 2008). To form films by this method, typically a polymer-plasticizer blend is first prepared in a mixing chamber before thermoplastic processing.

Thermoplastic processes commonly used in edible film production include compression molding and extrusion. Compression molding uses both high temperature and high pressure. The high temperature and pressure turns the polymer-plasticizer blend into a viscoelastic melt. Upon cooling, the films are formed through interaction between the chains. For proteins, these interactions include hydrogen, ionic, hydrophobic, and covalent interactions. Polysaccharides form films mainly through hydrogen bonding via the hydroxyl groups of the polymer chains. Ture and others (2012) produced a compression molded antimicrobial wheat gluten film in a two-step process including; (1) pre-mixing followed by (2) compression molding. During the premixing step the
antimicrobial agent was dissolved in a water/glycerol mixture and stirred for 10 mins. Wheat gluten was then added and mixed under ambient conditions for 5 min at 150 rpm. The prepared dough consisting of gluten, glycerol, water and the antimicrobial agent was subsequently molded by compression at 130°C using 400 kN pressure for 5 min in a Fontijne Press to obtain the films.

Extrusion is sometimes used in combination with compression molding, with the later acting as a precursor to extrusion or sometimes used after extrusion to reduce film thickness. In extrusion, the polymer material is continuously added through a hopper. It then travels through the barrel of an extruder and is finally pushed through a die by a screw. While going through the barrel, a viscoelastic biopolymer melt is produced and it is subsequently forced through the die to form a sheet or film depending on the thickness. Important factors that can affect the extruded film properties include the ingredient conditions, screw speed, and barrel temperature (Li and others 2011b). For protein-based films, a high barrel temperature aids denaturation. This exposes the functional groups, and enhances crosslinking. Such crosslinking helps to improve properties of the film such as tensile strength, flexibility or toughness (Hernandez-izquierdo and Krochta 2008). Pelissari and others (2011) studied the influence of different extrusion temperatures (120, 130 and 140°C) and screw speeds (25, 35 and 35 rpm) on the properties of an active film developed from starch-chitosan blend and oregano essential oil. Increasing the screw speed increased the water vapor permeability and decreased the tensile strength and elongation at break of the chitosan-starch film. Also, low die temperatures (120°C) resulted in increased tensile strength, elongation at break, Young’s modulus and water
vapor permeability of the films. The processing conditions of 140°C and 25 rpm resulted in the lowest WVP. The higher heat facilitated gelatinization of the starch. This exposes the hydroxyl group which interacted with chitosan thereby decreasing its availability, hence the decreased WVP. Films produced at 120°C and 25 rpm had a modulus of 77.40 MPa, while those produced using the same screw speed but a die temperature of 140°C reduced the value to 66.40 MPa. Under 120°C and 25 rpm extrusion conditions, the elongation was 41.49% while at 140°C and 45 rpm, this value reduced to 16.55%.

Pushpadass and others (2009) extruded a native corn starch, plasticized with water, glycerol and stearic acid. During the extrusion, the granular starch was transformed into a thermoplastic matrix with the aid of plasticizers.

2.9. Properties of Edible films

The application of edible films is affected by its mechanical, barrier and thermal properties. Factors affecting the properties of the film have been outlined by Janjarasskul and Krochta (2010). These factors are: (1) the structure and chemistry of the biopolymer, to include molecular weight, branching, and polarity; (2) film forming methods e.g. solvent casting versus thermal processing; (3) nature of the additives added during the film formation such (example type and concentration of plasticizers); and (4) film forming parameters such as temperature, pressure, solvent type, dilution, application and drying techniques.
2.9.1 Mechanical properties

The mechanical property of an edible film is important because it determines how the film will behave during handling and storage. It is a critical parameter that determines the durability of the films and hence its ability to enhance the mechanical integrity of foods (Ghanbarzadeh and Almasi 2011). For food packaging applications, films must be durable, stress resistant, flexible and elastic. A tensile or tension test is therefore commonly used to determine the mechanical properties of a material. This is a simple test where a sample of the material is pulled in opposite direction and the applied force measured. From the tensile testing, a stress-strain curve can be obtained (Figure 2.10) and it provides valuable information concerning the flexibility, toughness and elongation of the film (Hernandez-izquierdo and Krochta 2008). Tensile strength, percentage elongation and elastic modulus derived from the stress-strain curve thus describes the mechanical resistance of the films. Tensile strength (TS) is the maximum stress a film can withstand, and it is determined by dividing the maximum load on the film before failure ($\sigma_Y$) by the initial cross sectional area (S). The percent elongation at break, $E$ which measures the film’s extensibility ($\varepsilon_B$), can be expressed as the percentage change in the original length of the film before breaking (Skurtys and others 2010). The elastic modulus $Y$, measures the film’s stiffness and it is determined from the slope of the stress-strain curve created during the tensile testing.

The applications of edible films are limited because of their poor mechanical properties compared to synthetic films. Several researchers have investigated the mechanical properties of edible films, the factors that affect these properties and the
means to improve their tensile properties. For example, Azeredo and others (2009) used cellulose nanofibers (CNF) to improve the mechanical properties of mango puree film. The film was incorporated with the cellulose nanocomposite and the results obtained showed that the films had a higher tensile strength, and young modulus.

![Stress-strain curve](image)

Figure 2.10. Typical stress-strain curve (Skurtys and others 2010)

The results from that study showed that the modulus increased by more than 100% with a CNF loading of 10 g/100 g, and more than 1500% (a change of modulus from 19.85 to 322.05 MPa) with a loading of 36 g/100g of mango puree. These results were due to the formation of a fibrillar network with the polymer matrix as a result of hydrogen bonding.
of the cellulose fibers. Incorporation of nanoparticles can lead to increased matrix/filler interfacial interaction, which affects the molecular mobility and consequently the mechanical properties of the material (Azeredo and others 2009, Duncan 2011). The high surface area of nanocomposites provides reinforcing effects to the polymeric material (Dalmas and others 2007, Azizi Samir and others 2005). Additionally, the use of crosslinking agents blended with composite films have been used as a means to produce a high strength starch-based films with improved mechanical properties (Ghanbarzadeh and Almasi 2011). In that study, corn starch films with different concentrations (0–20%, w/w) of citric acid as crosslinking agent and carboxymethyl cellulose (CMC) were produced by the solvent casting method. The tensile strength was improved significantly as the percentage of citric acid increased from 0 to 10%. Citric acid with its carboxyl groups acted as a crosslinking agent by forming strong hydrogen bonds with the hydroxyl groups of the starch. The study showed that tensile strength increased from 6.57 MPa for films without CMC to 16.11 MPa for films containing 20% CMC (Ghanbarzadeh and Almasi 2011).

Drying of the film forming solution to obtain edible films is a very important step that affects the mechanical properties of edible films. Fernandez-Pan and others (2010) studied the effect of drying temperatures on polysaccharide-based chitosan films. Higher tensile strength and elongation at break were obtained with slow drying cycles (i.e. lower drying temperature). Mayachiew and Devahastin (2008) also obtained higher tensile strength for chitosan films at lower temperatures of drying. Similarly, Srinivasa and others (2008) reported that films obtained by a faster drying method i.e. infrared drying
had lower tensile strength (i.e. 49.58 MPa) compared to that of ambient-dried films which had tensile strength of 56.78 MPa. Faster drying at high temperature resulted in a less ordered structure with few hydrogen bonds due to a more intense structural collapse. On the contrary, during slower drying, the polymer chains are able to rearrange and form a more ordered structure resulting in a higher tensile strength (Fernandez-Pan and others 2010).

For protein-based films however, drying temperatures have a different effect compared to polysaccharide-based films. Denavi and others (2009) reported improved tensile strength at a high drying temperature of soy protein isolate films. The soy protein isolate solution was prepared without subjecting it to thermal treatment before drying, hence the drying step also served as the denaturation step. Other researchers have also reported improved mechanical properties of protein-based films with increasing drying rates and temperatures (Jangchud and Chinnan 1999; Alcantara and others 1998; Gennadios and others 1996). These results are due to that fact that heat is a strong protein denaturing factor. During drying, the removal of water can results in proteins conformation changes. The degree of protein unfolding would therefore determine the type and proportion of covalent (S–S bonds) or non-covalent (hydrophobic interactions, ionic and hydrogen bonds) interactions that may occur. Drying at high temperatures thus enhances the interaction between the protein functional groups, resulting in improved mechanical properties. Chiou and others (2009) however, reported that when gelatin was dried at a temperature above the gelation temperature, lower tensile strength and elongation values were obtained. Therefore, even though heat can improve mechanical
properties, the optimum amount heat for a given polymer needs to be investigated if the positive effect of drying is to be obtained.

2.9.2 Barrier Properties

An important requirement of edible coatings or films on a food is its ability to maintain the quality of the food. Barriers to moisture and gases are therefore important properties that affect the performance of edible coatings and films. Edible films however, are poor barriers compared to synthetic films. The poor barrier performance of edible films is partly due to the addition of plasticizers to improve flexibility. These plasticizers act by opening up the polymeric chains and thus allowing increased moisture and gas permeability. This is mainly due to the hydrophilic nature of plasticizers and the biopolymer itself.

The moisture sensitivity of edible films can be investigated using measures such as water vapor transmission rate, contact angle, moisture sorption isotherms and water solubility tests for examples. To improve barrier properties, incorporation of hydrophobic compounds into edible films has been used (Pol and others 2002). Additionally, Ghasemloua and others (2011) studied the impact of oleic acid, a hydrophobic compound, on moisture barrier properties of kefiran, a microbial polysaccharide. The authors incorporated 15-35% (w/w) of oleic acid into the hydrophilic film with tween 80 acting as an emulsifier. The film’s moisture content, solubility in water, contact angle and water vapor permeability (WVP) were then determined. The results showed that adding oleic acid decreased the moisture content of the film from 17.95% to 12.36%. Also there was a reduction in the film’s solubility in water and water vapor transmission rate.
Furthermore, the contact angle was higher for the oleic acid-incorporated films. The results obtained were due to the increased hydrophobicity of the film due to the incorporation of the lipid. Consequently, interaction of oleic acid with kefiran resulted in a decrease in available hydroxyl groups. There was a reduction in moisture sensitivity as recorded by water vapor transmission rate, solubility, contact angle and moisture content analyses. In yet another example, Siripatrawan and Harte (2010) also reported reduced water vapor permeability when green tea extract was incorporated in a chitosan-based film. This was due to the interactions between the green tea polyphenols and chitosan. In addition to reducing the available hydroxyl groups, the polyphenols also acted as crosslinking agents. The polyphenol tied up the hydroxyl groups of chitosan to prevent hydrogen bonding with water. Crosslinking agents can also increase barrier because of increase in the film’s density by lowering the void spaces (Gonzalez and others 2011).

An investigation of the effect of crosslinking soy protein isolate (SPI) film with genipin, a naturally-derived crosslinker, showed that the WVP value decreased by 29.5% when the film was incorporated with 2.5% (w/w of SPI) of genipin (Gonzalez and others 2011).

Moisture sorption isotherms (MSI) have also been used to characterize the water absorption property of edible films (Shih and others 2010). Information from MSI helps to predict stability and quality changes during the packaging of food products as well as establishing the range of water activity conditions needed to obtain desirable film properties (Abugoch and others 2011; Shih and others 2010). For example, in a study by Shih and others (2010), MSI was used to examine the impact of rice wax on the water sorption characteristics of pullulan films. Data obtained from the study were fitted into
the Brunauer-Emmet-Teller (BET) and Guggenheim-Anderson-de Boer (GAB) models to give sorption parameters that describe the hydration properties of the films. In that instance, a decreased in the monolayer moisture content reflected a reduced hydration capacity of the given test films.

2.9.3 Thermal properties

The thermal behavior of edible packaging is important since storage and end-use conditions of edible coatings and films can vary from sub-zero to cooking temperatures. Packaged foods can be stored under these temperature conditions for few days to several weeks. Differential Scanning Calorimetry (DSC), and Thermogravimetric analysis (TGA), are two methods commonly employed to evaluate the thermal behavior of polymers.

DSC is a technique that measures the thermal properties of a given material to establish a link between temperature and specific physical properties of substances. It directly measures the enthalpy associated with a process of interest. As a thermal analysis apparatus, the device determines the temperature and heat flow that is associated with material transitions as a function of temperature (Haynie 2008; Haines and other 1998). In a DSC experiment, heat energy is introduced simultaneously into a sample cell containing the material of interest and a reference cell. Temperatures of both cells are raised identically over time and the difference in the energy required to match the temperature of the sample to that of the reference would be the amount of excess heat absorbed (endothermic process) or released by the molecules in the sample (exothermic process). Different thermal transitions of polymers, including the glass transition
temperature ($T_g$), crystallization, and melt temperatures can be detected by DSC. Below the $T_g$, the polymer will exist in the glassy where the molecules are held tightly together by intermolecular forces. In this state, molecular mobility is restricted but when the polymer is heated above $T_g$, the polymer gains more energy and moves from the glassy state into the rubbery state. In the rubbery state, segmental mobility begins but molecular mobility is forbidden. Segmental motion refers to the movement of small chain segments above the glass transition temperature. This is the stage where the individual molecules don't move, but they move together as small segments. When the polymer is heated much more above the $T_g$, molecular mobility ensues where the molecules get more energy to move resulting in the flowing of the melt polymer like a viscous liquid. The temperature below which the polymer exists in the rubbery state and above which it exists as a viscous liquid is called the melting temperature. Upon cooling, crystals forms and arranged in an ordered lattice in a process called crystallization, which is an exothermic process.

In edible film applications, DSC can be used to determine how different type of plasticizers or their concentrations have altered the properties of the material. For example, in a study by Kim and Ustunol (2001), DSC was used to investigate changes in the glass transition temperature ($T_g$) of glycerol-plasticized whey protein isolate films and that of sorbitol-plasticized films. The results showed that glycerol-plasticized films had a lower $T_g$ than those of the sorbitol-plasticized films. This difference was due to the lower molecular weight of glycerol as explained by the authors. Julkapli and Akil (2010) also investigated the thermal properties of kenaf filled chitosan biocomposites using DSC.
Additionally, DSC was used to study the crystallization of the chitosan film as it was influenced by different electrical treatments of the film forming solution (Souza and others 2009). The authors observed higher melting temperature ($T_m$) and melting enthalpy ($\Delta H_m$) values of the treated sample because of the crystallization of the chitosan films, as was subsequently confirmed by X-ray diffraction and scanning electron microscopy analyses. In another study, Al-Hassan and Norziah (2012) used DSC to measure the reductions in $T_g$, $T_m$ and $\Delta H_m$ when a starch-gelatin blended film was plasticized with sorbitol. The researchers explained that sorbitol interacted with the hydroxyl groups of both starch and gelatin resulting in a reduced interaction between the starch and gelatin. Differential Scanning Calorimetry can also be used to determine the miscibility or compatibility of polymer blends by determining the $T_g$ of the blend. A single $T_g$ gives information on compatibility of the blends (Al-Hanssan and Norziah 2012). A single $T_g$ indicates a homogenous blend whereas two separate $T_g$ values signify that there is heterogeneity.

Thermogravimetric analysis (TGA) measures the weight changes that take place in a sample while increasing its temperature at a controlled rate and atmosphere. Since weight loss is indicative of degradation of components within the sample, TGA can therefore be used to study how different additives affect the thermal stability of edible films and coatings. In TGA, upon heating the sample, a descending TGA thermal curve is displayed that indicates a weight loss has occurred. Weight losses could be due to decomposition, oxidation and dehydration of the material. TGA is an important instrument for optimizing the film’s properties. A determination of the thermal stability
can also be very important for commercial film casting or thermoplastic processing, where higher temperatures are used. Du and others (2008), for example, reported film casting conditions in a pilot plant labcoater for edible films as 100-132°C for 12min. Abugoch and others (2011) used TGA to investigate the thermal stability of chitosan (CH), quinoa protein extract (PE) and CH/PE blend. From that study, PE/CH blends had a lower decomposition temperature with 2 weight losses occurring at 171°C and 271°C. In comparison, PE film decomposition occurred at a temperature of 230 and 285°C, whilst decomposition of the CH film occurred at 304°C. These results showed that the thermal stability of the CH/PE blend decreased when compared with the individual homogenous films. Also, the effect of the addition of nanoparticles to HPMC films was studied by de Moura and others (2009) using TGA for analysis. From the TGA analysis, the authors reported that the chitosan/tripolyphosphat (CH/TPP) nanoparticles increased the thermal stability of the HPMC films. Weight losses corresponding to the beginning of thermal degradation occurred at 232°C for HPMC and 271°C for HPMC with 110nm CH/TPP nanoparticles. These nanoparticles, according to the authors, occupied the empty space in the pores of the HPMC thereby increased the collapse of the pores. This resulted in an increased heat stability of the film.
CHAPTER 3

VIRUCIDAL AND BACTERICIDAL ACTIVITY OF CHITOSAN-BASED FILM ENRICHED WITH GREEN TEA EXTRACTS

3.1 Abstract

The virucidal/antiviral activity of green tea extract (GTE) dissolved in deionized water (aqueous extract solution), and incorporated into chitosan film forming solutions (FFS) and chitosan edible films were evaluated against murine norovirus (MNV-1). Additionally, the antibacterial activity of the GTE enriched films was tested against \textit{L. innocua} and \textit{E. coli} K12. The aqueous and film forming solutions containing the extract were incubated with the virus suspensions (~10^7 PFU/ml) for 3 h at 23 ± 1°C. Also, the chitosan films with or without the extract were incubated with the virus suspensions for at 23 ± 1°C with sampling times after 4 and 24 h exposure. The viral infectivity after treatments was measured by plaque assays. The 1, 1.5 and 2.5% aqueous GTE solutions significantly (p<0.05) reduced MNV-1 plaques by 1.69, 2.47, and 3.26 log cycles after 3 h exposure, respectively. Similarly, the FFS containing 2.5 and 5.0% GTE reduced MNV-1 counts by 2.45 and 3.97 log_{10} PFU/ml respectively after 3 h exposure. Additionally, the edible films enriched with the GTE (5, 10 and 15%) were also effective
against MNV-1. After 24 h incubation period, the 5 and 10% GTE films significantly (p<0.05) resulted in MNV-1 titer reductions of 1.60 and 4.50 log_{10} PFU/ml, respectively. The film containing 15% GTE reduced norovirus plaques to undetectable levels in 24 h. Finally, the antibacterial activity of the GTE incorporated chitosan films was investigated. Here, the edible films enriched with the GTE (5, 10 and 15%) were also effective against *L. innocua* and *E. coli* K12. All the GTE films reduced *L. innocua* and *E. coli* K12 populations to undetectable levels in tryptic soy broth after 24 h exposure. Green tea extract have both antiviral/virucidal and antibacterial efficacies. Therefore, films and coatings enriched with GTE had great potential to be used as wrapping film or coating for ready-to-eat meat and fresh produce.

3.2 Introduction

Foodborne illnesses caused by bacteria and viruses have emerged as a major public health concern worldwide. In the United States, an estimated 48 million people get sick every year (CDC 2011a). From this number, 128,000 are hospitalized and 3,000 die of foodborne diseases. The human norovirus alone accounts for about 21 million cases. This makes the virus the leading cause of gastroenteritis in the United States. The virus is normally transmitted via the fecal oral-route. Other means of transmission includes contaminated water and food, persons to persons, vomitus and aerosols (Koopmans and Duizer 2004). The seriousness of norovirus outbreaks is further heightened by its ability to persist in the environment, including food contact surfaces. Moreover, it has a low infection dose (10-100 virions) and large numbers of virions are shed in the feces (Kuusi and others 2002; Cheesbrough and others 2000). Clinical
symptoms of norovirus infection include diarrhea, nausea, vomiting, abdominal cramps, headaches, fevers, and chills lasting between 1-3 days (Atmar and Estes 2006). Common risk foods that can result in outbreaks include minimally processed and ready-to-eat foods such as berries and oysters. These foods can either be cross-contaminated by food handlers or contaminated with unsanitary water at both the pre-harvest or post-harvest stages. In addition to noroviruses, outbreaks from bacterial pathogens such as *L. monocytogenes* and *E. coli* O157:H7 have created a need for adequate control measures while maintaining the quality of ready to eat and processed foods.

An active food packaging (such as antimicrobial packaging) can serve as a nonthermal means of controlling foodborne pathogens. Brody and others (2001) defined active packaging as one intended to sense an internal or external environmental change and respond by changing its own properties or its internal environment. Thus, the package, the product, and the environment interact to prolong shelf life, enhance safety or sensory properties. In antimicrobial packaging, preservatives are added to the material either during the film fabrication process or applied as a coating on the surface. Several researchers have used edible films incorporated with antimicrobial agents to minimize bacterial growth in selected ready-to-eat foods (Zhang and others 2004; Shin and others 2010).

Additionally, there has been increased interest in the use of naturally-derived antimicrobial agents incorporated into active packaging. In addition to its perceived safety, naturally derived plant extracts can have broad spectral activities such as antibacterial, antifungal and antiviral. Work on the antiviral properties of polyphenolic
compounds such as catechins and proanthocyanidins found in plant extracts have been reported (Isaac and others 2011, Li and others 2012b). However, little is reported on the use of edible films and coatings with antiviral efficacy.

Human norovirus studies are limited by the fact that it is not able to grow in cell culture. Consequently, murine norovirus (MNV-1) which has similar properties to human norovirus is therefore used in cell culture studies as a surrogate. The objective of this study was to investigate the antiviral activity of green tea extracts incorporated into edible films and as a coating against MNV-1. The GTE films were also tested on two bacteria surrogates, *E. coli* K12 and *L. innocua* for comparison.

### 3.3 Materials and methods

#### 3.3.1 Materials

Murine norovirus strain (MNV-1) used as a human norovirus surrogate was obtained from Herbert W. Virgin IV, Washington University School of Medicine. The murine macrophage cell line RAW 264.7 (ATCC, Manassas, VA) was used to grow the MNV-1. High-glucose Dulbecco’s Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA) and used for growing the cells. Six-well plates were obtained from Corning Life Sciences (Wilkes-Barre, PA) and used to culture cells for plaque assay. *Escherichia coli* K12 (ATCC 29181) and *Listeria innocua* (ATCC 33090) were purchased from the American Type Culture Collection (Manassas, VA). Tryptic soy broth (TSB) and tryptic soy agar purchased from Difco (Sparks, MD) were used to grow the bacteria. Medium molecular weight chitosan powder
(94% purity, 75% deacetylation) was obtained from Huantai Goldenlake Carapace Products Co., Ltd (Tsingtao, China) and used to form the films. Glycerol was obtained from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ) and used as a plasticizer. Glacial acetic acid was purchased from J.T Baker (Phillipsburg, N.J) and used as a solvent. The green tea extract (GTE), (standardized 98% polyphenols, 80% catechins, 50% epigallocatechin gallate and 0.7% caffeine) was purchased from Herbstore USA (Walnut, CA).

### 3.3.2 Film formation

To make the films, chitosan (2% w/w) was dissolved in 1% acetic acid solution with glycerol as a plasticizer (Figure 3.1). Green tea powder at different concentrations (0-15%) was then added to the chitosan film forming solutions (FFS) (Table 3.1). The solutions were stirred using a magnetic stirrer for 30 min to dissolve the powdered extract. The film forming solutions were then degassed in a water bath sonicator. A 20 g aliquot of the FFS was poured onto a polypropylene sheet attached to a glass plate and a drawdown bar used to spread the solution into a thin film. After drying in an oven at 45°C for 2 h, the films were peeled and stored for further testing.

### 3.3.3 Virus Stock preparation

The MNV-1 was grown in a monolayer of RAW 264.7 cell line cultured in a DMEM that was supplemented with 10% FBS at 37°C under a 5% CO₂ atmosphere. Confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h of incubation at 37°C, 20 ml of serum-free DMEM was added and
incubated at 37°C under 5% CO₂. The virus was harvested after 2 days of incubation by three freeze-thawing cycles and centrifuging at 3,000g for 20min at 4°C. The supernatant was collected and used as a stock.

2% chitosan

Dispersed in deionized water

Addition of 1% of acetic acid (v/v) to dissolve chitosan

Glycerol added as plasticizer

Green tea addition

Solution casting on Polypropylene film and spreading into a film using a drawdown bar

Dried at 45°C for 2 h

Figure 3.1   Flow chart of chitosan/GTE blend and film formation
Table 3.1 Formula of GTE antimicrobial films

<table>
<thead>
<tr>
<th>Antimicrobial film</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chitosan</td>
</tr>
<tr>
<td>Chitosan only</td>
<td>2</td>
</tr>
<tr>
<td>5%GTE film</td>
<td>2</td>
</tr>
<tr>
<td>10%GTE film</td>
<td>2</td>
</tr>
<tr>
<td>15%GTE film</td>
<td>2</td>
</tr>
</tbody>
</table>

3.3.4 Quantification of virus titer by plaque assay

The MNV-1 plaque assay was performed on RAW 264.7 cells seeded into six-well plates and incubated for 24 h at 37°C under a 5% CO₂ atmosphere (Wobus and others 2006). The cell monolayers were then infected with 400µl of appropriate serial dilutions of the virus suspension and the plates incubated for 1 h at 37°C and shaken gently every 15min to allow for the virus attachment. After removal of the inocula, the cells were overlaid with 2ml of Eagle Minimum Essential Medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine (Invitrogen). After 48 h
of incubation at 37°C and 5% CO₂, the plates were fixed and stained with 10% formaldehyde and crystal violet. The plaques were then counted.

3.3.5 **Virucidal/Antiviral testing of GTE in aqueous and film forming solutions**

The green tea extract was dissolved in deionized water and filter sterilized using a 0.2µm filter. Concentrations of up to 2.5% GTE in deionized water were tested by adding equal volumes of the virus suspensions (10⁷ PFU/ml). Volumes of 0.5ml each of the virus suspensions and the aqueous extract solutions were mixed to give final extract concentrations of 1.0, 1.5 and 2.5%. The mixtures were incubated at 23 ± 1°C on a rotary shaker for 3 h. For the control, an equal volume of DMEM (0.5ml) was mixed with the virus suspension. After 3 h, 0.5ml of the treated samples and the control were withdrawn and diluted with 0.5ml DMEM. After 10-fold serial dilutions, the appropriate solutions were plaque assayed as previously described.

A similar procedure was followed for testing the reduction of virus infectivity by the film forming solutions (FFS). To perform this test, 5 and 10% GTE in FFS were mixed with equal volumes (0.5ml) of virus suspensions to yield final concentrations of 2.5 and 5.0% GTE in the FFS, respectively. The controls were DMEM and 2% chitosan solution individually mixed with equal volumes (0.5ml) of the virus suspensions.

3.3.6 **Virucidal/Antiviral testing of GTE in chitosan films**

In testing the virucidal activity of the films, the method of Haldar and others (2006) was used with slight modification. Film samples containing the different levels of GTE (5, 10 and 15%) and measuring 2.5cm X 2.5cm were individually placed at the
bottom of one of the wells of a 6-well plate and 2ml of the virus suspensions (10^7 PFU/ml) added to each film. They were incubated at 23 ± 1°C on an incubator shaker for 24 h. Virus suspensions with no added film and one with 2% chitosan film but with no added extract were used as controls. After 4 and 24 h, 0.5ml of the treated samples and controls were withdrawn and diluted with 0.5ml of DMEM. Ten-fold serial dilutions were then made and the appropriate solution plaque assayed as previously described. The experiments were done in duplicates.

3.3.7 *Antibacterial activity testing of films*

For the bacterial testing, only the antimicrobial films were used. The bacteria species were individually cultured by transferring a loopful of frozen (-80°C in 30% glycerol) *E. coli* K12 or *L. innocua* into 20ml of a sterile TSB with an inoculation loop. The organisms were incubated for 24 h at 37°C to reactivate them. Afterwards, a loopful of the revived bacteria was transferred to TSA slants and incubated for 24 h at 37°C. The slants were then refrigerated at 4°C and used as stock culture. Before each experiment, a loopful of the respective bacteria was taken from the slant and cultured in a 20ml sterile TSB for 24 h at 37°C. Bacterial suspensions containing approximately 10^7 CFU/ml were prepared in TSB from the overnight culture and used for the antibacterial testing of the films.

Film samples (2.5cm X 2.5cm) were placed in a dish and 2ml of each bacterial suspension (10^7 CFU/ml) added. Cultures without a film and another with the 2% chitosan film but no added extract were used as controls. The dishes were incubated at 37°C and shaken at 60 rpm in an incubator shaker for 24 h. Samples were taken at 0, 3, 6, 12, and
24 h intervals and diluted with 0.1% peptone. Appropriate dilutions were poured plated using TSA in duplicates. The plates were incubated for 24-48 h at 37°C and the number of colonies counted using a Darkfield plate counter (American Optical, Buffalo, NY).

3.3.8 **Statistical analysis**

All data were analyzed using the analysis of variance (ANOVA), and Tukey’s multiple comparisons test was used to determine significant differences between the means at a level of p<0.05. The statistical package used in the study was SPSS 20 for Windows. All the data represented are the means of duplicates. The statistical analysis was used to compare and determine the significant effect of the addition of GTE on (1) the antiviral efficacies of different concentrations of GTE dissolved in deionized water and chitosan film forming solutions on MNV-1; (2) the antiviral and antibacterial efficacies of chitosan films containing 3 levels (5, 10 and 15%) of GTE on MNV-1, and *E. coli* K12 and *L. innocua*, respectively. The Tukey’s test was therefore used to determine differences in means between the control and the GTE films.

3.4 **Results**

3.4.1 **Antiviral/virucidal effect of GTE in aqueous and film forming solutions**

The virucidal/antiviral effects of the green tea extract against MNV-1, a surrogate for the human norovirus, were tested. The extract was dissolved in deionized water (aqueous extract solution) and chitosan film forming solution (FFS). Concentrations of 1, 1.5 and 2.5% of the green tea extract dissolved in water reduced MNV-1 plaques by 1.69, 2.47, and 3.26 log cycles, respectively after 3 h (Figure 3.2). The green tea extract also
showed virucidal activity when incorporated into the chitosan film forming solutions. Chitosan solutions containing 2.5 and 5.0% of the green tea extract resulted in reductions of 2.45 and 3.97 log_{10} PFU/ml, respectively after 3 h (Figure 3.3). The chitosan film forming solution with no added green tea extract did not have antiviral/virucidal activity.

Figure 3.2 Infectivity of MNV-1 after treatment with different levels of green tea extracts (GTE) dissolved in deionized water as detected by plaque assay after 3 h of incubation. Error bars indicate the data range.
3.4.2 Antiviral/virucidal activity of chitosan/GTE films

Concentrations of 5, 10 and 15% GTE were added during the chitosan film fabrication process and its antiviral/virucidal activity evaluated against MNV-1 after 4 and 24 h of incubation. The results are presented in Figure 3.4. After 4 h of incubation, only the films containing 10 and 15% GTE resulted in significant (p<0.05) reductions of the MNV-1 titer with a decrease of 0.95 and $2.81 \log_{10}$ PFU/ml, respectively. However, after 24 h of incubation, all film types except the 2% chitosan film significantly reduced MNV-1 infectivity (p< 0.05). Log reductions of 1.60, and $4.50 \log_{10}$ PFU/ml were
obtained for the 5 and 10% GTE films, respectively. The film containing 15% GTE reduced the MNV-1 titer to undetectable levels.

Figure 3.4 Infectivity of MNV-1 after treatment with chitosan films with different levels of green tea extracts (GTE) as detected by plaque assay after 4 and 24 h of incubation. Error bars indicate the data range.

3.4.3 Antibacterial activity of chitosan film with GTE

The antibacterial activities of the chitosan/GTE films were investigated against the foodborne pathogen surrogates, *L. innocua* and *E. coli* K12. Bactericidal activity was
enumerated by the viable cell count method to give a quantitative estimation of the efficacy of the films. The inhibitory activities of the GTE incorporated chitosan-based films are shown in Figures 3.5 and 3.6. Samples were enumerated for bacteria viability at 0, 3, 6, 12, and 24 h of exposure to the films. Two different controls were tested. These were a control with no added film and a control with 2% chitosan film (2% CHI) with no added extract.

The bacteria strains used were highly susceptible to the films containing the green tea extract. For the GTE incorporated films, significant (p<0.05) reductions of both *L. innocua* and *E. coli* K12 occurred after 3 h of incubation when compared to the control. However, there was no significant differences (p>0.05) in log reductions between the different GTE films at 3 h after storage. The same was true for *E. coli* K12 after 6 h of incubation, where there was no difference between the treatments, even though the GTE films significantly reduced the growth of both bacteria. The GTE films reduced *E. coli* K12 and *L. innocua* counts by at least 2 and 2.5 logs after 3 h of incubation, respectively. At 6 h of incubation, *E. coli* K12 reductions of 3.73, 4.43 and 4.71 logs were recorded for the 5, 10 and 15% GTE films, respectively. The 5, 10 and 15% GTE films produced a reduction of 3.67, 4.72 and 5.98 log_{10} CFU/ml in Listeria after 6 h incubation, respectively. After 12 h exposure, the 5% GTE film reduced *E. coli* K12 and *L. innocua* populations by 5.04 and 6.88 log_{10} CFU/ml, respectively. However, 10 and 15% GTE films reduced the counts to undetectable levels.

Finally, all the GTE films (5, 10 and 15%) reduced both *E. coli* K12 and *L. innocua* counts to undetectable level after 24 h of contact. In contrast, the 2% chitosan
Figure 3.5 The effect of green tea extracts (GTE) incorporated into chitosan (CHI) films on the survival of *L. innocua* in tryptic soy broth. Error bars indicate the data range.

Figure 3.6 The effect of green tea extracts (GTE) incorporated into chitosan (CHI) films on the survival of *E. coli* K12 in tryptic soy broth. Error bars indicate the data range.
film alone (control) produced significant (p<0.05) in log reductions in *E. coli* K12 after 12 h exposure. However, reductions for *L. innocua* after 12 h were not significant. The reductions for both *E. coli* K12 and *L. innocua* were only significant after 24 h exposure with reductions of 1.67 and 1.34 log₁₀ CFU/ml, respectively.

3.5 Discussion

3.5.1 Antiviral/virucidal activity

In the present study, apart from investigating the virucidal/antiviral activity of GTE dissolved in sterile water (aqueous extract solutions), the extract was also tested to see if it would retain activity when incorporated into chitosan films or coating solutions. The GTE showed virucidal/antiviral activity in the aqueous extract solutions. Additionally, its antiviral/virucidal activity was retained against MNV-1 when incorporated into the film forming solutions and also in the resultant films. Moreover, the antibacterial activity of the edible films was enhanced against *L. innocua* and *E. coli* K12 when the GTE was incorporated. When an antimicrobial agent is incorporated into films or coatings, it can loss its efficacy especially if the functional groups responsible for its activity interacts with the polymer. Additionally, the functional groups of the antimicrobial agent may bind too tightly to the polymer such that its release is restricted (Han 2000).

Green tea extract contains catechins such as (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG) (Kajiya and others 2004) with EGCG been the predominant component (Zaveri 2006).
These polyphenolic compounds have been shown to have antiviral/virucidal efficacies (Isaac and others 2011, Weber and others 2003). Isaacs and others (2011) for instance investigated the effect of EGCG and EGCG dimers on both nonenveloped and enveloped viruses. From that study, the enveloped herpes simplex virus (HSV) and vesicular stomatitis virus (VSV) were reduced by about 4 and 2 log_{10} PFU/ml, respectively by EGCG. When dimers of EGCG were used, they reduced titers even more. For example, measles virus (MV) which was less sensitive to EGCG (with only 0.5 log_{10} PFU/ml reduction) was inactivated by more than 3 log_{10} PFU/ml by each of the following dimers: theasinensin A, theaflavin-3,3 digallate (TF-3) and theaflavin-3-gallate. However, EGCG itself was not effective in inactivating the non-enveloped enteroviruses. Also, even though, theasinensin A reduced the titer of coxsackie A9 virus by about 3 log_{10} PFU/ml, it was ineffective against other non-enveloped viruses including, coxsackie B4 and echovirus 6. The TF-3, on the other hand, was more effective in inactivating echovirus 6 and coxsackie A9 by 2 and 3.5 log_{10}, respectively. Regarding the mechanism of inactivation, Isaacs and others (2011) concluded that the activity of the EGCG dimers was due to the interaction of the catechin with the viral capsid of the nonenveloped viruses. For the enveloped virus such as HSV, inactivation was due to the catechin directly binding to fusion proteins or binding to the viral envelope glycoprotein. Additionally, Song and others (2005) reported the ability of polyphenolic compounds to inhibit influenza virus replication in cell culture and it also had a direct virucidal effect on the virus.
From the studies discussed above, the mechanisms responsible for the activity of GTE on MNV-1 can range from interaction with the virus protein capsid, interference with viral attachment or adsorption and/or interference with replications. It also appears that there may be differences in antiviral/virucidal activities of the components of the extracts against specific nonenveloped viruses. Chitosan had no antiviral/virucidal activity in this study. This agrees with the work of Su and others (2009) who also found that chitosan was ineffective against MNV-1, although it significantly reduced other surrogates, including feline calicivirus F-9 (FCV-F9), and bacteriophage. Davis and others (2012) also reported the resistance of MNV-1 to chitosan.

3.5.2 Antibacterial activity

The GTE used in this study was very efficient in reducing bacterial counts of the surrogates used. Other researchers also used GTE against foodborne bacteria. Theivendran and others (2006) for example, incorporated GTE into soy protein-based film and tested against *Listeria* in PBS. The soy film containing only 1% GTE was effective in reducing *L. monocytogenes* counts by about 2 logs after 48 h. In that study, when GTE was combined with nisin it resulted in an increased activity against *Listeria*. In addition, Friedman and other (2006) also studied the antimicrobial activity GTE and aqueous solutions of tea catechins (found in the extracts), on *Bacillus cereus*. Their study demonstrated that polyphenols present in the GTE ((-)-gallocatechin-3-gallate, (-)-epigallocatechin-3-gallate and theaflavin-3’-gallate) had antimicrobial activity at nanomolar levels against the *Bacillus cereus*. Furthermore, the ability of polyphenols
from GTEs to inhibits both gram-positive and gram-negative bacteria have also been reported (Gadang and others 2008). This present study also found that GTE incorporated into the chitosan was effective against both the gram negative *E. coli* K12 and the gram positive *L. innocua* bacteria.

According to Utlee and others (2002), polyphenols act on the outer cell membrane of bacteria as the main site of interaction. The active groups in the polyphenols responsible for such interaction are the hydroxyl groups, conjugated double bonds and galloyl groups. Since the membrane maintains the integrity of the cell, damage caused to it by this interaction can result in the death of the bacterium. This happens because of dissipation of the proton motive force (PMF) and/or inhibition of membrane-associated enzyme activity (Shimamura and others 2007, Juven and others 1994, Davidson and Naidu 2000). EGCG and ECG are reportedly the most effective green tea catechins in terms of its antibacterial activity. This is so because of the presence of the galloyl functional groups in these catechins (Shimamura and others 2007). The deteriorating effects of the galloyl moieties in a catechin on the lipid bilayer membrane have also been reported by Cox and others (2001) and Ikigai and others (1993). In the GTE used for this study, the EGCG content (according to the manufacturer) was 50% of the total catechins. This might have accounted for the high inactivating capacity of the GTE incorporated films. Several mechanisms have been cited as responsible for the antimicrobial activity of chitosan by itself. These includes increasing cell permeability due to chitosan’s interaction with the negative charges on the cell surface; interaction with DNA and messenger RNA and acting as chelating agent that binds metals and nutrients making
them unavailable to micro-organisms (No and others 2006, Tripathi and others 2010, Rabea and others 2003). The antimicrobial activity of chitosans however, influence by factors such as the degree of deacetylation and molecular weight as well as the pH of the medium (Devlieghere and others 2004).

3.6 Conclusions

The green tea extract (GTE) was effective in reducing the infectivity of murine norovirus (MNV-1) in aqueous solutions, edible coatings and the resultant dried films. These films also had antibacterial activity against *L. innocua* and *E. coli* K12. This study demonstrated that plant extracts have antiviral/virucidal and antibacterial properties. Therefore edible films and coating solutions enriched with the GTE have great potential to be used as wrapping films or coatings for ready-to-eat meats and fresh produce. However, additional research is needed to investigate the effect of the films or coatings against viruses and bacteria in food systems. Additionally, individual components of the GTE also need to be investigated to ascertain its respective antiviral/virucidal activity. This will help in an optimization of these compounds in films and coatings used to limit microbial growth in foods.
CHAPTER 4

VIRUCIDAL AND BACTERICIDAL ACTIVITY OF CHITOSAN-BASED FILM
ENRICHED WITH GRAPE SEED EXTRACTS

4.1 Abstract

Edible coatings and films based on chitosan containing grape seed extract (GSE) were developed and its activity tested against murine norovirus (MNV-1), a human norovirus surrogate. Also, the films were tested against *Listeria innocua* and *Escherichia coli* K12, surrogates for *L. monocytogenes* and *E. coli* O157:H7, respectively. Grape seed extract concentrations of 1, 1.5 and 2.5% dissolved in deionized water resulted in MNV-1 plaque reductions (p<0.05) of 1.75, 2.60 and 3.58 log$_{10}$ PFU/ml, respectively after 3 h. The chitosan solutions incorporated with the GSE (2.5 and 5%) also reduced MNV-1 titers significantly (p<0.05) by 2.68 and 4.00 log$_{10}$ PFU/ml, respectively after 3 h. Additionally, incorporation of the GSE into the chitosan films enhanced its efficacy against MNV-1, *L. innocua* and *E. coli* K12. The chitosan films containing 5, 10, and 15% GSE caused reductions of 0.92, 1.89 and 2.27 log$_{10}$ PFU/ml, respectively after 4 h of incubation. Also after 24 h, the 5 and 10% GSE films reduced MNV-1 titers by 1.90 and 3.26 log$_{10}$ PFU/ml, respectively, while the 15% GSE film reduced MNV-1 to undetectable levels. For *E. coli* K12, there were reductions of 2.28, 5.18 and 7.14 log$_{10}$
CFU/ml after 24 h exposure by the 5, 10, and 15% GSE films, respectively. Also, *L. innocua* counts were reduced by 3.06, 6.15 and 6.91 log_{10} CFU/ml by the 5, 10 and 15% GSE films, respectively. This study demonstrated that GSE in edible films and coatings is effective against the organisms tested and potentially effective against foodborne microbes of public health significance.

### 4.2 Introduction

Food contamination with pathogenic microorganisms is a serious threat to public health and safety. The increased consumption of fresh and minimally processed foods and lifestyle changes are among factors driving high levels of foodborne illnesses worldwide. Ready-to-eat foods can easily become recontaminated or cross contaminated with foodborne pathogens by food handlers and equipment surfaces during processing or packaging.

Human norovirus is a common foodborne virus and belongs to the Caliciviridae family. It causes gastroenteritis, symptoms of which include vomiting, diarrhea, nausea, abdominal cramps and fever. The virus is highly contagious and transmitted through the fecal-oral route. Also, enteric foodborne bacteria including *Escherichia coli* O157:H7 and *Listeria monocytogenes* can also cause illnesses. *Escherichia coli* O157:H7 is gram-negative and has been associated with foodborne diseases such as bloody diarrhea, hemolytic uremic syndrome, and hemorrhagic colitis (Griffin and Tauxe 1991). Outbreaks of *E. coli* O157:H7 are normally associated with bovine products; however, other food products such as leafy greens have been linked to this bacterium (Feng 1995). *Listeria monocytogenes* is ubiquitous microorganism that can be found in environments
such as soil, vegetation, and animals. It is a gram positive, facultative anaerobe and intracellular bacterium that causes listeriosis. This is a potentially lethal disease in immune compromised individuals, such as the elderly, pregnant women and infants (Schuppler and Loessner 2010). *L. monocytogenes* is also able to grow at refrigerated temperatures and this makes foods stored in this environment susceptible to this organism.

Interests in edible films are on the increase because, in addition to reducing environmental waste, they also serve as carriers of functional additives such as antimicrobial agents. Various plant derived compounds are been used as preservatives in food processing. These naturally derived extracts are safe to use in foods and are excellent source of natural phenolic compounds that have antimicrobial activity. Grape seed extract (GSE) (*Vitis vinifera*), as an example, can have antifungal, antiviral and antibacterial activities. Recently, it was reported that GSE can inactivate murine norovirus (MNV-1), a surrogate for human norovirus (Li and others 2012b; Su and D’Souza 2011). The antibacterial activities of GSE have also been demonstrated against *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Staphylococcus aureus* (Rhodes and others 2006; Baydar and others 2006; Kao and others 2010). Corrales and others (2009) incorporated GSE in a pea starch film and tested its antibacterial activity on pork loins infected with *Brochothrix thermosphacta*. The film containing 1% GSE reduced the bacterial growth on the pork loins by 1.3 log_{10} CFU/ml after 4 days at 4°C.
Edible films and coatings with added antimicrobial agents have been demonstrated to have efficacies against several foodborne bacteria (Ravishankar and other 2009; Siripatrawan and Harte 2010; Siripatrawan and Noipha 2012; Martins and others 2012). However, the use of edible films and coatings to control foodborne viruses has not been explored. Therefore, in this study, chitosan film forming solutions and films enriched with GSE were investigated to establish its virucidal activity on MNV-1. For comparison, the films were also investigated against L. innocua and E. coli K12.

4.3 Materials and methods

4.3.1 Materials

Murine norovirus strain (MNV-1) used as a human norovirus surrogate was obtained from Herbert W. Virgin IV, Washington University School of Medicine. The murine macrophage cell line RAW 264.7 (ATCC, Manassas, VA) was used to grow the MNV-1. High-glucose Dulbecco’s Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA) and used for growing the cells. Six-well plates were obtained from Corning Life Sciences (Wilkes-Barre, PA) and used to culture cells for plaque assay. Escherichia coli K12 (ATCC 29181) and Listeria innocua (ATCC 33090) were purchased from the American Type Culture Collection (Manassas, VA). Tryptic soy broth (TSB) and tryptic soy agar purchased from Difco (Sparks, MD) were used to grow the bacteria. Medium molecular weight chitosan powder (94% purity, 75% deacetylation) was obtained from Huantai Goldenlake Carapace Products Co., Ltd (Tsingtao, China) and used to form the films. Glycerol was obtained
from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ) and used as a plasticizer. Glacial acetic acid was purchased from J.T Baker (Phillipsburg, N.J) and used as a solvent. Leucoselect® contained ≥95.0% ≤105.0% proanthocyanidins, as determined by Gel permeation chromatography, and ≥13.0% ≤19.0% catechin and epicatechin, as determined by high pressure liquid chromatography.

4.3.2 **Film formation**

To make the films, chitosan (2% w/w) was dissolved in 1% acetic acid solution with glycerol as a plasticizer (Figure 4.1). Grape seed powder at different concentrations (0-15%) was added to the chitosan solution (Table 4.1). The solutions were then stirred using a magnetic stirrer for 30min to dissolve the extract. The film forming solutions was degassed in a water bath sonicator. A 20g aliquot of the solution was poured onto a polypropylene sheet attached to a glass plate and a drawdown bar used to spread the solution into a thin film. After drying in an oven at 45°C for 2 h, the films were peeled and stored for further testing.

4.3.3 **Virus Stock preparation**

The MNV-1 was grown in a monolayer of RAW 264.7 cell line cultured in DMEM that was supplemented with 10% FBS at 37°C under a 5% CO₂ atmosphere. Confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h of incubation at 37°C, 20ml of serum-free DMEM were added and incubated at 37°C under 5% CO₂. The virus was harvested after 2 days of incubation by
three freeze-thawing cycles and centrifuging at 3,000g for 20mins at 4°C. The supernatant was collected and used as a stock.

4.3.4 Quantification of virus titer by plaque assay

The MNV plaque assay was performed in RAW 264.7 cells seeded into six-well plates and incubated for 24 h at 37°C under a 5% CO₂ atmosphere (Wobus and others 2006). The cells monolayers were then infected with 400µl of appropriate serial dilutions of the virus suspension and the plates incubated for 1 h at 37°C and shaken slightly every 15min to allow for the virus attachment. After removal of the inocula, the cells were overlaid with 2ml of Eagle Minimum Essential Medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine (Invitrogen). After 48 h of incubation at 37°C and 5% CO₂, the plates were fixed and stained with 10% formaldehyde and crystal violet. The plaques were then counted.

Table 4.1 Formula of GSE antimicrobial films

<table>
<thead>
<tr>
<th>Antimicrobial film</th>
<th>Amount (g)</th>
<th>Amount (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chitosan</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Chitosan only</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>5% GSE film</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>10% GSE film</td>
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<td>6.5</td>
</tr>
<tr>
<td>15% GSE film</td>
<td>2</td>
<td>8.5</td>
</tr>
</tbody>
</table>

GSE: green seed extract
4.3.5 **Virucidal/Antiviral testing of GSE in aqueous and film forming solutions**

The grape seed extract was dissolved in deionized water and filter sterilized using a 0.2μm filter. Concentrations of up to 2.5% GSE dissolved in deionized water (aqueous solutions) were tested by adding equal volumes of the virus suspension (10^7 PFU/ml).
Volumes of 0.5ml of both the virus suspensions and aqueous extract solutions were mixed to give final extract concentrations of 1.0, 1.5 and 2.5%. The mixtures were incubated at 23 ± 1°C on a rotary shaker for 3 h. For the control, an equal volume of DMEM was mixed with virus suspension. After 3 h, 0.5ml of the treated samples and the control were withdrawn and diluted with 0.5ml DMEM. After 10-fold serial dilutions, the appropriate solutions were plaque assayed as previously described.

A similar procedure was followed for testing the reduction of virus infectivity by the film forming solution (FFS). To perform this test, chitosan solutions containing 5 and 10% GSE were mixed with equal volume of virus suspensions to yield final concentrations of 2.5 and 5.0% GSE, respectively. The controls were DMEM and 2% chitosan solution mixed with equal volumes of the virus suspensions.

4.3.6 Virucidal/Antiviral testing of chitosan/GSE films

In testing the virucidal activity of the films, the method of Haldar and others (2006) was used with slight modification. Film samples containing the different levels of GSE (5, 10 and 15%) and measuring 2.5cm X 2.5cm were individually placed in the bottom of one of the wells of a 6-well plate and 2ml of the virus suspension (~10^7 PFU/ml) added to each film. They were then incubated at 23 ± 1°C on an incubator shaker for 24 h. Virus suspensions with no added film and one with 2% chitosan film but with no added extract were used as controls. After 4 and 24 h, 0.5ml of the treated samples and controls were withdrawn and diluted with 0.5ml of DMEM. Ten-fold serial dilutions were then made and appropriate dilutions plaque assayed as previously described. The experiments were done in duplicates.
4.3.7 *Antibacterial activity testing of films*

For the bacterial testing, only the antimicrobial films were used. The bacterial species were individually cultured by transferring a loopful of frozen (-80°C in 30% glycerol) *E. coli* K12 and *L. innocua* into 20ml of sterile TSB with an inoculation loop. The organisms were incubated for 24 h at 37°C to reactivate them. Afterwards, a loopful of the revived bacteria was transferred to a TSA slants and incubated for 24 h at 37°C. The slants were then refrigerated at 4°C and used as a stock culture. Before each experiment, a loopful of the respective bacteria was taken from the slant and cultured in a 20ml sterile TSB for 24 h at 37°C. Bacterial suspensions containing approximately $10^7$ cfu/ml were prepared from the overnight culture and used for the antibacterial testing of the film.

A similar method used for the virucidal/antiviral testing was also used to quantify the bacteria survival. Film samples measuring 2.5cm x 2.5cm were placed in a dish to which 2ml of each ($10^7$ cfu/ml) bacteria suspensions added. Cultures without a film and another with the 2% chitosan film but no added extracts were used as controls. The dishes were incubated at 37°C and shaken at 60 rpm in an incubator shaker for 24 h. Samples were taken at 0, 3, 6, 12, and 24 h intervals and diluted with 0.1% peptone. Appropriate dilutions were pour plated using TSA in duplicates. The plates were incubated for 24-48 h at 37°C and the number of colonies counted using a Darkfield plate counter (American Optical, Buffalo, NY).
4.3.8 **Statistical analysis**

All data were analyzed using the analysis of variance (ANOVA), and Tukey’s multiple comparisons test was used to determine significant differences between the means at a level of p<0.05. The statistical package used in the study was SPSS 20 for Windows. All the data represented are the means of duplicates. The statistical analysis was used to compare and determine the significant effect of the addition of GSE on (1) the antiviral efficacies of different concentrations of GSE dissolved in deionized water and chitosan film forming solutions on MNV-1; (2) the antiviral and antibacterial efficacies of chitosan films containing 3 levels (5, 10 and 15%) of GSE on MNV-1, and *E. coli* K12 and *L. innocua*, respectively. The Tukey’s test was therefore used to determine differences in means between the control and the GSE films.

4.4 **Results**

4.4.1 **Antiviral/virucidal effect of GSE in aqueous GSE and film forming solutions**

Figure 4.2 shows the result of the virucidal effects of GSE against MNV-1 when dissolved in deionized water (aqueous extract solutions). The results show that log reductions of 1.75, 2.60 and 3.58 log$_{10}$ PFU/ml were obtained by the 1.0, 1.5 and 2.5% GSE aqueous solutions, respectively after 3 h. The antiviral/virucidal efficacy of the extract was further assessed when they were incorporated into the chitosan film forming solutions. The results shows that the 2.5 and 5.0% GSE samples presented in Figure 4.3 produced MNV-1 titer reductions of 2.68 and 4.00 log$_{10}$ PFU/ml, respectively.
Figure 4.2. Infectivity of MNV-1 after treatment with different levels of grape seed extract solution as detected by plaque assay after 3 h. Error bars indicate the data range.

Figure 4.3. Infectivity of MNV-1 after treatment with chitosan film forming solutions (FFS) with different levels of grape seed extract (GSE) as detected by plaque assay after 3 h. Error bars indicate the data range.
4.4.2 Antiviral/virucidal effect of the GSE films

In Figure 4.4, the results of the chitosan films incorporated with the different levels of GSE are presented. The films results show that they significantly (p< 0.05) reduced MNV-1 titers. The films containing 5, 10, and 15% GSE resulted in reductions of 0.92, 1.89 and 2.27 log_{10} PFU/ml, respectively after 4 h incubation. After 24 h of incubation, titer reductions of 1.90 and 3.26 log_{10} PFU/ml were obtained for the 5 and 10% GSE films, respectively. The 15% GSE film reduced MNV-1 titers to undetectable levels after 24 h.

![Figure 4.4 Infectivity of MNV-1 after treatment with Chitosan film with different levels of grape seed extract (GSE) detected by plaque assay after 4 and 24 h. Error bars indicate the data range.](image-url)
The antibacterial efficacy of the GSE films was investigated against *L. innocua* and *E. coli* K12. In conducting the experiment, two different controls were tested. These were the one having no added film and the other having 2% chitosan. Figures 4.5 and 4.6 summarize the results obtained. Bacterial viability was assessed at times of 0, 3, 6, 12, and 24 h of incubation. After 3 h of incubation, the 5, 10 and 15% GSE films reduced *L. innocua* counts significantly (p<0.05) by 1.70, 1.88 and 2.32 log\(_{10}\) CFU/ml, respectively. Although, GSE films reduced *L. innocua* after 6 h, there were no differences between the 10 and 15% GSE films, which recorded reductions of 2.75 and 2.84 log\(_{10}\) CFU/ml, respectively. The 5% GSE film resulted in 1.88 log\(_{10}\) CFU/ml reductions of *L. innocua* after 6 h. Also, *L. innocua* reductions of 2.39, 3.60 and 3.69 log\(_{10}\) CFU/ml were recorded at 12 h. Reductions of 3.06, 6.15 and 6.91 log\(_{10}\) CFU/ml at 24 h by the 5, 10 and 15% GSE films, respectively.

A similar trend was obtained for *E. coli* K12. At 3 h, reductions of about 2 log cycles were recorded for the 10 and 15% GSE films. After 6 h of incubation, reductions of 2.28, 2.70 and 2.82 log\(_{10}\) CFU/ml were recorded by the 5, 10 and 15% GSE films, respectively. There were no significant differences between the different levels of GSE in the films at 6 h of incubation. However, the GSE films significantly (p<0.05) reduced *E. coli* K12 levels compared to the control. The 5, 10 and 15% GSE films reduced the population by 2.36, 3.02, and 3.62 log\(_{10}\) CFU/ml, respectively after 12 h. Finally, after 24 h of incubation, the films containing 5% GSE reduced *E. coli* K12 counts by 2.28, the 10% GSE film by 5.18 and the 15% GSE film by 7.14 log\(_{10}\) CFU/ml.
Figure 4.5. The effect of GSE incorporated films on *L. innocua* in tryptic soy broth. Each column represents average of duplicate experiments. Error bars indicate the data range.

Figure 4.6. The effect of GSE incorporated films on *E. coli* K12 in tryptic soy broth. Each column represents average of duplicate experiments. Error bars indicate the data range.
4.4.5 Discussion

4.4.5.1 Antiviral/virucidal activity of GSE

Nonenveloped viruses tend to be resistant against commonly used preservatives in the food industry (Li and others 2012b). In this study, we evaluated the antiviral/virucidal activity of GSE. Since GSE is known to enhance the inactivation of foodborne bacterial pathogens, our main interest was to see if it can also inactivate MNV-1. Therefore, we incorporated GSE into chitosan film forming solutions and films and tested them on their antiviral/virucidal activities. Aqueous extract solutions were tested. From the results obtained, the aqueous GSE solutions inactivated MNV-1. Also, when incorporated into the chitosan films or film forming solutions, the GSE enhanced the antiviral/virucidal activity of the FFS and the resultant film. Grape seed extracts are rich in oligomeric proanthocyanadin (OPC) which are the dimeric, trimeric and tetrameric forms of the catechins. Su and D’Souza (2011) studied the effect GSE against viral surrogates including MNV-1, FCV, Feline Calicivirus, and bacteriophage MS2 in solution and reported reductions of viral infectivity. Additionally, Iwasawa and others (2009) demonstrated the effect of proanthocyanidins on feline calicivirus and coxsackie A7. Furthermore, Li and others (2012b) recently confirmed the virucidal activity of GSE. These authors investigated the anti-norovirus activity of GSE on MNV-1, human norovirus virus-like particles (VLP), human norovirus P particles and human norovirus GII.4. Results reported by Li and others (2012b) included the reductions of binding levels of the human norovirus GII.4 and the P particles. They also reported MNV-1 titers reductions after incubation with GSE. When Transmission Electron Microscopy (TEM)
was used to assess the morphology of the VLP, the structure of the untreated VLP sample was where spherical in shape. However, the GSE treated VLP samples had deformations and inflations which signifies that GSE damaged the capsid proteins (Li and others 2012b). Su and D’Souza (2011) also reported that GSE can affect viral adsorption and replication using pre- and post-infection viral studies, respectively. Other polyphenols and polyphenol rich products such as pomegranates, cranberry juice, and gallic acid have also reduced titers of norovirus surrogates (Su and others 2010a, 2010b; Su and D’Souza 2011, D’Souza and others 2011). The mechanism(s) responsible for the antiviral/antiviral activity of GSE therefore includes disruption of capsid protein, interfering with viral attachment/adsorption and/or interfering with viral replications.

4.4.5.2 Antibacterial activity of GSE

The quest for antimicrobial agents with broad spectral activities has resulted in an increased interest in the use of naturally-derived plant extracts. Additionally, consumers are increasingly demanding foods that are free from synthetic chemicals. In this study, the antibacterial activities of the GSE films were also tested against L. innocua and E. coli K12. Here too, GSE addition enhanced the efficacy of the edible films. Corrales and others (2009) incorporated 1% GSE in a pea starch film. The results showed a reduction in the growth of Brochothrix thermosphacta on pork loins by 1.3 log_{10} CFU/ml after 4 days at 4°C. Additionally, Moradi and others (2011) reported that 1% GSE incorporated into chitosan films reduced aerobic mesophilic and lactic acid bacteria populations on sausage by 1.1 and 0.7 log_{10} CFU/ml respectively, after 21 days of storage. In yet another example, Gadang and others (2008) incorporated 3% GSE into protein coatings and
reported a reduction of *Salmonella typhimurium* counts by 2 log$_{10}$ CFU/ml. In some instances, GSE are used in combination with other preservatives such as nisin, oregano oil, EDTA and malic acid (Moradi and others 2011, Gadang and others 2008, Theivendran and others 2006). For instance, soy protein edible films containing 1.0% GSE combined with 1.0% nisin resulted in 2.8 log$_{10}$ CFU/ml reductions against *L. monocytogenes* in turkey frankfurter (Theivendran and others 2006). In this present study, high levels of GSE were incorporated into the chitosan films and this enhanced the film’s efficacy against *L. innocua* and *E. coli* K12.

In explaining the mechanism of action for GSE, Lin and others (2004) stated that the partial hydrophobic nature of polyphenolic compounds enhances their accumulation and attachment to the bacterial cytoplasmic membrane. Therefore, polyphenolic compounds are expected to initiate antibacterial activity through interactions with the outer cell membrane of the bacterium. The antibacterial efficacy of these polyphenols correlates with the presence of reactive groups such as the hydroxyl groups, galloyl moieties and conjugated double bonds (Utlee and others 2002, Ikigai and others 1993). The presence of these reactive groups enhances the efficacy of the polyphenols. Consequently, the interaction of these active groups with the cell membrane would lead to cell death through the physical disruption of the membrane, dissipation of the proton motive force (PMF) and/or inhibition of membrane-associated enzyme activity (Shimamura and others 2007, Juven and others 1994). This increases membrane permeability and leakage of the cellular constituents. Once the membrane is damage, the polyphenols can also enter into the interior of the cell and subsequently impairs
enzymatic activity and destroy genetic materials (Ikigai and others 1993). Also, chitosan by itself is known to inhibit bacterial growth by increasing cell permeability, interacting with DNA and messenger RNA and acting as chelating agent that binds metals and nutrients (Martinez-Camacho and others 2010, Tripathi and others 2010, Rabea and others 2003).

4.4.5 Conclusion

It can be concluded that GSE was successfully incorporated into chitosan-based films. Also GSE had antiviral/virucidal and antibacterial efficacy when incorporated into of the chitosan films. When in the film forming solution, GSE was also effective as an antimicrobial agent. This showed that compounds in the film solution did not significantly interfere with the antimicrobial properties of the GSE.
CHAPTER 5

GREEN TEA AND GRAPE SEED EXTRACTS INCORPORATED INTO CHITOSAN FILMS AND THEIR EFFECTS ON THE MECHANICAL AND MOISTURE BARRIER PROPERTIES OF THE MATERIAL

5.1 Abstract

The objective of this study was to investigate the effect of green tea extract (GTE) and grape seed extract (GSE) on the mechanical and water barrier properties of chitosan film. The chitosan film was fabricated with 5, 10 and 15% GTE or GSE. Glycerol was also added in order to produce a film with more flexibility. To optimize the quantities of glycerol, GTE and GSE in the chitosan film samples, the tensile strength (TS), percent elongation at break (%E), thickness, moisture content, water vapor permeability and solubility were determined for each treatment combination. The results showed that, TS significantly (p<0.05) decreased after the incorporation of glycerol. The tensile strength of 2% chitosan (used as the control) was 48.09 ± 5.17 MPa. The films incorporated with GTE or GSE and glycerol were significantly (p<0.05) lower in TS compared to the control. Values of 11.07 ± 2.50, 6.42 ± 0.68 and 6.55 ± 0.80 MPa were recorded for the 5, 10, and 15% GSE films, respectively. For the 5, 10 and 15% GTE films, TS values of
12.48 ± 2.52, 5.43 ± 0.59 and 5.07 ± 1.27 MPa were obtained, respectively. Also, the %E obtained for the 2% chitosan control film was 4.98 ± 0.94. The 5, 10 and 15% GSE films produced %E values of 16.75 ± 2.02, 30.95 ± 6.92 and 30.22 ± 4.98%, respectively. The values obtained for the 5, 10, and 15% GTE films were 23.77 ± 3.30, 39.12± 4.27 and 43.49 ± 1.06%, respectively. The WVP of chitosan film without the extract was 0.349 ± 0.039 g.mm/m².h. kPa. The 5, 10, and 15% GTE films produced WVP values of 0.417 ± 0.007, 0.457 ± 0.005 and 0.569 ± 0.025 g.mm/m².h. kPa, respectively. Also, the 5, 10 and 15%GSE films recorded WVP values of 0.422 ± 0.085, 0.496 ± 0.083 and 0.524± 0.029 g.mm/m².h. kPa, respectively.

5.2 Introduction

Edible films and coatings represent examples of environmentally friendly packaging and contribute to the reduction of environmental pollution. Additionally, edible films and coatings can serve as carriers for active and functional ingredients including antioxidants, antimicrobials, minerals and nutraceuticals. The mechanical and barrier properties of the film are two very important factors that are known to affect the performance of edible films. Factors that can influence these properties are the structure and chemistry of the biopolymer, the film forming methods, and the nature of the additives incorporated during the film formation (Janjarasskul and Krochta 2010).

Mechanical properties are known to influence how films behave during handling and storage. These determine the durability of the films and hence the applications to which they can be put to use (Ghanbarzadeh and Almasi 2011). For food packaging applications, the film must be durable, stress resistant, flexible and elastic. Tensile testing
can be used to determine mechanical properties such flexibility, toughness and elongation of the film (Hernandez-izquierdo and Krochta 2008).

A barrier to moisture is also a very important property that affects the performance of edible coatings and films. When compared with their synthetic counterparts, edible films are poor barriers to moisture because of the hydrophilic nature of the hydrocolloids used to form them. Also, to improve the flexibility of edible films, plasticizers are typically added. Unfortunately, this also contributes to the poor barrier properties of these films. Additionally, the hydrophilic nature of plasticizers contributes to a reduction of the film’s barrier properties. Moisture sensitivity of edible films is commonly measured by determining the water vapor transmission rate (WVTR) through a film of known area and thickness over time. From the WVTR, the water vapor permeability (WVP) of the film is then computed.

The film’s solubility also affects its end use. Some applications may require that the film is highly soluble, whereas others may require them to be less permeable to water. For instance, when films are used on high-moisture foods, they must be insoluble, whereas films used for water soluble pouches must be readily soluble (Sothornvit and Krochta 2000). When additives such as antimicrobial and antioxidants are added, it is important that the film release these additives to the packaged product in a timely manner. This is so because the additives are designed to migrate to the packaged food. In an aqueous environment, this involves the film’s ability to adsorbed water and become plasticized in the process. This helps to open up void spaces between the polymeric chains and cause the release of additives that were initially incorporated into the polymer.
Chitosan is derived from chitin by deacetylation (removal of acetyl groups). In the deacetylation process, chitin is heated with an aqueous 40–45% (w/v) NaOH solution at 90–120°C for 4–5 h (Dash and others 2011). The amide and hydroxyl groups on chitosan provide reactive sites for various reactions and this makes it possible to alter the biological and functional properties of the biopolymer (Dash and others 2011). This also allows the incorporation of polyphenols such as those found in green tea and grape seed extracts. Polyphenols interact with chitosan through hydrogen and covalent bonding (Zhang and Kosaraju 2007). Siripatrawan and Harte (2010) reported on the interaction of green tea polyphenolic compounds via the hydroxyl and amino groups in the chitosan matrix.

Since the incorporation of additives may alter the mechanical and barrier properties of the film, it is important to investigate these properties anytime a film is developed. Therefore, the objective of this study was to investigate how the addition of glycerol, grape seed and green tea extracts to chitosan films affects its tensile strength, percentage elongation at break, water solubility and WVP.

5.3 Materials and Method

5.3.1 Materials

Medium molecular weight chitosan powder (94% purity, 75% deacetylation) was obtained from Huantai Goldenlake Carapace Products Co., Ltd (Tsingtao, China) and used to form the films. Glycerol was obtained from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ). Glacial acetic acid was purchased from J.T Baker (Phillipsburg, N.J).
Commercial green tea extract (GTE), (standardized 98% polyphenols, 80% catechins, 0.7% caffeine and 50% epigallocatechin gallate) was purchased from Herbstore USA (Walnut, CA). Commercial grape seed extract (GSE), Leucoselect® was obtained from Indena USA (Seattle, WA). Leucoselect® contained $\geq 95.0\%$ $\leq 105.0\%$ proanthocyanidins, as determined by Gel permeation chromatography, and $\geq 13.0\%$ $\leq 19.0\%$ catechin and epicatechin, as determined by high pressure liquid chromatography.

5.3.2 Film formation

To make the films, chitosan (2% w/w) was dissolved in 1% acetic acid solution with glycerol as a plasticizer (Figure 5.1). Green tea and grape seed powders at different concentrations (0-15%) were added to the chitosan solution (Table 5.1).

<table>
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<tr>
<th>Antimicrobial film</th>
<th>Amount (g)</th>
<th>Amount of deionized water (ml)</th>
</tr>
</thead>
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<td></td>
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<td>Glycerol</td>
</tr>
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</tr>
<tr>
<td>5%GTE film</td>
<td>2</td>
<td>3</td>
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<tr>
<td>10%GTE film</td>
<td>2</td>
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</tbody>
</table>

GTE: Green tea extract, GSE: Grape seed extract
The solutions were stirred using a magnetic stirrer for about 30min to dissolve the extracts. The film forming solution was then degassed using a water bath sonicator. A 20g aliquot of the solution was poured onto a polypropylene sheet attached to a glass plate and a drawdown bar used to spread the solution into a thin film. After drying in an oven at 45°C for 2 h, the films were peeled and stored for further testing.

**5.3.3 Film thickness**

The film thickness was measured with a micrometer (Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) around the film at 5 random positions. The means of the 5 values
of the film thicknesses were used in the calculations for water vapor permeability and mechanical testing.

5.3.4 Film Solubility

The method of Jangchud and Chinnan (1999) was used to measure the film’s solubility with slight modification. Dried film pieces, weighing 100 ± 5mg were immersed into 30ml of distilled water in 50ml screw cap centrifuge tubes. The tubes were capped and placed at 23 ± 1°C for 1 h and shaken intermittently. The solutions and film pieces were then poured onto a Whatman #1 qualitative filter paper, rinsed with 10 ml distilled water, and dried at 105°C in an oven for 24 h to determine the dry mass. Triplicates measurements were done for each film type. The film solubility (FS) was determined using Equation 5.1:

\[
FS = \frac{(\text{Initial film weight} - \text{final film weight})}{\text{Initial film weight}} \times 100 \quad (5.1)
\]

5.3.5 Mechanical testing

Prior to testing the mechanical properties, the films were conditioned for 24 h at a relative humidity (RH) of 50 ± 1% and 24 ± 1°C (environmental condition in the room where testing was done). Tensile strength (TS) and percent of elongation at break were determined using an Instron Universal Testing Machine (Instron Corporation, Canton, MA) equipped with a 500N load cell. Rectangular strips (1 inch wide and 3.5 inches in length) were cut from each film type to measure the mechanical properties. The cross-
head speed was set at 0.5 mm/s. Tensile strength expressed in MPa was calculated by dividing the maximum load \( F_{\text{max}} \) necessary to break the film sample by the cross-sectional area \( A \) (m\(^2\)) based on Equation 5.2. Percentage elongation at break \( (%E) \) was calculated by dividing the film’s elongation \( \Delta l \) at the moment when the film ruptured by the initial grip length of samples \( l_0 \) multiplied by 100 (Equation 5.3). A total of at least 5 samples were tested for each film type.

\[
TS = \frac{F_{\text{max}}}{A} \quad (5.2)
\]

\[
%E = \frac{\Delta l}{l_0} \times 100 \quad (5.3)
\]

5.3.6 Water vapor permeability

Water vapor permeabilities (WVP) of the films were evaluated using a gravimetric test according to ASTM E96 (1993), using the desiccant method. Test cups of diameter 5 cm were filled with 10 g anhydrous calcium sulfate to produce a RH of 0% inside the cup. The film samples were placed on top of the cup and sealed with an o-ring. A high vacuum silicone sealant was applied between the o-ring and the film samples, between the sealing lip of the cup and the sample, before clamping them with 4 screws. The cups containing the desiccants were weighed to give the initial weight and then placed in a humidity chamber at \( 25 \pm 1 \) °C and RH of \( 55 \pm 2\% \). At regular time intervals the cups were weighed until a steady state was reached. The amount of water vapor transmission rate (WVTR) through the film was estimated from the linear portion of the
plot of weight gained versus time and the slope divided by the film exposure area according to Equation 5.4. Three replicates per film were tested. The WVP of the film was calculated by multiplying the WVTR by the film’s thickness and dividing that by the water vapor pressure difference across the film (Equation 5.5).

\[
WVTR = \frac{\text{Slope}}{A} \ldots (5.4)
\]

\[
WVP = \frac{WX}{At(P2-P1)} (5.5)
\]

Where \( w \) is the weight gain of the cup (g), \( x \) is the film thickness (mm), \( A \) is the area of exposed films (m\(^2\)), \( t \) is the time of gain (s), and \( (P_2-P_1) \) is the vapor pressure differential across the film (MPa). The water vapor pressure \( (P_1) \) on the desiccant side of the film was assumed to be zero and the water vapor pressure \( (P_2) \) in the humidity chamber was calculated from the vapor pressure of pure water at the selected condition.

5.3.8 Statistical analysis

All data were analyzed using the analysis of variance (ANOVA), and Tukey’s multiple comparisons test was used to determine the significant differences between the means at a level of \( p<0.05 \). The statistical package used in the study was SPSS 20 for Windows. The statistical analysis was used to compare and determine the significant effect of the addition of glycerol and GTE or GSE on the thickness, solubility, moisture content, and the mechanical and barrier properties of the chitosan films. The Tukey’s test was therefore used to compare the control to the different levels of the GSE or GTE and glycerol on the film’s properties.
5.4 Results and discussion

5.4.1. Thickness, water solubility and moisture content

The thickness of the films were averages from 5 readings and are shown in Table 5.1. The film thicknesses ranged from 0.024 ± 0.002mm for pure chitosan film to 0.113 ± 0.005mm for 15% GSE film. The thickness of the chitosan films significantly increased (p < 0.05) after the addition of the glycerol and, green tea and grape seed extracts. Film thickness is affected by the amount of the total dissolved solids in the film forming solution. It was therefore expected that with the increasing concentration of glycerol and the plant extracts, the final thickness of the GTE/GSE films would be greater than the control chitosan film, as long as the same volume of solution is used for each formula.

Also, presented in Table 5.1 are the moisture content values of the chitosan films with and without the plant extracts. From the results obtained, chitosan film was highest with a moisture content value of 31.53 ± 2.91%. Contrary to the results obtained in this present study, Rubilar and others (2013) reported an increased in moisture content when GSE was added to chitosan films. However, Wang and others (2013) reported that increasing total polyphenols content in chitosan films reduced the moisture content which agrees with the results obtained in the present study. According to the authors, the interaction between chitosan and the polyphenols reduced the availability of amino and hydroxyl groups in chitosan for hydrogen bonding with water. Also, glycerol as a humectant can chemically bind to water and thereby inhibiting its evaporation (Enrione and others 2007). Therefore, it is possible that the levels of glycerol affected the moisture evaporation. Since the loss of water was used in the calculation of moisture
Table 5.1 Film moisture content and thickness

<table>
<thead>
<tr>
<th>Type of film</th>
<th>Glycerol added (%)</th>
<th>Dissolved solids (%)</th>
<th>Film thickness (mm)</th>
<th>Moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%CHI</td>
<td>0.6</td>
<td>2.53</td>
<td>0.024 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.53 ± 2.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%GTE</td>
<td>3</td>
<td>7.41</td>
<td>0.061 ± 0.007&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.11 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%GTE</td>
<td>5</td>
<td>13.04</td>
<td>0.078 ± 0.009&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.13 ± 0.86&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15%GTE</td>
<td>7</td>
<td>18.03</td>
<td>0.108 ± 0.003&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19.84 ± 0.62&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%GSE</td>
<td>4</td>
<td>8.26</td>
<td>0.063 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.52 ± 1.71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%GSE</td>
<td>6.5</td>
<td>14.16</td>
<td>0.073 ± 0.005&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.60 ± 0.66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15%GSE</td>
<td>8.5</td>
<td>19.02</td>
<td>0.113 ± 0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.28 ± 0.81&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> In a given column, values with same letters are not significantly different (p>0.05), different letters are significantly different (p<0.05).

Fig 5.2 Percent solubility of chitosan films incorporated with green tea and grape seed extracts with different levels of glycerol compared with 2% chitosan film control (2% CHI).
contents, inhibiting evaporation will result in lower loss of water and thus a lower moisture content of the sample.

The solubilities of the films are also presented in Figure 5.2. Here too, the films containing the same levels of GSE or GTE did not significantly differ in solubilities (p>0.05). Solubility is important because it affects the biodegradability of films when used as packaging materials (Gnasambadam and others 1997). It is also important for potential applications because sometimes high water solubility is desired, especially when the film would be consumed as part of the food. However, low solubility is necessary, when used in the packaging of high moisture foods (Rhim and others 1998). The pure chitosan film had the lowest solubility (23.97 ±3.19%). Significant (p<0.05) solubility values of 56.94 ± 7.80 and 65.62 ± 4.90% were obtained for the 5% GTE and GSE films, respectively. The films containing 10 and 15% GSE or GTE recorded solubilities above 90%. The higher solubilities of the films were as a result of the water binding ability of the gycerol plasticizer.

5.4.2 Film Mechanical properties

Tensile testing provides valuable information about the strength and elasticity of the film. The mechanical properties (TS and %E) of chitosan films with or without GTE and GSE are presented in Figures 5.3 to 5.6. From the results obtained, the TS of 2% chitosan, used as control was 48.09 ± 5.17 MPa. Films incorporated with GTE or GSE and glycerol were significantly (p<0.05) lower in TS compared to the control. Values of 11.07 ± 2.50, 6.42 ± 0.68 and 6.55 ± 0.80 MPa were recorded for the 5, 10, and 15% GSE
films, respectively. For the 5, 10 and 15% GTE films, TS values of 12.48 ± 2.52, 5.43 ± 0.59 and 5.07 ± 1.27 MPa were obtained, respectively. The films containing 5% GSE or GTE was significantly higher (p<0.05) in TS compared to the 10 and 15% films. However, the films containing 10 and 15% GTE or GSE did not differ significantly (p>0.05). A previous study by Siripatrawan and Harte (2010) reported an increased in TS with the addition of GTE polyphenols in chitosan. However, in that study they used aqueous tea infusions as the source of green tea polyphenols and they were able to keep the concentration of glycerol plasticizer at the same lower level as that of the control. The tea infusion used in that study was not high in polyphenol content. In this present study, we used commercial extracts with high levels of concentrated polyphenols. Therefore, the glycerol concentration was increased in order to produce a film that was more flexible. Consequently, the glycerol reduced the TS. Another important factor responsible for the TS of films is its crystallinity. Higher crystallinity comes from higher interchain interaction and regularity within the polymer matrix. Stiffer films would be obtained when there is an increased crosslinking between the polymer chains. However, when additives such as plasticizers are added, they are known to disturb the cohesiveness of the polymer chains. Plasticizers are known to increase the free volume that this results in the lowering of the glass transition temperature (Han 2005; Daniels 2009). Although polyphenols in plant extracts have been used as crosslinking agents to increased TS at lower levels (Orliac and others 2002; Gómez-Guillén and others 2007), the high levels of plant extracts used in this present study required the addition of high amounts of glycerol plasticizer.
As expected, increasing the level of glycerol plasticizer increased the %E of the films. The %E obtained for the 2% chitosan control film was 4.98 ± 0.94%, which was significantly lower (p<0.05) compared to the films containing GTE or GSE. The 5, 10 and 15% GSE films produced %E values of 16.75 ± 2.02, 30.95 ± 6.92 and 30.22 ± 4.98%, respectively. The values obtained for the 5, 10, and 15% GTE films were 23.77 ± 3.30, 39.12 ± 4.27 and 43.49 ± 1.06%, respectively. The 10 and 15% films did not differ significantly (p> 0.05) in %E. The %E was due to reduced interaction (reduced crosslinking) between the polymer chains due to added glycerol.

From the literature, disparities in TS and %E values of pure chitosan films are reported. These are typically due to differences in molecular weight, degree of deacetylation and concentration of chitosan used, the type and concentration of plasticizer, and the type of the solvent used (Nunthanid and others 2001; Martins and others 2012; Sánchez-González and others 2009). Increasing molecular weight, concentration of chitosan and degree of deacetylation increases the TS and decreases %E. This is because of higher entanglement of the polymer network, which can result in high amount of chitosan and molecular weight. Also, removal of bulky acetyl groups in deacetylation would result in higher interchain interaction (Nunthanid 2001) and this would increase TS and decrease %E. Rubilar and others (2013) reported values of 49 MPa and 28% for TS and %E, respectively. Souza and others (2010) on the other hand reported lower values (TS = 13 MPa and %E = 16%). In yet another example, Vargas and others (2009) reported TS and %E values of 12 MPa and 17%, respectively. Moreover, Altiok and others (2010) reported %E value of 2% chitosan film as low as 4.8%.
Fig 5.3 Tensile strength (MPa) of chitosan films incorporated with green tea extract (GTE) and glycerol (GLY) compared with 2% chitosan film control (2% CHI).

Figure 5.4. Tensile strength (MPa) of chitosan films incorporated with grape seed extract (GSE) and glycerol (GLY) compared with 2% chitosan film control (2% CHI).
Figure 5.5. Percentage elongation of chitosan films incorporated with grape seed extract (GSE) and glycerol (GLY) compared with 2% chitosan film control (2% CHI).

Figure 5.6. Percentage elongation of chitosan films incorporated with grape seed extract (GSE) and glycerol (GLY) compared with 2% chitosan film control (2% CHI).
5.4.3 Film moisture barrier properties

The moisture vapor permeabilities of the films are represented in Figures 5.7 and 5.8. As can be seen from the plots, WVP increased with increasing levels of glycerol. From the results obtained, the WVP of the chitosan film without the extract was 0.349 ± 0.034 g.mm/m².h. kPa. The 5, 10, and 15% GTE films produced WVP values of 0.417 ± 0.008, 0.457 ± 0.006 and 0.569 ± 0.028 g.mm/m².h. kPa, respectively. Also, the 5, 10 and 15%GSE films recorded WVP values of 0.422 ± 0.069, 0.496 ± 0.069 and 0.524 ± 0.024 g.mm/m².h. kPa, respectively. The concentrations of glycerol in the GTE films were 3, 5 and 7% for the 5, 10 and 15% GTE, respectively. The GSE films on the other hand contained 4, 6.5 and 8.5% glycerol for the 5, 10 and 15% GSE, respectively.

Different WVP values of chitosan films have been reported due to factors such as molecular weight, degree of deacetylation, type and amount of plasticizers and film preparation method (Wang and others 2013, Siripatrawan and Harte 2010, Park and Zhao 2004). For example, Wang and others (2013) and Siripatrawan and Harte (2010) reported WVP values of pure chitosan of 0.496 and 0.256 g.mm/m².h. kPa, respectively. Siripatrawan and Harte (2010) reported that GTE inclusion in chitosan reduce WVP because the resultant films were denser than the control due to an increased intermolecular interactions. Similarly, Wang and others (2013) found a reduction of WVP values because tea polyphenols resulted in a denser film which resulted in a reduced interstitial space in the film matrix. Additionally, the interaction between polyphenols and chitosan reduced the hydrophilic hydroxyl groups. Other researchers also found that compounds such as mineral and vitamins incorporated into edible films
increased interchain interactions and resulted in a reduction in WVP (Park and others 2004; Gómez-Guillén and others 2007).

As in the case of the TS data, as a result of the high concentrations of the polyphenolic compounds in the commercial extracts used in this study, glycerol also at high amounts was needed to produce a more flexible film. Without the addition of a plasticizing agent, edible films tend to become brittle because of extensive intermolecular interactions between the functional groups within biopolymer (Nur Hanani and others 2013). However, plasticizers such as glycerol can also increase the film’s WVP. For instance, Farahnaky and others (2013) reported that glycerol reduce film’s crystallinity. This was due to a reduction in the attractive intermolecular forces and an increased in free volume. Therefore, there was a greater space for the water molecules to migrate through the film matrix (Sothornvit and Krochta 2000).

The increasing WVP values as observed for the films with increasing glycerol levels can also be attributed to the hydrophilic nature of the films due to the plasticizer (McHugh and Krochta 1994). Consequently, water diffusion rate within the film increased. The barrier properties of a film matrix are dependent on its chemical structure and morphology, compatibility with the permeant and the environmental temperature (Siripatrawan and Harte 2010). In this study, the chemical structure and morphology of the films accounted for the differences in WVP values recorded.
Figure 5.7. The effect of incorporation of green tea extracts (GTE) and glycerol (GLY) on the water vapor permeability of chitosan films.

Figure 5.8. The effect of incorporation of grape seed extracts and glycerol (GLY) on the water vapor permeability of chitosan films.
5.4.5 Conclusions

Green tea and grape seed extracts rich in polyphenols were successfully incorporated into the chitosan films. Even though, flexible films were able to be produced with the aid of glycerol as plasticizer, the films were lower in tensile strength but were more elastic than the control films. Increasing glycerol levels increased the film’s solubility and WVP. The films can be used as edible coating as part of a multilayer film system or in applications where high solubility is required.
CHAPTER 6

CONCLUSIONS AND FUTURE WORK

Grape tea and grape seed extracts, rich in polyphenolic compounds, were successfully incorporated into chitosan-based edible coatings and films. The antiviral/virucidal and antibacterial efficacies of the chitosan films were enhanced by the incorporation of the plant extracts. Flexible films were able to be produced with the aid of glycerol as plasticizer. However, the films were lower in tensile strength because of the added glycerol but were more elastic compared to the control film. Glycerol also caused an increase in the water vapor permeability of the films.

Future work should be aimed at improving the mechanical strength or lowering the solubility, especially for applications that requires more rigid films but with greater solubility. In such cases, it might be necessary to blend the edible film with other bio or synthetic polymers. Also, the incorporation of hydrophobic compounds could help to decrease the water solubility and permeability. Additionally, the use of reinforcing nano-sized particles could also help to increase the tensile strength if required. Moreover, there is the need to investigate the effect of the films or coatings on viruses and bacteria in foods. This is so because this study was limited to in vitro testing.
REFERENCES


Embuscado ME and Huber KC. 2009. Edible films and coatings for food applications. Springer Science, Business Media, LLC, New York, USA.


Kolodziejska I, Piotrowska B. 2007. The water vapour permeability, mechanical properties and solubility of fish gelatin–chitosan films modified with transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and plasticized with glycerol”, Food Chemistry, 103: 295-300.


Li D, Baert L, Zhang D, Xia M, Zhong W, Van Coillie E, Jiang X, Uyttendaele M. 2012b. Effect of grape seed extract on human norovirus GII.4 and murine norovirus 1 in viral suspensions, on stainless steel discs, and in lettuce wash water. Applied Environmental Microbiology. 78(21):7572-8


Pérez-Pérez C, Regalado-González C, Rodríguez-Rodríguez CA, Barbosa- Rodríguez JR, Villaseñor-Ortega F. 2006. Incorporation of antimicrobial agents in food packaging films and coatings. Advances in Agricultural and Food Biotechnology 193-216.

Perumalla AVS and Hettiarachchy NS. 2011. Green tea and grape seed extracts e potential applications in food safety and quality. Food Research International 44:827-839.


Soliman KM, Badeaa RI. 2002. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chemical Toxicology. 40:1669-1675.


Theivendran S, Hettiarachchy NS and Johnson MG. 2006. Inhibition of Listeria monocytogenes by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. *Journal of Food Science* 71:39–44.


