DEVELOPMENT OF ANTIMICROBIAL COMPOSITE MATERIALS FOR FOOD PACKAGING APPLICATIONS

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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ABSTRACT

Due to increasing environmental concerns regarding plastic waste, the development of composite film materials from biodegradable polymers and natural food additives is gaining more attention. Over the last few decades, the safety of synthetic additives that are commonly added as food additives has also been questioned because of their potential toxicity, carcinogenicity and teratogenicity effects. Natural antimicrobial agent could be a promising and versatile component in food packaging materials to improve their physiochemical, antioxidant and antimicrobial properties and hence, improve food quality and shelf life. In this study, novel chitosan/acetylated starch and polyvinyl alcohol/chitosan composite films were developed via a solution casting technique. In addition, a novel *Escherichia* phage, CAM-21, was isolated from a slurry lagoon at a local dairy farm before incorporating into soy protein isolate-based films. The results indicate that the chitosan/acetylated starch films incorporated with cinnamon and clove essential oils displayed enhanced light, water vapor, and oxygen barrier characteristics, as well as excellent antibacterial activity against spoilage bacteria and the pathogen, *Escherichia coli* O157:H7, on beef. Polyvinyl alcohol/chitosan films incorporated with aminosilane-modified bacterial nanocellulose and 4-hexylresorcinol exhibited improved mechanical, water vapor barrier, antioxidant, and antimicrobial properties against spoilage bacteria on

vacuum packaged refrigerated raw beef. Also, CAM-21 has a broad host spectrum against various serotypes of Shiga toxin-producing *E. coli*. The growth of *E. coli* O157:H7 was effectively controlled in phage-treated milk, ground beef and baby spinach. Soy protein isolate films incorporated with CAM-21 showed excellent antimicrobial activity against *E. coli* O157:H7 in broth and refrigerated beef products. This project demonstrates the potential application of natural antimicrobial agents to produce novel composite films for antimicrobial food packaging.

Chapter 1 Introduction

1.1 Background

Food safety and quality are major concerns for both the food industry and consumers alike. Food spoilage may occur due to various physical, chemical, and microbiological changes that affect the food organoleptic properties and safety of the food for consumption. Food stability is determined by the changes in food components, including carbohydrate, lipid, protein, and water molecules, as a result of environmental and processing conditions (Cha & Chinnan, 2004). For instance, food spoilage caused by microbial growth can lead to a significant economic loss, as nearly a quarter of all the food products are contaminated before consumption (Chawla, Sivakumar, & Kaur, 2021). Microbial contamination can dramatically affect food quality by causing undesirable loss of texture, color, aroma, odor and nutritional value. Conventional food preservation techniques, such as irradiation, freezing, drying, and thermal processing have been widely utilized to eliminate the growth of spoilage and pathogenic bacteria. However, foods contaminated by foodborne pathogens are constantly recalled because they pose a significant hazard to public health. Thus, the development of novel food packaging materials with greater functionality are being investigated to address consumer demand for food safety and quality (Chawla et al., 2021).

Food packaging is one of the most critical parts of protecting food from microbial contamination and other detrimental physical factors, such as light, physical damage, pests, dust, moisture and oxygen, in order to maintain food quality and safety before consumption (Firouz, Mohi-Alden, & Omid, 2021; Yildirim et al., 2018). The shelf life of foods highly depends on both the intrinsic (e.g., water activity, pH, redox potential and nutritional

content) and extrinsic factors (e.g., gas composition, humidity and storage temperature) (Zinoviadou, Koutsoumanis, & Biliaderis, 2016). The primary role of food packaging is to act as a protective barrier that can retard the deterioration rate of food products during food processing, handling and storage, resulting in increased shelf life and reduced losses of food products (Cha & Chinnan, 2004; Han, Ruiz-Garcia, Qian, & Yang, 2018).

Because of the increasing global population, the field of food packaging is expanding tremendously in both the industrial and scientific areas to fulfil the laws and regulations on food safety that are constantly being revised and improved. Also, due to increasing concerns about the safety of conventional food additives, consumers are demanding preservatives-free foods that are neither processed nor minimally processed, and have an optimal shelf life (Yildirim et al., 2018). As a result, the protective role of food packaging has been modified and enhanced to meet higher consumer expectations. Over the last few decades, a variety of novel packaging materials and technologies have been implemented, such as biodegradable packaging, edible packaging, and antimicrobial packaging, as detailed by researchers in several review articles (Bhargava, Sharanagat, Mor, & Kumar, 2020; Huang, Mei, Chen, & Wang, 2018; Petkoska, Daniloski, D'Cunha, Naumovski, & Broach, 2021; Yildirim et al., 2018). All the above-mentioned packaging materials not only enhance the shelf life, quality, and safety of food products, but they also make the packaged food more appealing to retailers and customers.

More attention has recently been drawn to the global issue of plastic pollution, with the announcement that more than 340 million tons of plastic waste are produced worldwide (Wu, Misra, & Mohanty, 2021). Most critically, the packaging industry was responsible for 46% of this waste. Thus, the environmental concerns about packaging waste,

particularly non-biodegradable polymer materials have grown dramatically. Most of the packaging materials are derived from conventional petroleum-based polymers, such as polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP), high-density polyethylene (HDPE), low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), polystyrene (PS), polyvinyl chloride (PVC) and polyolefins, which are not recycled by consumers after just a single use, resulting in a global economic loss of about $80-120 billion per year (Wu et al., 2021; Zhong, Godwin, Jin, & Xiao, 2020). In recent years, there has been greater emphasis on developing biodegradable polymers from renewable sources, such as cellulose, starch, chitosan, lipid and protein, to combat these problems (Mangaraj, Yadav, Bal, Dash, & Mahanti, 2019; Maraveas, 2020). Despite their ability to degrade in bioactive environments, those natural polymers have excellent barrier properties against moisture, aroma and gasses. Thus, these biodegradable polymers have gained popularity, particularly in food packaging applications due to their renewable, low-cost, eco-friendly and biocompatible properties (Mangaraj, Yadav, et al., 2019; Wróblewska-Krepsztul et al., 2018). Natural-based materials, such as polysaccharides, lipid and protein can be utilized to produce edible films as solid laminates for food packaging applications (Mohamed, El-Sakhawy, & El-Sakhawy, 2020; Petkoska et al., 2021). Because edible packaging materials show excellent tensile and barrier properties, they can be used to avoid physical damage during transportation and improve the shelf life of food products.

Due to consumer concerns about food quality and safety, many researchers have developed bio-based active packaging materials by reinforcement with different additives, such as antimicrobial and antioxidant compounds (Asgher, Qamar, Bilal, & Iqbal, 2020;

Firouz et al., 2021). Microbial growth occurs mainly at the surface of most processed and fresh foods, leading to food spoilage. Thus, an antimicrobial film is one of the promising active food packaging that can be utilized to extend the food shelf life by avoiding or delaying the growth of foodborne bacteria (Al-Tayyar, Youssef, & Al-Hindi, 2020; Sofi et al., 2018). As compared to direct surface applications of antimicrobials onto foods, the use of antimicrobial packaging films may be more effective due to the controlled release of the antimicrobial agents onto the food surface over an extended period, especially during food storage, transport and distribution (Quintavalla & Vicini, 2002; Yildirim et al., 2018). Due to environmental concerns about non-biodegradable plastic pollution, the development of antimicrobial films made from natural-based polymer materials that are renewable and biodegradable is extremely important (Chawla et al., 2021; Motelica et al., 2020).

Furthermore, consumer concerns about the safety of synthetic antimicrobials used in food have led to the development of packaging materials using natural antimicrobials, such as essential oils (EO), plant extracts, chitosan (CS), bacteriocins and bacteriophages, which are biodegradable and degrade easily in the environment (Irkin & Esmer, 2015; Punia Bangar et al., 2021). These antimicrobials can provide bioactive properties to the biodegradable packaging materials, making them more appealing to consumers. For example, they can be utilized as bio-preservatives in a food packaging material as their antimicrobial properties have been demonstrated in the literature (Punia Bangar et al., 2021; Sofi et al., 2018). As most of the natural antimicrobials have the generally recognized as safe (GRAS) status, they are safer than other synthetic additives. Thus, the development of antimicrobial packaging fortified with natural-based active agents could be a useful technique to improve the shelf life of foods, leading to better food quality and safety.

1.2 Objectives

The objectives of this study were to develop and characterize novel composite food packaging materials incorporated with antimicrobial agents. The specific objectives were to:

(a) develop food packaging materials incorporated with EO.

i. To develop novel edible CS/starch films incorporated with cinnamon and clove EO.
ii. To determine the physiochemical and antimicrobial properties of the films.
iii. To analyze the shelf life of beef covered with the films.

(b) develop food packaging material containing 4-hexylresorcinol (4-HR).

i. To synthesize novel non-edible polyvinyl alcohol/CS/modified bacterial nanocellulose (mBNC) films incorporated with 4-HR.
ii. To study the physiochemical, antioxidant, and antimicrobial properties of the films.
iii. To evaluate the shelf life of beef wrapped with the films.

(c) isolate lytic bacteriophages against foodborne pathogens.

i. To isolate a novel *Escherichia* phage from the environment.
ii. To evaluate the morphological, biological, and genetic characteristics of the phage.
iii. To determine the biocontrol abilities of the phage on *Escherichia coli* O157:H7 in vitro and in foods.

(d) synthesize food packaging material incorporated with *Escherichia* phage CAM-21.

i. To synthesize novel soy protein isolate (SPI)-based edible films incorporated with CAM-21.
ii. To evaluate the physiochemical and antimicrobial properties of the film.
iii. To determine the shelf life of beef treated with the film.

Chapter 2 Literature Review

2.1 Antimicrobial packaging system

Antimicrobial packaging has gained more attention from the food packaging industry in recent decades due to its ability to reduce and prevent the growth of spoilage and pathogenic bacteria in foods, which results in increased food shelf life (Chawla et al., 2021). Irkin and Esmer (2015) reported that antimicrobial packaging can be utilized to decrease microbial growth in non-sterile foods and reduce the contamination risk in pasteurized products through migration of antimicrobials. For such a packaging system to suppress microbial growth, its antimicrobial activity typically relies on a resulting increased lag period of bacterial growth and decreased viable counts of certain bacteria. Thus, an antimicrobial packaging can be used to protect perishable food products, including fruits, vegetables, and dairy and meat products, that are often spoiled by microbial contamination (Chawla et al., 2021).

An antimicrobial packaging system can be developed by various approaches, as detailed by Fang, Zhao, Warner, and Johnson (2017) and Khaneghah, Hashemi, and Limbo (2018). The first method is to incorporate antimicrobials directly into packaging materials by a traditional thermal treatment technique, including co-extrusion of polymer films with the active agents. Non-thermal techniques, such as solution casting, electrospinning and solvent compounding have a great potential to replace the former technique due to a reduced loss of bioactive components from the packaging materials. Also, the packaging system can be developed by applying an antimicrobial coating layer onto packaging materials, leading to the migration of volatile and non-volatile antimicrobials to the foods through evaporation and diffusion, respectively (Irkin & Esmer, 2015).

The packaging system can also be produced using natural polymers that are inherently antimicrobial, such as polylysine and chitosan (CS) (Chawla et al., 2021). The antimicrobial activity of such packaging materials is primarily owing to the interaction of positively charged amine groups of the polymers with the negative charges on the bacterial cell membrane, resulting in cell leakage and, eventually, cell death. Also, the packaging system can be formed by incorporating antimicrobials into a pad or sachet in the food package. In this packaging system, the antimicrobial pad and sachet can produce bioactive compounds *in situ* or serve as antimicrobial carriers prior to their release into the foods. For example, pads are mostly utilized in trays for meat products to absorb excess fluids, while at the same time, antimicrobials in the pads inhibit microbial growth (Fang et al., 2017).

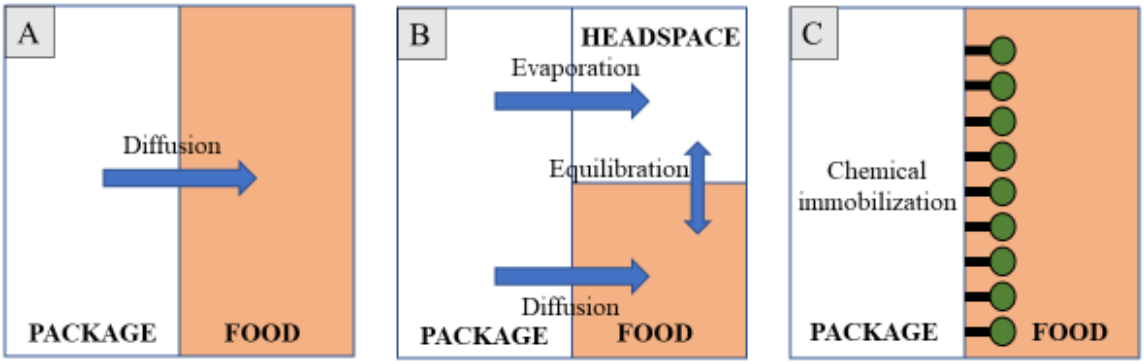


Figure 2.1 Classification of antimicrobial food packaging systems, including (A) migration of antimicrobials without headspace, (B) migration of antimicrobials with headspace and (C) non-migrating types (Quintavalla & Vicini, 2002).

Antimicrobial packaging systems are classified as the migrating and non-migrating types, depending on the antimicrobials used and their interactions with the packaging

material and food products (Khade, 2009). Antimicrobials in migrating packaging material could be transferred to the solid or liquid food products, that do not have a headspace through direct contact (Fig. 2.1A). Antimicrobials may be incorporated into packaging films, followed by their diffusion into the foods. Also, migrating packaging materials, such as flexible packaging and cups, can release antimicrobial vapors into the headspace of packaged food products through both diffusion and equilibrium evaporation (Fig. 2.1B) (Quintavalla & Vicini, 2002). In such a packaging system, volatile antimicrobials can be incorporated into the packaging material to allow for their migration to the headspace of the food and packaging material. For non-migrating packaging, non-food grade antimicrobials could be covalently immobilized onto the surface of packaging materials, and the inhibition occurs when foodborne bacteria come in contact with these active agents (Fig. 2.1C) (Irkin & Esmer, 2015).

Based on Quintavalla and Vicini (2002), multiple variables must be taken into consideration in the development of antimicrobial packaging films, including conditions of casting, chemical nature of films, and residual antimicrobial activity. For instance, the incompatibility of the antimicrobials with the packaging material and their low thermal stability during extrusion, are often limiting factors in selecting the active agents (Diblan & Sevim, 2018). Meanwhile, residual antimicrobial activity refers to the effective antimicrobial activity of the components after the casting and converting steps. The efficiency, release kinetics, and chemical stability of antimicrobials in the packaging material are strongly influenced by the physiochemical factors of food products, which include the pH, water activity, salt concentration and many others (Van Long, Joly, & Dantigny, 2016; Wu, Wang, Hu, & Nerín, 2018). Other important parameters include the

mass transfer coefficients, storage temperature and physical properties of packaging materials (Quintavalla & Vicini, 2002).

Antimicrobial packaging has benefits over the direct addition of food preservatives since the packaging system requires only low amounts of antimicrobials, whereas the conventional method requires a large dose of antimicrobials (Irkin & Esmer, 2015). Slow migration of antimicrobials from the packaging film to the food surface could also help to maintain their concentrations at higher levels, allowing them to inhibit the growth of foodborne bacteria for longer periods. Khade (2009) reported that the release of antimicrobials from packaging materials could be systematically controlled. Also, the direct addition of antimicrobials may reduce their activities due to leaching into the food matrix and undesirable reaction with other food components, including proteins and lipids (Irkin & Esmer, 2015). Thus, controlled release of antimicrobials from packaging materials into the foods not only inhibits the foodborne bacteria, but also improves their residual activity during food storage and distribution (Almasi, Jahanbakhsh Oskouie, & Saleh, 2021).

2.2 Food packaging materials used for the delivery of antimicrobials

Food packaging containing antimicrobials can be classified into two major types, which include non-biodegradable and biodegradable packaging according to the nature of the polymer matrix (Fig. 2.2). Plastics are estimated to account for approximately one-tenth of worldwide waste with single-use plastics, such as food containers and straws, accounting for 70% of marine litter objects. If the current trends continue, the figure is expected to grow considerably over the next decade (Omerović et al., 2021). Currently, most of the

commercial plastics are still produced from petrochemicals due to their high production speed, cost-effectiveness, excellent tensile and barrier properties and abundant availability (Asgher et al., 2020). Examples of non-biodegradable polymers used in the development of antimicrobial food packaging are polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyethylene (PE), polystyrene (PS), polyolefin (PO), linear low-density polyethylene (LLDPE) and many others. Although these polymers showed excellent plastic film characteristics for packaging application, they are either non-biodegradable or non-recyclable in nature. Thus, plastic waste can result in enormous landfill depletion and environmental pollution (Chawla et al., 2021). Hence, it is critically necessary to find an alternative biodegradable polymer material to replace those plastics that are not biodegradable.

In general, biodegradable packaging is a plastic that degrades rapidly into smaller molecular weight fragments due to the action of naturally occurring microorganisms, including bacteria, algae, and fungi, under certain conditions (Sofi et al., 2018). Biodegradable packaging could be produced from non-renewable resources since the chemical structure of the polymer, rather than its origin, is a primary factor that determines the biodegradability of the material. The biodegradation rate mainly depends on the chemical bonding in the polymer matrix. Despite the fact that the majority of synthetic polymers are not biodegradable, packaging materials made from synthetic polymers, such as polylactic acid (PLA), polyvinyl alcohol (PVOH) and polyglycolic acid (PGA), poly lactic-co-glycolic acid (PLGA), have been reported to be biodegradable (Asgher et al., 2020). These synthetic polymers can be developed through ring-opening polymerization

or condensation (Taherimehr, YousefniaPasha, Tabatabaeekoloor, & Pesaranhajiabbas, 2021). Also, biodegradable polymers, such as bacterial cellulose, polyhydroxybutyrate (PHB) and polyhydroxyalkanoates (PHA), can be made by incubating different types of bacterial species in a growth medium supplemented with the required nutrients (Taherimehr et al., 2021).

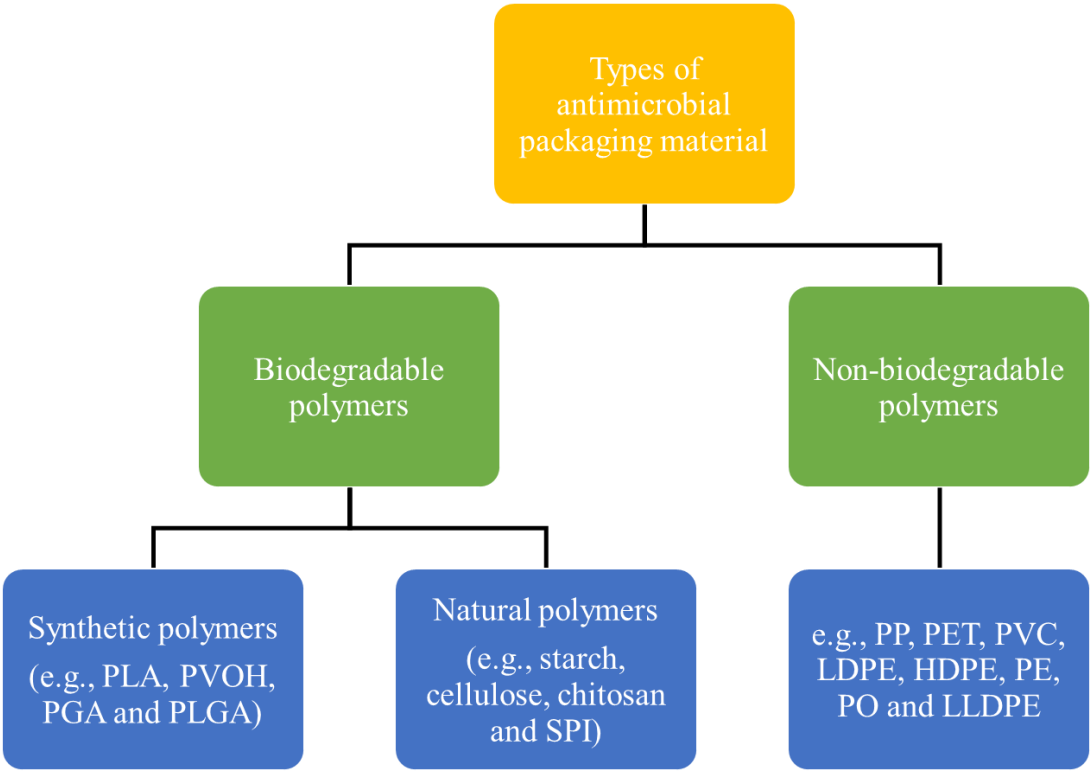


Figure 2.2 Classification of antimicrobial packaging materials based on their nature.

However, packaging materials produced from natural resources, such as biomass and agricultural materials are still the main priority when striving for less hazardous and more sustainable food packaging (Omerović et al., 2021). Examples of biodegradable natural polymers are polysaccharides (e.g., CS and starch), protein and lipids made from animals or plants. Among these polymers, polysaccharides and proteins and their

derivatives are most used for biodegradable packaging production due to their excellent mechanical and gas barrier properties. Biopolymers are a great alternative for non-biodegradable polymers because they are environmentally friendly, renewable, and non-toxic. However, the hydrophilicity of natural-based biopolymer films presents a major challenge for direct application in food packaging systems (Omerović et al., 2021). As compared to traditional polymers, their relatively higher cost, and poor barrier and tensile properties, limits their use in food packaging. To improve the physiochemical characteristics of biopolymers, various approaches have been studied, such as surface modification and via incorporation of organic and inorganic compounds (Taherimehr et al., 2021). The incorporation of plasticizers, lipid, and nanomaterials can improve the mechanical (e.g., extensibility and flexibility), water vapor barrier, thermal and functional properties of a biopolymer film (Basumatary, Mukherjee, Katiyar, & Kumar, 2020; Omerović et al., 2021).

Due to the non-biodegradable properties of conventional polymers, researchers and industries have increased their efforts at producing antimicrobial packaging from biopolymers. The biopolymers can be used as carriers for controlled delivery of active compounds to improve the shelf life of packaged foods. Also, the release rate and concentration of antimicrobials should be controlled to minimize undesired impacts on toxicological and sensory properties (Khaneghah et al., 2018). To ensure appropriate protective activity during the intended shelf life, a balance between microbial growth kinetics and controlled release rate should be maintained. The most important aspects are the types of incorporation method, the nature of the polymer matrix containing the antimicrobials, the food properties, and the release mechanism (Khaneghah et al., 2018).

To develop controlled released methods for antimicrobials, slight chemical and physical changes can be made to the host materials to achieve the criteria of food packaging materials. Small molecules, such as antimicrobial components may be able to take advantage of the activity of the synthetic and natural biopolymers.

2.2.1 Synthetic biopolymers

Due to technological advancements in recent decades, synthetic biopolymers, that are chemically produced from bio-based monomers have been widely developed. Synthetic biopolymers have great polymeric characteristics, including high gloss, improved flexibility, mechanical strength, durability and clarity, as well as the potential to develop a sustainable industry (Othman, 2014). Due to all their benefits, synthetic biopolymers have received increased attention for the formation of antimicrobial packaging. For instance, PLA is one of the most promising biodegradable polymers that can be produced by two main techniques, which include direct polycondensation of lactic acid or ring opening of lactide (Sampath, Ching, Chuah, Sabariah, & Lin, 2016). Lactic acid is a monomer synthesized by fermentation of sugar feedstock, such as dextrose, or through chemical processing. Sugar feedstocks can be produced from agricultural resources, such as sugar beets and sugar cane, or through conversion of starches from rice, wheat, potato, corn or other agricultural residues (Kamarudin et al., 2022). Hence, PLA is considered a renewable polymer. In addition to its low cost and abundant availability, PLA exhibits great thermal and tensile characteristics that are nearly identical to those of conventional thermoplastics, such as PET (Tang, Kumar, Alavi, & Sandeep, 2012). Unfortunately, the food packaging application of PLA is only limited to short-life packaged foods because of certain

drawbacks, such as low gas and vapor barrier properties (Y. Huang et al., 2018). To improve the properties of the material, PLA is often blended with other polymers or incorporated with fillers, such as chitosan, starch and cellulose, as detailed by Zhong et al. (2020).

Consumer demand for natural foods with minimal preservatives and reduced microbial contamination using sustainable packaging has generated more interest in the application of PLA in antimicrobial packaging. In recent years, researchers have widely examined the potential of PLA for antimicrobial packaging applications (Gao et al., 2017; Tawakkal, Cran, Miltz, & Bigger, 2014). To provide antimicrobial activity, PLA-based packaging material has been incorporated with various antimicrobials, including essential oils (EO) (e.g., cinnamon, oregano, curry, kesum, thymol) (Anuar et al., 2017; Llana-Ruiz-Cabello et al., 2016; Mohamad et al., 2020), plant extracts (e.g., green tea and propolis extracts) (Martins et al., 2018; Safaei & Roosta Azad, 2020), bacteriocins (e.g., nisin) (Shiroodi et al., 2016), organic acids (e.g., salicylic acid) (Yin, Sun, Tian, & Wang, 2018) and metal nanoparticles (e.g., silver nanoparticles) (Li et al., 2017). Also, Tawakkal et al. (2014) have reported that PLA is an effective carrier of antimicrobials without causing any problem on the procedures of composting or biodegradation. For instance, the biodegradability of PLA was significantly improved with the addition of metal oxides, such as titanium dioxide, magnesium oxide and zinc oxide, as reported by Ghozali et al. (2020).

PVOH is another important synthetic polymer in the food packaging industry that has abundant availability and low production cost. Commercial PVOH is produced from a radical polymerization of vinyl acetate, followed by hydrolysis on the acetate group of polyvinyl acetate under alkaline conditions (Musetti et al., 2014). PVOH is a lightweight,

non-toxic, water-soluble, highly crystalline, renewable, biodegradable and biocompatible polymer. Also, it has excellent physiochemical properties (e.g., mechanical strength, flexibility and chemical resistance) and good film-forming ability due to the formation of a strong intermolecular hydrogen bonding between the hydroxyl groups (Choo, Ching, Chuah, Julai, & Liou, 2016). Due to the strong carbon-carbon linkages on its backbone, PVOH has a lower biodegradation rate. Hence, a polymer blending technique or incorporation of nanofiller into the polymer is often required to improve its biodegradability and tensile properties (Yazik & Tukiran, 2021). As PVOH film does not have antimicrobial activity, the addition of antimicrobial agents can improve the functional properties of the packaging. Previous studies reported the development of PVOH-based antimicrobial packaging by incorporating with cinnamon, oregano and clove EO, for the inhibition of bacterial growth and lipid oxidation (Chen et al., 2018; Lin et al., 2020; Pan, Ai, Shao, Chen, & Gao, 2019). Chowdhury, Teoh, Ong, Zaidi, and Mah (2020) have also reported the use of crosslinking agents, such as glutaraldehyde and glyoxal, to improve the physiochemical and antimicrobial properties of PVOH films.

PGA is a biodegradable polymer derived from petroleum-based resources that can be produced through various chemical pathways, which include polycondensation of glycolic acid or ring-opening polymerization of glycolide. However, the production of PGA is low because of its high production cost. Recently, an environmentally friendly technology has been utilized by converting carbon monoxide into dimethyl oxalate, followed by hydrogenation and polymerization to produce PGA (Jem & Tan, 2020). The carbon dioxide emissions and production cost are decreased due to the use of waste gases as raw material. Although PGA and PLA are similar in chemical structure, the polymer

exhibits improved mechanical, thermal and barrier properties (Vibha et al., 2022). Also, PGA has a shorter degradation time than other biodegradable polyesters because it degrades through chemical hydrolysis rather than enzymatic hydrolysis (Samantaray et al., 2020). However, PGA has several disadvantages, such as short hydrolysis time, and low degradation temperature and brittleness, limiting its food packaging application (Samantaray et al., 2020). To solve these problems, copolymerization can be utilized by combining PGA with another polymer, such as PLA. PLGA is an aliphatic linear copolymer of PLA and PGA produced through a ring-opening polymerization technique of lactide and glycolide in the presence of a metal catalyst (Sampath et al., 2016). The US Food and Drug Administration (FDA) has approved PLGA for use as a food contact material. Also, PLGA possesses excellent barrier properties against oxygen and water vapor, as detailed by Murcia Valderrama, van Putten, and Gruter (2020).

In recent years, PLGA has reportedly been having a significant impact on antimicrobial packaging. Incorporation of antimicrobials in the PLGA copolymer has significantly improved the antimicrobial activity of the PLGA films (Mlalila, Hilonga, Swai, Devlieghere, & Ragaert, 2018). For instance, PLGA incorporated with natural antimicrobials (Correia et al., 2015; Gomes, Moreira, & Castell‐Perez, 2011; Hill, Taylor, & Gomes, 2013) or metal nanoparticles (Armentano et al., 2010) has been reported to have excellent antimicrobial activity against foodborne spoilages and pathogens. Other biodegradable petroleum-based polymers, including polybutylene adipate-co-terephthalate (PBAT) (de Andrade et al., 2020), polybutylene succinate (PBS) (Petchwattana, Covavisaruch, Wibooranawong, & Naknaen, 2016), polybutylene succinate adipate (PBSA)

(Suwanamornlert et al., 2020) and polycaprolactone (PCL) (Yahiaoui et al., 2015), have been reported for antimicrobial food packaging applications.

2.2.2 Natural biopolymers

Polysaccharides, such as starch, cellulose and CS are derived from monosaccharides that are joined together by glycosidic bonds. These polymers are widely utilized in food packaging applications because they are non-toxic, cheap and widely available (Taherimehr et al., 2021). Also, they are selectively permeable to oxygen and carbon dioxide, slowing the respiration and ripening of many fresh produces (Cha & Chinnan, 2004). Starch is the most abundant type of carbohydrate stored in plants, including potato, corn, wheat and rice. It contains two forms of alpha-glucose polymer, known as amylose and amylopectin. The contents of linear amylose and branched amylopectin may be different, depending on the plant origin (Taherimehr et al., 2021). Starches from different resources have been utilized for biodegradable packaging applications, either alone or in combination with other polymers, to improve the shelf life of food products (Asgher et al., 2020). Othman (2014) claimed that the biodegradability of non-biodegradable polymers can be improved with the addition of biodegradable starch into the polymer matrix. Since starch and its derivatives are edible materials, they are safe for food packaging applications. Films made from native starch exhibit moderate oxygen barrier properties but low mechanical and water vapor barrier properties, limiting their wide applications (Tang et al., 2012). Native starch films are poorly processable, hydrophilic and brittle. Thus, the film properties can be enhanced by a surface modification technique or incorporation of additives, including other polymers, nanofillers and

plasticizers (Taherimehr et al., 2021). Many antimicrobial agents, such as EO, plant extracts, food preservatives, organic compounds and metal nanoparticles, are compatible with starch, and the antimicrobial starch-based films have been found to be effective against a wide range of foodborne bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and many others (Mlalila et al., 2018).

Cellulose is a most abundant renewable polymer, and it is present in plant cell walls, marine life and invertebrates (Vibha et al., 2022). For instance, wood, which consists of about 40-50% (w/w) cellulose, is the most important source of cellulose (Tang et al., 2012). Cellulose can be produced in two steps, which include removal of contaminant molecules (e.g., hemicelluloses and lignin) by alkali and acid-chlorite treatments, followed by cellulose extraction using mechanical treatment, enzymatic and acid hydrolysis (Basumatary et al., 2020). Also, it can be produced by fungi, algae and Gram-negative bacteria (e.g., *Gluconoacetobacter xylinus* and *Acetobacter xylinum*). Although cellulose produced by bacteria has a similar chemical structure to plant-based cellulose, contaminant molecules are absent (Sampath et al., 2016). Cellulose is a linear polymer, that comprises of repeated D-glucose units linked together by β-1,4 glycosidic bonds. As it is a biodegradable, environmentally friendly, low cost, lightweight and non-toxic polysaccharide, it has great potential as a packaging material. Films made from cellulose have excellent tensile strength, toughness, transparency and high surface gloss (Asgher et al., 2020). Due to its high crystallinity, fibrous and hydrophilic nature and insolubility in water, native cellulose is undesirable for food packaging production (Taherimehr et al., 2021).

Instead, derivatives of cellulose, such as carboxymethyl cellulose (CMC), hydroxypropyl cellulose (HPMC), and methylcellulose (MC) are used to develop water-soluble packaging films. Also, thermoplastic materials can be produced from cellulose-based materials, including cellulose acetate (CA) and cellulose acetate butyrate, through an esterification technique (Basumatary et al., 2020). To improve the tensile strength and water vapor barrier properties, inorganic nanoparticles, such as silver (Chen, Xiao, Shi, & Cai, 2020) and zinc oxide (Jebel & Almasi, 2016), are incorporated into cellulose-based films. Also, cellulose nanostructures can be utilized as a nanofiller in food packaging materials due to its large surface area, high aspect ratio and availability of surface hydroxyl groups (Basumatary et al., 2020). For instance, incorporation of nanocelluloses, such as cellulose nanofiber (CNF), cellulose nanocrystal (CNC) and bacterial nanocellulose (BNC) improved tensile, thermal and barrier properties of the nanocomposite films for food packaging applications, as reported by many researchers (Abral et al., 2021; Kang, Xiao, Guo, Huang, & Xu, 2021; Mohammadi Sadati, Shahgholian-Ghahfarrokhi, Shahrousvand, Mohammadi-Rovshandeh, & Shahrousvand, 2021). However, incorporation of unmodified nanocelluloses in the hydrophobic polymer matrix may have a negative impact on the mechanical properties of developed films. Saedi, Garcia, Kim, and Shin (2021) and Kumar, Rai, Gahlyan, and Kumar (2021) detailed the incorporation of nanocellulose that has been modified physically (e.g., ultrasonic, electric discharge, irradiation, etc.) or chemically (e.g., oxidation, esterification, polymer grafting, etc.) to enhance the physiochemical properties of food packaging films, including tensile, barrier, optical and water absorption capabilities. Also, introduction of surface functional groups, including quaternary ammonium and

aldehyde, to nanocelluloses improves the biocompatibility and antimicrobial properties of food packaging films (Norrrahim et al., 2021).

CS is a linear polysaccharide produced by partial deacetylation of chitin, which is found in the crustacean shells, including shrimps, insects and crabs. CS, the second most abundant natural-based polymer, is renewable, non-toxic, biodegradable and biocompatible (Choo et al., 2016). It has repeating units of 1,4-linked 2-amino-deoxy-β-D-glucan. CS is soluble in acidic solution, but insoluble in water. It is an inherent antimicrobial biopolymer that can function as a polymer substrate and an antimicrobial agent (Y. Huang et al., 2018). Films made with CS also have great antioxidant, antifungal, tensile, gas barrier, optical and film-forming properties (Tang et al., 2012). Due to their excellent antimicrobial activity against bacteria, yeasts and molds, they can be used as food packaging materials to extend the shelf life and quality of food products. However, they exhibit poor water vapor barrier properties and long-term stability. Different techniques, including blending of chitosan with other biopolymers (e.g., starch, whey protein, PVOH, PLA, etc.) and incorporation of plasticizer (e.g., glycerol, sorbitol, etc.), have been used to overcome the disadvantages for food packaging applications, as reported by researchers (Bie et al., 2013; Brink, Šipailienė, & Leskauskaitė, 2019; Choo et al., 2016). Basumatary et al. (2020) reported that the incorporation of nanomaterials, such as nano clay (e.g., montmorillonite (MMT)) and metal nanoparticles (e.g., silver, zinc, etc.) has improved the barrier and tensile properties of chitosan films. CS films have also been incorporated with various antimicrobial agents, such as plant extracts, EO and nanoparticles to further enhance their antimicrobial activity against foodborne bacteria, such as *E. coli*, *B. subtilis*

and *P. aeruginosa* (Kaya et al., 2018; Priyadarshi, Kumar, Deeba, Kulshreshtha, & Negi, 2018; Sani, Pirsa, & Tağı, 2019).

In recent decades, protein-based biopolymers have gained more attention due to their biodegradability, inexpensive nature and film forming properties. The biopolymers can be produced from soy protein, whey protein, corn zein, pea protein, gelatin, casein, and others. A protein is a heteropolymer made up of over a hundred amino acids bonded together by peptide linkages. As compared to polysaccharides, protein-based biopolymers have greater mechanical properties, such as elasticity, strength and toughness because of their unique structure. Still, their mechanical strength is weaker when compared to synthetic plastics (Mangaraj, Yadav, et al., 2019). Although protein-based films have great oxygen barrier properties, they exhibit poor water vapor barrier properties as a result of the hydrophilicity of most proteins (Tang et al., 2012).

Soy protein is a globulin composed primarily of both polar (e.g., acidic and basic) and non-polar amino acids. For instance, β-conglycinin and glycinin are two important components of soy protein. Soy protein isolate (SPI), soy protein concentrates (SPC) and soy flour are commercially available biodegradable products derived from soybean seeds. As SPI has a higher protein content than the others, it has better film-forming properties. Tang et al. (2012) detailed the incorporation of plasticizers, such as propylene glycol, ethylene glycol and glycerol, to enhance the film processability and flexibility. Also, soy protein is blended with other biopolymers to improve its water uptake and tensile properties (Basumatary et al., 2020). In addition, incorporation of nanocelluloses, such as CNF and CNC, improved the mechanical, moisture and oxygen barrier properties and reduced water susceptibility of SPI films (González, Gastelú, Barrera, Ribotta, & Igarzabal, 2019; Han,

Yu, & Wang, 2018b; Martelli-Tosi et al., 2018). Because SPI films do not possess antimicrobial properties, certain additives, such as plant extracts, food preservatives, metal oxide nanoparticles, bacteriocin, phenolic and acidic-based components, can be incorporated to improve its antimicrobial activity and, hence, extend the food quality and shelf life (Ferreira et al., 2021; Sivarooban, Hettiarachchy, & Johnson, 2008; Tao, Sedman, & Ismail, 2022; Xiao, Liu, Kang, Wang, & Xu, 2020).

2.3 Techniques used for film formation

The techniques for preparing biodegradable film materials have gained attention from researchers and industry. The viability of the technique is evaluated by its cost time and process difficulty (Cheng et al., 2021). Also, packaging films are usually strengthened by the hydrogen, covalent, electrostatic and hydrophobic bonding in the polymer matrix. The processing techniques of packaging material can be categorized into wet and dry processing. Wet processing, such as solvent casting method, strongly depends on the type and pH of solvent used while dry processing, such as extrusion and thermal processing, depend on the thermoplastic characteristics of polymers (Asgher et al., 2020). Solvent casting technology is a traditional processing technique utilized for production of thin films. However, after the 1950s, film extrusion methods became more popular and replaced other production techniques. In the past few decades, the casting method has reemerged because of its benefits, which include the ability to develop films with high transparency and uniform thickness, as well as the ability to process thermosensitive polymers containing active agents (Velásquez et al., 2021). Although the solvent casting method is simple to use, it can only be utilized for small-scale production in the laboratory due to its low yield

and time intensive, which increases the production cost. Film extrusion processing technology can be utilized in large-scale manufacturing and has a high production speed, but it consumes a lot of energy (Cheng et al., 2021). Other conventional techniques include compression molding, injection molding, mixing and compounding.

Among all of the processing methods discussed above, compression molding the is most commonly used processing technique for green composite materials (Faruk & Sain, 2014). Due to its simplicity, versatility, high reproducibility and production rate, and low cost and reduced cycle time, the compression molding method is a common method to produce polymer materials (Park & Lee, 2012). However, the processing technique exhibits several disadvantages, such as the need for expensive tools and equipment, development of untransparent polymer films and imperfections of polymer surface (e.g., waviness and pitting). In comparison to the conventional methods, modern processing techniques, such as electrospinning, 3D printing, supercritical impregnation and reactive extrusion, not only improve the characteristics and manufacturing performance of biodegradable materials, but also offer other processing options, including allowing for different functions and shapes (Cheng et al., 2021). For instance, electrospinning can be utilized to generate a multi-layer thin film structure with strong adhesion between the hydrophobic layer and the hydrophilic natural polymer.

2.3.1 Casting method

Film solvent casting is a simple film preparation technique that has often been utilized, particularly for the development of biodegradable plastics. For film formation using this method, polymers and additives are usually dissolved in an appropriate solvent

before the film forming solution is casted (Asgher et al., 2020). Antimicrobials, antioxidants, plasticizer, cross-linking agents and nanofillers can be incorporated to improve the film properties, such as tensile strength, flexibility, antioxidant and antimicrobial properties. Ultrasonication and mechanical stirring are often utilized to assist the biopolymer dissolution in the solvent. For a biopolymer like protein, heating and pH adjustments are required to promote cross-linking (Yu, Dhital, et al., 2019). Films are typically formed via strong intra- and inter-molecular interactions or cross-linking of polymer chains, resulting in semi-rigid three-dimensional networks. The polymer structure, additives, temperature and solvent are responsible for the film cohesiveness (Abdul Khalil et al., 2018). To avoid air bubbles in the finished films, film-forming solution, especially for protein suspension, must be degassed prior to casting for film formation. Then, the film-forming solution is poured onto a smooth and flat surface, including plastic or glass plate before drying at certain temperatures and relative humidity to evaporate the solvent. Abdul Khalil et al. (2018) suggested that the tensile properties of the films are determined by the temperature and rate of drying. The dried films are eventually peeled off from the plate and stored under specific conditions for optimal film performance.

Nevertheless, if the film components change, the film processing technique may be altered. For instance, an emulsifying agent, such as Tween 20 or Tween 80 was added into a film forming solution to improve the solubility of hydrophobic compounds (e.g., EO) in a hydrophilic polymer matrix. The tensile strength of emulsified films produced by a solvent casting technique has been reported to be lower than those of control films without the presence of hydrophobic components (Song, Zuo, & Chen, 2018; Zhou, Wu, Chen, & He, 2021). Solvent casting technique has been used to produce various biodegradable films

from synthetic and natural biodegradable polymers, such as PVOH, PLA, starch, CS, cellulose and soy protein (Ali et al., 2019; Anuar, E'zzati, Izzati, Inani, & Ali, 2018; Choo et al., 2016; Tao et al., 2022). Solvent casting method can be used to develop various types of antimicrobial films. For instance, antimicrobials can be incorporated into the film-forming solution before casting onto a plate. The other method is the coating of a film-forming solution containing antimicrobial agents onto a base polymer layer to produce an antimicrobial bilayer film (Fu & Dudley, 2021). Although the solvent casting technique has a low installation cost, it is not suitable for large scale production. The uniformity and thickness of finished films are difficult to control using the film processing technique. Its high production cost also limits the use of solvent casting technology for large scale production of biodegradable films in the packaging industry.

2.3.2 Compression molding

Compression molding is one of the conventional melting-based techniques used to develop biodegradable films with antimicrobial capabilities. It is a flexible method that can handle a wide range of design complexity, thicknesses and lengths. Compression and flow compression molding are the two techniques in use. The methods are different in terms of the tool structure and processing, type of semi-finished product employed and its cutting (Faruk & Sain, 2014). Melt blending with an internal mixer is a typical method for producing polymer materials (Velásquez et al., 2021). Several variables are manipulated during the melt blending, including mixing speed, time and temperature, which is maintained above the melting point of the components. The melted blend is subjected to a heated mold cavity after a homogeneous mixture is obtained. Controlled variables,

including curing time, heating temperature and compressive pressure, are critical in the development of composites with desired and superior properties (Jaafar, Siregar, Tezara, Hamdan, & Rihayat, 2019). When the mold is filled, it is sealed and pressured to force the preheated blend into all parts of the mold cavity. Rani and Kumar (2019) suggested that compression molded polymer films exhibit greater mechanical and optical properties, and a smoother surface, as compared to those produced by solvent casting, which have weaker water resistance and lower hydrophobicity.

Numerous studies have been carried out to determine the potential of employing natural fibers as reinforcing agents in composite polymer materials by compression molding (Faruk & Sain, 2014; Jaafar et al., 2019). For instance, biopolymer blends, such as thermoplastic starch/PLA (P. Zhou et al., 2021) and PVOH/starch (Nazrin, Sapuan, & Zuhri, 2020) have been mixed with cellulose nanofibrils to produce biodegradable composites that exhibit excellent mechanical properties. Although compression molding can be used to produce antimicrobial packaging materials, it may cause losses of antimicrobial agents as a result of their high volatility (Velásquez et al., 2021). Various antimicrobial agents, such as chitosan (Valencia-Sullca, Atarés, Vargas, & Chiralt, 2018), silver-copper nanoparticles and cinnamon EO (Ahmed et al., 2018), and oregano EO (da Costa et al., 2020), have been incorporated into different biopolymer matrixes for antimicrobial food packaging applications. Compression molded antimicrobial biopolymers can be developed by direct addition of antimicrobials in the melt state, or encapsulating the antimicrobials in carrying agents, including β-cyclodextrin (Dobrzyńska-Mizera et al., 2021) and oleic acid (Talón, Vargas, Chiralt, & González-Martínez, 2019), before incorporating them into a melted biopolymer. Velásquez et al. (2021) suggested that

the encapsulation method not only improve the oxidative and thermal stability and shelf life of volatile antimicrobials, but also enhances their color stability during aging. Also, incorporation of encapsulated antimicrobials has shown to improve the stiffness and water vapor and oxygen barrier properties of a biopolymer matrix (Talón et al., 2019).

2.3.3 Extrusion

Extrusion, a conventional technique used to prepare biopolymer films, is a dry processing technique, that is based on the thermoplastic characteristics of biopolymer. In general, several steps are required to produce the biopolymer films by extrusion. First, different compositions of polymer and additives are combined in a mixer before transferring to a screw extruder. In a large-scale industrial production, the extrudates are usually cut into particles by a pelletizer before drying in an oven. Different batches of dried pellets are blended in the extruder for subsequent production as required (Wang, Qian, & Ding, 2018). The rotating screw then pushes the melted film-forming material to the die heads, which come in a variety of shapes. To make a biopolymer film, the extrudate is processed through various techniques, which include extrusion injection molding, extrusion compression molding and extrusion blowing (Cheng et al., 2021). Both extrusion compression molding and extrusion injection molding include extrusion and molding throughout the manufacturing process. For the extrusion compression molding, biopolymer films are produced by hot pressing and cooling of the extruded film-forming material. Meanwhile, extrusion injection molding involves the injection of melted film-forming material into the mold, followed by a cooling process to obtain the final product. This method is widely utilized in polymer processing due to its ability to develop a large amount

of sophisticated plastic items at a cheaper price. For the extrusion blowing, biopolymer films are produced by blowing, pressing and cooling the extruded film-forming materials.

Extrusion is an environmentally friendly and cost-effective method for producing biopolymers with good characteristics. For instance, Wang et al. (2018) reported that films produced by extrusion often exhibit great thermal resistance and decent tensile properties. The extrusion temperature is customized based on the polymer properties, such as the melting and degradation temperatures of the components, to obtain the greatest film characteristics. The morphologies of developed films are similar to those produced by the solvent casting method (Cheng et al., 2021). However, poor polymer interactions may have a detrimental impact on the tensile properties, resulting in film brittleness (Wang et al., 2018). Extrusion has been used by many researchers to make films from synthetic and natural biopolymers (de Oliveira et al., 2021; Ferreira et al., 2021; Rodrigues et al., 2021). As starch lacks the material characteristics required for commercial extrusion, it is often mixed with various biopolymers (e.g., PVOH, PLA, etc.) and plasticizers to develop biopolymer films for food packaging applications (Ge, Lansing, & Lewis, 2021; Mangaraj, Mohanty, Swain, & Yadav, 2019). Extrusion has also been utilized to make biodegradable films containing antimicrobial agents, such as grapefruit seed extract (Lyu, Lee, & Han, 2019), sodium metabisulfite (Machado et al., 2021) and curcumin (de Campos et al., 2019), for antimicrobial packaging applications. However, the antimicrobial concentration of the film is often lesser than the antimicrobial concentration in the raw mixture, due to antimicrobial component loss during melt processing (Velásquez et al., 2021). Thus, higher amounts of antimicrobials are required to be incorporated into the biopolymers to overcome the negative impact. Also, the food storage temperature can have an impact on

the rate of release and the stability of the antimicrobial packaging system (Khaneghah et al., 2018). As a result, direct incorporation of volatile antimicrobials into polymers during extrusion is still very limited.

2.4 Antimicrobials used in food packaging

Food packaging material is primarily used as a protective barrier to isolate food products from the external environment and to protect them against deterioration caused by mechanical stresses, dust, water vapor, gases, aromas and microorganisms. Microbial contamination is often the leading cause of food spoilage. When foods are contaminated by microorganisms, their shelf life, quality and safety are greatly reduced, leading to an increase in consumer health concerns. Traditional food preservation techniques, such as thermal processing, freezing, drying and many others, have been utilized, but each has drawbacks when applied to fresh meats and produce (Sung et al., 2013). In addition, there is no effective strategy for avoiding outbreaks of foodborne pathogens. Thus, the current trend in food packaging is to develop more novel techniques to inhibit spoilage of food products.

In the last few decades, researchers have developed a variety of active packaging by incorporating functional properties, such as antimicrobial and antioxidant properties, to the packaging materials, and thus, improving the food quality and safety (Vibha et al., 2022). Antimicrobial agents are biological active substances that can be classified into synthetic and natural classes, according to their physiologies and sources (Fig. 2.3) (Kamarudin et al., 2022). These substances play a vital role in the food packaging and processing industries, particularly in eliminating or inhibiting the growth of

microorganisms. Antimicrobial packaging, one of the active packaging, has been developed by incorporating various antimicrobial agents into packaging materials to inhibit foodborne spoilages and pathogens, such as Shiga toxin-producing *E. coli* (STEC), *Salmonella*, *L. monocytogenes*, *B. cereus*, *S. aureus* and many others.

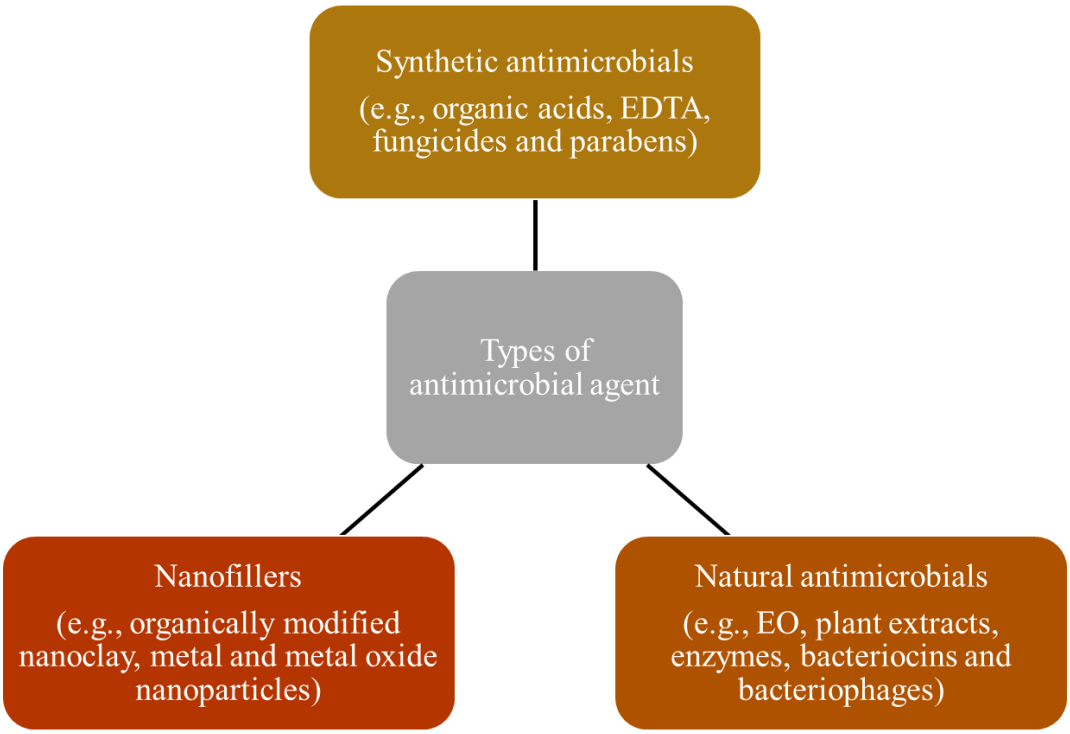


Figure 2.3 Classification of antimicrobial agents based on their physiologies and sources.

Despite consumer demand for healthy foods that are free of chemical preservatives, synthetic antimicrobials are widely utilized in active packaging because of their availability and low cost, as well as excellent thermal resistance (Fu & Dudley, 2021). Synthetic antimicrobials are classified into two major groups, which include organic (e.g., organic acids and their salts, chelating agents, fungicides, and parabens) and inorganic compounds (e.g., metal and metal oxide nanoparticles) (Kamarudin et al., 2022). In recent years,

natural-based antimicrobials in packaging has received increased attention from researchers due to their high potential as alternatives to chemical preservatives (Vasile & Baican, 2021). Natural-based antimicrobials, derived from three main resources, including plants (e.g., EO and plant extracts), animals (e.g., enzymes) and microorganisms (e.g., bacteriocins and bacteriophages), provide a significantly lower risk to consumer health (Irkin & Esmer, 2015). The effectiveness and antimicrobial properties of these antimicrobial agents in food packaging have also been reported in many literatures (Becerril, Nerín, & Silva, 2020; Fu & Dudley, 2021; Sofi et al., 2018).

2.4.1 Synthetic antimicrobials

Weak organic acids, such as acetic acid, citric acid, sorbic acid, benzoic acid, propionic acid, lactic acid, tartaric acid and many others, and their salts (e.g., propionates, benzoates, sorbates, etc.) are the most commonly used synthetic antimicrobials due to their excellent antimicrobial activity against a broad range of microorganisms, such as bacteria, molds and yeasts (Becerril et al., 2020). The preservatives offer an optimum antimicrobial activity at an acidic environment (pH < 6). Weak acid in a dissociated form then diffuses through the cell membrane of microorganisms, leading to acidification of the cell cytoplasm (Corrales, Fernández, & Han, 2014). However, Gram-negative bacteria are more resistant to the preservatives due to their outer membrane, which acts as a protective barrier. In addition, sorbic acid and its salt are reported to avoid bacterial spore germination and proliferation (Cha & Chinnan, 2004). These tasteless and colorless chemicals are accepted as “Generally Recognized as Safe (GRAS)”, and they have long been utilized as preservatives in the food, pharmaceutical and cosmetic sectors (Becerril et al., 2020).

Weak organic acids and their derivatives have traditionally been applied to the food matrix using spraying or dipping techniques. However, due to the possible reduction of antimicrobial activity through evaporation or dilution, the preservatives have been incorporated in packaging materials to offer antimicrobial activity over a longer period and extend the shelf life of food products (Corrales et al., 2014). For example, poly(butylene adipate-co-terephthalate) (PBAT)/thermoplastic starch (TPS) films incorporated with potassium sorbate and sodium benzoate had a significant antifungal activity against the growth of molds, such as *Aspergillus niger* and *Penicillium*, extending the shelf life of fresh noodle (Wangprasertkul, Siriwattanapong, & Harnkarnsujarit, 2021). Also, active polypropylene films containing sorbic acid showed excellent antimicrobial activity against *E. coli, S. aureus* and *A. niger* (Fasihnia, Peighambardoust, Peighambardoust, & Oromiehie, 2018).

Ethylenediaminetetraacetic acid (EDTA) is an economical and food-grade chelating agent that possesses strong antimicrobial activity against Gram-negative and Gram-positive bacteria (Morsy, Elsabagh, & Trinetta, 2018). For instance, this molecule can bind to divalent cations, such as calcium and magnesium ions, in the lipopolysaccharides (LPS) of Gram-negative bacteria, altering the integrity of the outer membrane and increasing its permeability to other antimicrobials (Huang et al., 2021). EDTA has been used together with other antimicrobials, such as nisin, lysozyme, zinc oxide nanoparticles, weak organic acid and bacteriophages (Huang et al., 2021; Morsy et al., 2018; Sangcharoen, Klaypradit, & Wilaipun, 2017), to enhance the antimicrobial activity against Gram-negative bacteria. EDTA is permitted for use in a wide range of foods at 36-500 ppm after health risk assessments by the World Health Organization and the US

FDA (Huang et al., 2021). In addition, EDTA has been incorporated in combination with other antimicrobials, such as nisin and lysozyme, into food packaging films to inhibit the microbial growth and extend the shelf life of food products (Abarca, Medina, Alvarado, Ortiz, & Carrillo López, 2022; Bhatia & Bharti, 2015; Chang et al., 2021; Leelaphiwat, Pechprankan, Siripho, Bumbudsanpharoke, & Harnkarnsujarit, 2022). Incorporation of EDTA in chitosan films containing LAE enhanced the antimicrobial activity against Gram-negative bacteria, such as *E. coli* O157:H7 and *Salmonella enterica* (Ma, Zhang, & Zhong, 2016).

Fungicide, such as imazalil, is utilized as an antimycotic agent in food packaging. Imazalil is a fungal sterol biosynthesis inhibitor, which is commonly used to control a broad range of molds on fresh produces (Cha & Chinnan, 2004). It possesses antimicrobial activity at relatively low concentrations and is stable at high temperatures (Corrales et al., 2014). Imazalil has been incorporated into low density polyethylene (LDPE) films to inhibit the growth of molds, such as *Penicillium* sp., and *Aspergillus toxicarius*, on the surface of Cheddar cheese (Weng & Hotchkiss, 1992). In addition, Vartiainen, Skytta, Ahvenainen-Rantala, and Enqvist (2003) found that LDPE films containing both imazalil and EDTA had an excellent antimicrobial activity against *A. niger*, but was not active against *E. coli* O157:H7. All the above findings suggest that packaging films containing imazalil can successfully limit mold growth on food products. In Europe, imazalil is permitted for use as a fungicide to avoid decay in post-harvest fruits, such as apples, bananas, pears and citrus. However, overuse of imazalil may result in the formation of fungicide-resistant strains, increasing consumer concerns about the safety of the fungicide (Huang, Qian, Wei, & Zhou, 2019).

Parabens have been utilized in the cosmetic industry due to their low toxicity and strong antimicrobial properties (Corrales et al., 2014). Methyl and propyl parabens are permitted for use as food preservatives with a GRAS status. Other parabens, such as ethyl, butyl and heptyl parabens, also exhibit antimicrobial activity against a wide range of bacteria and molds. For instance, propyl paraben possesses excellent antifungal activity against molds and yeasts between pH 4 and 8. Incorporating phenolic compounds, such as parabens and their salts into packaging materials is an effective method to enhance food quality and safety. Under controlled release conditions, a styrene-acrylate copolymer coating containing propyl paraben was found to be effective in inhibiting the growth of *Saccharomyces cerevisiae* (Chung, Chikindas, & Yam, 2001).

2.4.2 Natural antimicrobials

The use of naturally-derived antimicrobials as alternative food preservatives to replace synthetic antimicrobials has attracted the interest of researchers in recent years. Due to consumer demand for safer products, natural antimicrobials as active ingredients in food packaging are becoming more popular because they pose a lower health risk to consumers. Natural antimicrobial compounds in food packaging can help to avoid food degradation while also improving the shelf life and safety of food products (Punia Bangar et al., 2021). For thousands of years, plants extracts and EO have been employed for various applications, such as food preservation and taste and flavor enhancement. Many plant extracts and EO exhibit excellent antimicrobial activity due to their high amounts of phenolic compounds, including eugenol, thymol and carvacrol (Irkin & Esmer, 2015).

Hence, incorporation of plant extracts and EO into biopolymers could meet consumer demands for more biodegradable, natural, or disposable food packaging materials.

EO are natural extracts rich in volatile and hydrophobic components, such as terpenoids, non-terpenoids and phenolic compounds, that are responsible for their antimicrobial activity. They are secondary metabolites produced by plants to protect themselves from microbial invasion. EO are usually obtained through the fatty acid/polyketide, alkaloid, phenyl propanoid or isoprenoid pathways (Baldevraj & Jagadish, 2011). They are active compounds derived from herbs, spices and plant extracts, and most of them are categorized as GRAS by the US FDA. EO are isolated from different parts of the plant by physical treatments, including pressing and distillation (Varghese, Siengchin, & Parameswaranpillai, 2020). Antimicrobial activity of plant EO, such as cinnamon, clove, mustard, thyme, oregano, coriander, lemongrass, sage, onion, basil and garlic, against a wide spectrum of foodborne bacteria has been reported in the literature (Clemente, Aznar, Silva, & Nerín, 2016; Dobre, Gagiu, & Petru, 2011; Mith et al., 2014). However, Corrales et al. (2014) suggested that EO are more effective against Gram-positive bacteria than Gram-negative bacteria. Active components of EO increase the permeability of bacterial cells by disrupting the outer membrane and cytoplasmic membrane structures, leading to cellular content leakage and cell death (Fu & Dudley, 2021). EO containing a higher concentration of phenolic compounds exhibit a greater antimicrobial activity. For example, cinnamaldehyde, eugenol, and thymol are the major phenolic components in cinnamon, clove, and thyme EO, respectively.

EO have been added into packaging materials to inhibit foodborne microorganisms and improve the shelf life of food products. Volatile antimicrobials, such as EO, are

incorporated into food packaging through various techniques, such as extrusion, solvent casting, encapsulation and supercritical carbon dioxide impregnation (Beltrán Sanahuja & Valdés García, 2021). Due to their antimicrobial effect, the development of packaging materials incorporated with EO, such as cinnamon, basil and thyme, as food additives has been extensively reported in the last few decades (Amor et al., 2021; Syafiq, Sapuan, & Zuhri, 2021; X. Zhang et al., 2021). However, the efficiency of EO decreases with time because of their interaction with food components. Instead of adding more EO, blends of EO are incorporated into packaging materials to improve the properties of the films (Peng & Li, 2014; Ribeiro-Santos et al., 2017; Y. Wang et al., 2017). Currently, most of the EO-containing biopolymers are still produced in a small scale. Although active packaging containing EO have been widely studied, their commercial application is still very limited in the food industry, due to the high volatility, photothermal instability, strong aroma and high reactivity of EO with food components. Other considerations include the effect of EO on the physical and chemical properties of packaging materials, and the performance of this packaging system when produced under actual situations (Yildirim et al., 2018).

Plant extracts have attracted more interest because they contain significant amounts of polyphenolic compounds, such as alkaloids, tannins and saponins, with excellent antimicrobial and antioxidant properties (Mir, Dar, Wani, & Shah, 2018). They exhibit multiple antimicrobial mechanisms, which include disruption of membrane structure and function, suppression of the synthesis and function of DNA/RNA, coagulation of cytoplasmic materials and many others (Becerril et al., 2020). Thus, plant extracts have excellent antimicrobial activity against a broad range of microorganisms, particularly Gram-positive bacteria. Only a few plant extracts have been found to inhibit Gram-negative

bacteria due to their bacterial cell walls, which act as a protective barrier and avoid phenolic compounds from penetrating into the cell cytoplasm (Corrales et al., 2014; Liu, McKeever, & Malik, 2017). Due to consumer demand for natural products, plant extracts are used as alternative additives in packaging materials to improve the physiochemical, antimicrobial and antioxidant properties of the films.

In the food industry, animal-derived enzymes are used as antimicrobial agents, oxygen scavengers, bio-transformers and catalysts (Corrales et al., 2014). One of the most extensively used enzymes as a food preservative is lysozyme, which is isolated from hen egg white. Lysozyme exhibits antimicrobial activity against both Gram-negative and Gram-positive bacteria due to its ability to hydrolyze the β-1,4 glycosidic linkage between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan of the bacterial cell walls, leading to the leakage of intracellular materials and cell death (Huang et al., 2019). Lysozyme is more effective against Gram-positive bacteria because their cell wall contains 90% of peptidoglycan, whereas Gram-negative bacteria's membrane is made up of only 10% of peptidoglycan, which is covered by an outer membrane (Sofi et al., 2018). To enhance its antimicrobial activity against Gram-negative bacteria, lysozyme is usually coupled with chelators (e.g., EDTA) or other enzymes (e.g., lactoferrin). For instance, the antimicrobial activity of lysozyme is enhanced when EDTA is used to break the outer membrane of Gram-negative bacteria (Véronique, 2008). Lactoferrin, a protein present in human and cow milks, also increases the membrane permeability of Gram-negative bacteria by interacting with their LPS layer (Becerril et al., 2020).

Bacteriocins, low molecular weight proteins or peptides, are metabolic by-products generated by some lactic acid bacteria (LAB) to inhibit the growth of spoilage and

pathogenic microorganisms, particularly Gram-positive bacteria (Sofi et al., 2018). Because Gram-negative bacteria are protected by an outer LPS membrane, they can only be inhibited by combining with other antimicrobials, such as organic acids, EDTA and other enzymes (Corrales et al., 2014). Bacteriocins have been utilized as food preservatives for decades due to their GRAS status and low toxicity (Becerril et al., 2020). They are hypoallergic, stable across a broad range of pH and temperatures and easily inactivated in the human intestine by proteolytic enzymes (e.g., proteases) (Véronique, 2008). Some examples of bacteriocins include nisin, pediocin, propionicins, diplococcins, enterocins and lacticins (Baldevraj & Jagadish, 2011). Nisin, produced by *Lactococcus lactis*, is a commercially significant bacteriocin used as a food preservative due to its antimicrobial activity against lactic acid bacteria and other Gram-positive spoilages and pathogens, particularly the *Clostridia* sp. (Cha & Chinnan, 2004).

Due to consumer demand towards chemical-free products, bacteriophages have received attention as alternative natural antimicrobials in food products. In the food industry, lytic bacteriophages are utilized to inhibit foodborne bacteria by infecting and lysing target bacteria. Phage biocontrol is becoming more widely recognized as a natural and ecofriendly food preservation technique, targeting specific pathogens in food product, and thus, improving the food chain safety (Moye, Woolston, & Sulakvelidze, 2018). Since lytic bacteriophages do not interact with other microbes or eukaryotic cells in the environment, they are safe for human and animal consumptions with no health risk. The organoleptic characteristics of food products are not affected by the addition of bacteriophages (Moye et al., 2018). They are also simple and inexpensive to produce and have a long shelf life. The US FDA has granted the GRAS status to some phage-based

products for food safety and veterinary applications. Commercial phage-based products, including SalmoFresh™, ListShield™ and EcoShield PX™, are used to control foodborne pathogens, such as *Salmonella*, *L. monocytogenes* and STEC and non-STEC, respectively (Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015; Vikram, Tokman, Woolston, & Sulakvelidze, 2020; X. Zhang et al., 2019). However, the major drawback that limits the use of bacteriophages as food antimicrobials is their low stability in food environment conditions (Costa, Pastrana, Teixeira, Sillankorva, & Cerqueira, 2021). Biopolymer films containing bacteriophages have gained more attention due to their inexpensive cost and minimal food processing. Incorporation of bacteriophages into food packaging increased their stability and allowed antimicrobials to be released at specific areas. Bacteriophages are typically used in food packaging based on their target bacteria. Hence, the type of food product to be packaged is determined by the potential foodborne bacteria present in that food (Becerril et al., 2020).

2.4.3 Nanofillers

In the last few years, the rapid development of nanotechnology has led to the emergence of novel nanosized antimicrobial agents, such as organic (e.g., organically modified nanoclay) and inorganic nanofillers (e.g., metal and metal oxide nanoparticles), which offer numerous advantages to the food packaging industry. Nanofillers, at least one dimensions between 1-100 nm, can be classified into nanoparticles, nanotubes and nanolayers, based on their nanoscale dimensions (Vasile & Baican, 2021). Inorganic nanofillers exhibit higher thermal stability than organic antimicrobials, such as weak organic acids and enzymes. When compared to antibiotics, nanofillers often show excellent

antimicrobial activity against a wider range of microorganisms (Al-Tayyar et al., 2020). Nanofillers are also incorporated in biopolymers to improve the thermal, barrier, tensile and rheological properties of the films (Taherimehr et al., 2021). The high surface area of nanofillers leads to a large interfacial interaction between the nanofiller and polymer matrix, which reduces the molecular mobility of the polymer chains (Othman, 2014). Two types of antimicrobial nanocomposites can be developed, which include packaging materials incorporated with antimicrobial nanomaterials (e.g., silver and titanium oxide) or packaging materials incorporated with antimicrobial compounds and nanofillers (e.g., nanocellulose and nanoclay), which function as reinforcing agents for packaging materials (Vasile & Baican, 2021). Antimicrobial nanofillers are important components of antimicrobial food packaging because they extend the shelf life of packaged foods by reducing and inhibiting the growth of microorganism. Nanofillers have a stronger antimicrobial activity than their macro- or micro-scale counterparts because of their large surface area (Yildirim et al., 2018). Due to the inhalation of dust, some possible hazards may still exist if a large scale of nanomaterials are used for packaging production (Vasile & Baican, 2021).

In recent decades, clay minerals or nanoclays have been extensively used as nanofillers in food packaging materials due to their availability in nature and cost effectiveness. Montmorillonite (MMT), a member of the smectite group, is the most commonly used hydrophilic nanoclay because of its unique properties, such as large aspect ratio and surface area, great adsorption capacity, high ion exchange capacity and excellent compatibility with most of the organic thermoplastics (Bumbudsanpharoke & Ko, 2019; Othman, 2014). MMT has been incorporated in hydrophilic polymer materials, such as

polyvinyl alcohol (PVOH) and starch, soy protein isolate (SPI), to improve the oxygen barrier, mechanical and thermal properties (Guo, Tian, & Wu, 2019; Tee et al., 2013). However, inorganic nanoclay works poorly in organic environments.

Chemical modifications of MMT with hydrophobic components, such as organic cations (e.g., quaternary ammonium compounds) are needed to improve its compatibility with hydrophobic polymers (Bumbudsanpharoke & Ko, 2019). Organically modified MMT (OMMT) also has as stronger affinity for the polymer and lower surface energy than unmodified MMT. Incorporation of certain OMMT improved not only the physiochemical properties, such as thermal and mechanical, of polymer materials, but also their antimicrobial activity against both Gram-negative and Gram-positive bacteria. For instance, El Bourakadi et al. (2019) reported the antimicrobial activity of chitosan/PVOH films incorporated with thiabendazoluim-montmorillonite against *E. coli*, *S. aureus* and *P. aeruginosa*. Also, many researchers reported the addition of OMMT into polycaprolactone (PCL) improved the mechanical, thermal, barrier and antimicrobial properties of the packaging films (Hadj-Hamou, Metref, & Yahiaoui, 2017; Seyrek, Okur, & Saraçoğlu, 2021; Yahiaoui et al., 2015). Other clay nanoparticles, utilized as nanofillers in packaging materials, include cloisite (Sothornvit, Rhim, & Hong, 2009) and bentonite (Dogaru, Simionescu, & Popescu, 2020; Zheng, Tajvidi, Tayeb, & Stark, 2019).

In the past few decades, researchers are increasingly interested in inorganic nanoparticles, including metal and metal oxide nanoparticle, as synthetic antimicrobial agents due to their durability, great thermal stability and excellent antimicrobial activity against a wide range of bacteria and fungi (Becerril et al., 2020). Although metal and metal oxide particles are naturally abundant, their nanoparticles are often produced through

different physical and chemical treatments (Fu & Dudley, 2021). Metal-based nanoparticles have little toxicity to eukaryotes because they can distinguish bacterial cells from mammalian cells via the metalloproteins and metal transport system of bacteria (Becerril et al., 2020). Also, these nanoparticles inactivate the bacterial cell via three different pathways, which include destabilization of the phospholipid bilayer, binding to cytosolic proteins (e.g., DNA) and production of reactive oxygen species (e.g., singlet oxygen, superoxide and hydroxide radicals and hydrogen peroxide), resulting in oxidative stress, nucleic acid damage and enzyme inhibition (Gold, Slay, Knackstedt, & Gaharwar, 2018; Omerović et al., 2021). The most commonly used metal and metal oxide nanoparticles in food packaging materials are silver (Ag), gold (Au), titanium dioxide (TiO_2), zinc oxide (ZnO), copper oxide (CuO), magnesium oxide (MgO), alumina (Al_2O_3), silicon dioxide (SiO_2) and calcium oxide (CaO) (Hoseinnejad, Jafari, & Katouzian, 2018). Usually, incorporation of these nanoparticles at a low amount can enhance optical, tensile (e.g., strength and toughness), thermal and antimicrobial properties (Taherimehr et al., 2021).

Ag has long been utilized as an antimicrobial agent against a broad spectrum of bacteria, molds and viruses, with minimal toxicity to eukaryotic cells. It disrupts the metabolic activities of electron-transport and respiratory systems, as well as mass transfer across microbial cell membranes (Corrales et al., 2014). Similarly, Ag nanoparticles (Ag NP) are non-hazardous antimicrobial agents with high thermal stability and antimicrobial activity, allowing them to be used in a variety of commercial applications (Hoseinnejad et al., 2018). Ag NP can be produced by different methods, which include through the reaction of silver nitrate with polyethylene glycol (PEG) or via the photo-reduction of silver

nitrate using UV light (Fu & Dudley, 2021). The antimicrobial activity of Ag NP is accomplished through bacterial membrane adherence and cell penetration (Omerović et al., 2021). Ag NP are the most widely used nanofiller in active packaging materials to improve the film properties. Immobilization of these nanoparticles into packaging materials allows for the controlled release of toxic silver ions, thereby lowering their toxicity by limiting the migration of silver (Omerović et al., 2021). The amount of silver ions released into the system is mainly determined by both the nanoparticle properties (e.g., contact time, nanoparticle content, composition, shape and size) and external conditions (e.g., temperature, dissolved oxygen level, humidity, pH, ionic strength and microorganism type) (Hoseinnejad et al., 2018). Because of their high stability during film production and higher antimicrobial activity than their bulk counterparts, Ag NP are particularly appropriate for direct addition into packaging materials (Fu & Dudley, 2021).

In addition, Au nanoparticles (Au NP) are readily produced through top-down (e.g., irradiation, diffusion and thermal decomposition) and bottom-up (e.g., chemical reduction, electrochemical reaction and chemical polyol synthesis technique) approaches (Hoseinnejad et al., 2018). The antimicrobial activity of Au NP mainly depends on several factors, such as the type of target bacteria, functionalization and nanoparticle size. These nanoparticles inhibit the bacteria by adhering to the cell membrane and altering its potential, or by penetrating into cells and preventing tRNA binding to the ribosome or lowering the levels of adenosine triphosphate, leading to biological dysfunction (Omerović et al., 2021). As antimicrobial agents, these nanoparticles are considered safe due to their reactive oxygen species-independent antimicrobial mechanism.

TiO_2 and ZnO are the most widely studied metal oxide nanoparticles for antimicrobial and antifungal purposes. TiO_2 nanoparticles (TiO_2 NP), GRAS-approved additives by the US FDA, are often used in food, healthcare and cosmetic products to improve their appearance and brightness (Al-Tayyar et al., 2020). The sol-gel method is commonly used to develop these nanoparticles. TiO_2 NP can be used in various sectors due to their low toxicity and cost, longevity and availability. However, the European Food Safety Authority (EFSA) stated that these nanoparticles are no longer regarded safe as a food additive due to the possibility of genotoxicity and inflammation to human cells (Hoseinnejad et al., 2018). Also, France has issued a ban on the sale of food products containing TiO_2 by January 2020. TiO_2 NP have been widely utilized due to their high stability and antimicrobial activity against a broad range of microorganisms. The antimicrobial activity of the nanoparticles depends on the production of reactive oxygen species, such as superoxide and hydroxide radicals, upon exposure to UV light (Omerović et al., 2021). TiO_2 NP exhibit greater photocatalytic properties and antimicrobial activity than their bulk material counterparts, making them more attractive for food applications (Fu & Dudley, 2021). Because of their small particle size, they can easily penetrate the bacterial membrane and destroy them.

Inorganic oxides, such as ZnO, contain micronutrients essential to animal and human health (Corrales et al., 2014). ZnO is an odorless white powder and is used as a GRAS-approved food additive and nutritional supplement. ZnO nanoparticles (ZnO NP) have received increased attention due to their excellent antimicrobial activity against a wide range of bacteria, fungi and viruses, even at low concentrations. The antimicrobial activity of ZnO NP is achieved by three different pathways, which include the production

of reactive oxygen species, the disruption of microbial membrane and the release of active zinc ions in an aqueous solution (Al-Tayyar et al., 2020). These nanoparticles can be easily synthesized in a variety of shapes, including nanoheliz and nanorings (Al-Tayyar et al., 2020). In addition, researchers have developed various antimicrobial food packaging incorporated with nanoparticles of metal oxides, such as CuO (Saravanakumar, Sathiyaseelan, Mariadoss, Xiaowen, & Wang, 2020), Al_2O_3 (Ramesh, Sivasamy, Rhee, Park, & Hui, 2015), MgO (Hosseini, Pirsa, & Farzi, 2021), SiO_2 (Monadi Sefidan, 2018) and CaO (Silva et al., 2020).

2.5 Effects of antimicrobials on food packaging properties

The performance of an antimicrobial food packaging film is determined by several parameters, including type and nature of the biopolymer utilized as the base material, the type and amount of antimicrobials incorporated in the biopolymer, the incorporation techniques, the retention and release rate of the antimicrobials, the conditions of storage and the target microorganisms (Varghese et al., 2020). Different types of antimicrobial agents, when incorporated into packaging materials will have different effects on the tensile, barrier, thermal, antioxidant and antimicrobial properties of the films. When antimicrobial agents are not compatible with the biopolymers, their incorporation into packaging materials can have a negative impact on the film properties. Antimicrobial agents that are compatible with packaging materials can disperse homogeneously in the polymer matrix with a minimal impact on the film properties (Sung et al., 2013). Physical and chemical interactions between antimicrobials and biopolymers influence the polymer structure, hence, altering their functionality.

2.5.1 Effect on mechanical properties

The mechanical properties, including tensile strength, elongation at break, elastic modulus, and many others, of packaging materials are important in the development of antimicrobial food packaging because they indicate the ability of the packaging to protect food products against physical damage, including blackening, detaching and indentation (Abdul Khalil et al., 2018). Tensile strength is the force per unit applied when the film is broken, while the elongation at break indicates the film stretchability (Bastarrachea, Dhawan, & Sablani, 2011). These film properties highly depend on the types of polymers and antimicrobial agents used. The American Society of Testing and Materials (ASTM) D882 is the most widely used standard method for measuring the tensile properties of packaging films. Based on the standard method, the mechanical properties of packaging films are influenced by film preparation technique, film thickness, type of grip, distance of grip separation and test speed (Taherimehr et al., 2021). The method can be utilized to determine the efficiency of food packaging materials during practical use, storage and handling (Cheng et al., 2021).

The addition of antimicrobials often reduces the tensile strength of packaging materials because the additives are typically small components that can occupy between polymer chains, causing slippage when external pressures are applied, due to the plasticizing effect (Sung et al., 2013). The tensile strength of packaging materials are not greatly affected when only small quantities of antimicrobial agents are incorporated or when the molecular weight of the antimicrobial agent is less than that of polymer materials (Bastarrachea et al., 2011). For instance, the addition of 2.00-2.50% (w/w) of malic acid did not significantly affect the tensile strength of zein films (Sözbilen, Çavdaroğlu, &

Yemenicioğlu, 2022). Similar results were obtained when low concentrations of cinnamon, thyme or lemon EO was incorporated into biodegradable films (Peng & Li, 2014; Syafiq et al., 2021). However, the tensile strength of zein films significantly decreased when 3.0% (w/w) or higher concentrations of lactic acid, malic acid and tartaric acid were incorporated. Similar reductions in tensile strength were also observed in packaging films incorporated with a combination of cinnamon EO, citrus extract (Shankar, Khodaei, & Lacroix, 2021) and nisin (Monjazeb Marvdashti, Koocheki, & Yavarmanesh, 2019). It could be due to the development of heterogeneous film structure with discontinuities, resulting in weaker interactions between polymer chains and, hence, a lower tensile strength (Varghese et al., 2020). Incorporation of inorganic nanoparticles, such as Ag NP (Shankar et al., 2021), Au NP (Chowdhury et al., 2020), ZnO NP (Hezma, Rajeh, & Mannaa, 2019) and TiO_2 NP (Malathi & Singh, 2019), improved the tensile strength of packaging films due to the formation of strong hydrogen bonds between the nanoparticles and the polymer matrix. A similar trend of tensile strength was observed with SPI and fish gelatin films containing 1-5% (w/w) of mango kernel extract due to crosslinking between protein molecules and polyphenolic compounds, resulting in an increase in film stiffness (Adilah, Jamilah, & Hanani, 2018). The tensile strength of packaging films strongly depends on the distribution and density of the intra- and inter-molecular interactions between the polymer chains (Jafarzadeh & Jafari, 2021).

Zein films containing organic acids, such as lactic acid, malic acid and tartaric acid, exhibited greater elongation at break than control films due to their plasticizing effect (Sözbilen et al., 2022). Khaneghah et al. (2018) and Varghese et al. (2020) suggested that the incorporation of most EO and plant extracts to polymer materials typically reduces the

tensile strength and improves the elongation at break of food packaging films. The tensile strength of packaging films is often reduced when additives other than crosslinking agents are incorporated (Mir et al., 2018). For instance, chitosan films incorporated with 5-9% (w/w) of lemongrass EO showed reduced tensile strength and increased elongation at break (Han Lyn & Nur Hanani, 2020). The EO droplets act as plasticizers, which decrease the cohesion of the polymer network forces, resulting in an increase in film flexibility (Varghese et al., 2020). Similar trends were also found in hydrophobic PLA films incorporated with fenugreek (Subbuvel & Kavan, 2022) or anise EO (Noori, Khanjari, Rezaeigolestani, Karabagias, & Mokhtari, 2021). The film flexibility improved significantly when a high concentration (10% (w/w)) of black cumin seed extracts was added into sago starch films (Ekramian, Abbaspour, Roudi, Amjad, & Nafchi, 2021). However, the SPI and fish gelatin films containing 5% (w/w) of mango kernel extract displayed lower film flexibility because of the crosslinking effect, which reduces the film mobility by decreasing the plasticizing effect (Adilah et al., 2018). In addition, incorporation of nisin (Monjazeb Marvdashti et al., 2019) and inorganic nanoparticles, such as copper sulfide nanoparticles (CuS NP) (F. Li et al., 2020), Ag NP (Cano, Cháfer, Chiralt, & González-Martínez, 2016), ZnO NP (Hezma et al., 2019), and TiO_2/Ag NP (Li et al., 2017), improved the elongation at break of antimicrobial packaging films.

2.5.2 Effect on barrier properties

The incorporation of antimicrobial agents to polymer materials can affect the barrier properties of packaging films. Transmissions of water vapor and oxygen between the environment and food products must be avoided to achieve effective food packaging

due to their significant impact on the quality, appearance, taste, odor and shelf life of packaged food products. (Taherimehr et al., 2021). Thus, packaging materials with excellent barrier properties can help to maintain or extend the shelf life of food products. Inappropriate packaging can induce weight and moisture losses and shrinkage in food products during long-term storage (Hoseinnejad et al., 2018). Hence, it is critical that the barrier properties can be measured and controlled. Water vapor permeability (WVP) and oxygen permeability (OP) of packaging materials are typically used to determine their barrier properties.

Water vapor barrier properties are determined by measuring WVP coefficients and water vapor transmission rate (WVTR) based on the amount of water vapor that penetrates and passes through a packaging material (Sung et al., 2013). The ASTM E 96-95 gravimetric analysis technique is commonly used to determine the WVP and WVTR of biopolymer films (Abdul Khalil et al., 2018). The openings of glass containers or aluminum permeation cans containing a hygroscopic substance (e.g., anhydrous calcium chloride) are covered with film samples and sealed with paraffin to ensure that water migration happens only through the covered area. They are placed in a desiccator with a saturated sodium chloride solution (75% relative humidity) and their weight changes over time are measured (Cheng et al., 2021). One of the significant factors influencing food quality and safety is the equilibrium moisture content. A packaging material with good water vapor barrier properties keeps water vapor from the environment from migrating into foods and prevents water loss from foods. Food texture loss and microbial growth are reduced or inhibited by preventing or reducing water migration into packaged foods, and thus, extending the shelf life of foods (Monjazeb Marvdashti et al., 2019). The WVP of biopolymer films depends

on various factors, such as the degree of crosslinking, the presence of tortuosity, crack or voids in the polymer matrix, and the hydrophobic-hydrophilic ratio of the polymer matrix.

It was reported that the addition of 3.5% (w/w) of organic acid, such as lactic acid, increased the WVP of plasticized zein films, whereas the incorporation of malic acid and tartaric acid reduced their WVP (Sözbilen et al., 2022). Even though zein films are hydrophobic, their high WVP is primarily due to the weak chain interaction and the presence of voids in the structure. In addition, incorporation of nisin significantly increased the WVP of PVOH/seed gum composite films (Monjazeb Marvdashti et al., 2019). It could be related to the disruption of polymer network, which reduces the intermolecular interaction between the polymer chains of PVOH and seed gum. The incorporation of nisin also enhanced the free volume of the polymer matrix, resulting in a greater WVP. The incorporation of mango kernel extract decreased the WVP of SPI films (Adilah et al., 2018). The plant extract may fit between the protein-protein globular structure, causing the water vapor channel to be blocked or reduced, resulting in a lower WVP. However, the WVP of fish gelatin films increased with the incorporation of mango kernel extract. It could be due to the disruption of the ordered polymer network, which results in the formation of channels and voids for water vapor to permeate through, resulting in a greater WVP.

The incorporation of EO into polymer materials is expected to improve the water vapor barrier properties of films by increasing the hydrophobic-hydrophilic ratio of the polymer matrix and disrupting the hydrophilic polymer network, resulting in a tortuosity pathway for water vapor to permeate through the polymer matrix (Khaneghah et al., 2018). Also, the improved water vapor barrier properties could be attributed to a reduced hydrophilic region in polymer matrix, which reduces water vapor migration (Syafiq et al.,

2020). For instance, WVP was reported to be lower in chitosan films incorporated with EO, such as lemongrass (Han Lyn & Nur Hanani, 2020), cinnamon, thyme, lemon (Peng & Li, 2014) and apricot kernel (Priyadarshi, Kumar, Deeba, et al., 2018). However, the WVP of hydrophobic PLA films increased when anise (Noori et al., 2021) or fenugreek EO (Subbuvel & Kavan, 2022) was added. This could be related to the formation of a heterogeneous structure in the PLA/EO composites, resulting in a disruption of film uniformity, as a result, reducing the water vapor barrier properties of the films. Adding nanofillers into polymer materials alters the WVP of films. The water migration into foods can be avoided or reduced by incorporating low amounts of inorganic nanoparticles in packaging materials. For instance, Cano et al. (2016) found that when low levels of Ag NP were added, the WVP of starch/PVOH films decreased, but it increased when the amounts of Ag NP were increased. At a lower level of nanoparticles, the polymer films have a more compact structure than the control films, reducing the WVP of films. A higher level of Ag NP restricts macromolecule extension in the aqueous conditions, resulting in a less compact film structure.

Oxygen barrier properties are important in food packaging for improving the food shelf life during handling, distribution and storage (Taherimehr et al., 2021).The oxygen barrier properties of biopolymer films are determined by measuring the OP and oxygen transmission rate (OTR) using an oxygen transmittance rate analyzer (Cheng et al., 2021). They are used to determine the shelf life, nutritional value and sensory properties of packaged food products. The gaseous content in the headspace of packaged foods is one of the significant parameters of storage of minimally processed fruits and vegetables (Abdul Khalil et al., 2018). In such food packaging, an optimum percentage ratio of oxygen and

carbon dioxide gases is controlled to avoid undesirable food reactions (e.g., browning and oxidation) at a higher level of oxygen, and to prevent the growth of anaerobic bacteria and microaerophilic bacteria (e.g., lactic acid bacteria and *Listeria* sp.) and *Clostridium botulinum*, at a lower level of oxygen (Abdul Khalil et al., 2018). Also, an increased oxygen level in the food packaging may lead to nutritional value and quality losses, oxidative rancidity and changes in color, texture, aroma and flavor of food products containing oil (e.g., nuts) (Sözbilen et al., 2022). All these unfavorable effects dramatically reduce the quality and shelf life of food products. Hence, it is critical to monitor oxygen transmission in food packaging systems for maintaining the quality and safety of food products.

In general, the concentration of antimicrobial agents and the degree of crystallinity of the antimicrobial packaging have a significant impact on the oxygen barrier properties (Rojas et al., 2021). Some antimicrobial agents have been shown to decrease the OP of food packaging. For instance, the addition of potassium sorbate and sodium benzoate significantly reduced the OP of extruded PBAT/TPS films (Wangprasertkul et al., 2021). The hydrophilicity of these synthetic antimicrobials enhanced water affinity of the polymer matrix and, hence, avoid the diffusion of oxygen. On the other hand, PBAT/TPS films had a higher OP value when incorporated with nisin due to their ability to enhance polymer chain mobility and free volume and increase the dispersion of non-polar areas, causing an increase in oxygen permeation (Leelaphiwat et al., 2022). However, the addition of hydrophilic EDTA into the antimicrobial packaging reduced the diffusion of non-polar oxygen molecules. In addition, the incorporation of nisin slightly increased the OP of PVOH/seed gum composite films (Monjazeb Marvdashti et al., 2019). It could be attributed

to the film compactness, as there were no pores on the surface when the antimicrobial agent was added.

It was also reported that the effects of cinnamon EO and TiO_2 NP on the OP of sago starch films were in opposite directions (Arezoo, Mohammadreza, Maryam, & Abdorreza, 2020). For instances, the addition of cinnamon EO increased the OP, whereas the incorporation of inorganic nanoparticles reduced the OP. In addition, CS/MMT films incorporated with EO, such as ginger (Souza, Pires, Rodrigues, et al., 2019) and rosemary (Souza, Pires, Vieira, et al., 2019), displayed a higher OP value. This could be due to the plasticizing effect of hydrophobic EO, which increases polymer chain mobility and intermolecular space and, hence, allow oxygen gas to penetrate through the films. Also, the incorporation of pine needle extract decreased the OP of chitosan films (Kadam, Singh, & Gaikwad, 2021), which could be related to the crosslinking effect due to the formation of intermolecular hydrogen bonding between the plant extract and chitosan chains. The plant extract may also fill the voids of the polymer matrix, leading to a denser polymer structure. However, the addition of some plant extracts, such as winter jujube leaf powder, peanut shell powder, pine nutshell powder and cashew leaf extract, decreased the OP of chitosan films (Shiekh et al., 2022; Zhang, Lian, Shi, Meng, & Peng, 2020). Because inorganic nanoparticles are impermeable to gases, their incorporation is also expected to improve the oxygen barrier properties of packaging films. The incorporation of metal and metal oxide nanoparticles, such as Ag NP (Castro-Mayorga, Fabra, & Lagaron, 2016), Au NP (Pagno et al., 2015), ZnO NP (Ngo, Dang, Tran, & Rachtanapun, 2018) and TiO_2 NP (Arezoo et al., 2020), into packaging materials effectively improved the oxygen barrier properties of biopolymer films. This could be due to the good dispersion of nanoparticles in the polymer

matrix, which results in the formation of a more tortuous oxygen pathway and reduced free spaces and, hence, delaying the oxygen permeation. Other important factors that affect the oxygen barrier properties include the immobilization of polymer chains due to strong interactions between polymer and nanomaterials, and the increased degree of crystallinity of polymer films (Wang et al., 2018).

2.5.3 Effect on antioxidant properties

In addition to microbial contamination, food deterioration is caused by oxidation, which has a significant impact on the shelf life of food products. Oxidation has a negative impact on the energy content, nutritional values and organoleptic properties (e.g., color, flavor, aroma and texture) of food products due to the degradation of essential unsaturated fatty acids, lipid soluble vitamins and proteins, as well as the production of toxic aldehydes, which pose a health hazard to consumers (Sanches-Silva et al., 2014). Food products with a high level of fatty acids are susceptible to lipid oxidation. In the food industry, antioxidants are commonly added to the food products to neutralize free radicals, that contribute to cell membrane and DNA damage, as well as oxidative stress. However, once the active compounds are consumed, the protective efficacy dramatically diminishes, and the food quality deteriorates at a faster rate (Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, 2014). Despite the use of vacuum or modified atmosphere packaging, the oxygen that is dissolved in food is difficult to remove or cannot be completely removed and, hence, oxidation cannot be avoided (Sanches-Silva et al., 2014). Due to consumer demand for safer and healthier foods, researchers are searching

for new preservation methods, such as antioxidant packaging systems, to enhance the stability of food products that are prone to oxidation.

Biopolymer films with antioxidant properties exhibit a great potential to extend the shelf life of foods by retarding the natural processes. Antioxidants can be incorporated into polymers materials, allowing controlled migration of active agents to the food surface during handling, distribution and storage. For a non-migratory type of active food packaging, they are grafted onto the polymer surface via physical or chemical binding. The efficiency of antioxidant food packaging is evaluated using various main techniques, including 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radicals scavenging, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals scavenging and ferric reducing antioxidant power (FRAP) methods (Rangaraj, Rambabu, Banat, & Mittal, 2021). An antioxidant food packaging reduces the amounts of antioxidants needed and simplifies food processing by eliminating unnecessary steps, such as spraying, immersion or mixing of antioxidants (Sanches-Silva et al., 2014). Also, the conventional use of synthetic antioxidants, including tert-butylhydroquinone, butylated hydroxyanisole and butylated hydroxytoluene, for food applications remains controversial because of their potential health risk to consumers (Gómez-Estaca et al., 2014). Due to consumer demand for healthier and safer food products, natural antioxidants have the potential to replace their synthetic counterparts due to their higher safety and lower toxicity.

In recent decades, many studies have reported improved stability of oxidation-sensitive food products packaged with biopolymer films containing naturally derived components, such as plant extracts and EO, that exhibit both antimicrobial and antioxidant properties. For example, food packaging films incorporated with plant extracts, such as

olive leaves (da Rosa, Vanga, Gariepy, & Raghavan, 2020), basil leaves (Singh, Gaikwad, & Lee, 2018), gallnut (Aloui, Deshmukh, Khomlaem, & Kim, 2021), mango kernel extract (Adilah et al., 2018) and many others, showed a greater antioxidant activity as the concentration of plant extracts increases. The antioxidant activity of packaging films is directly correlated to the availability of phenolic compounds, which are responsible for quenching free radicals (Mir et al., 2018). The antioxidant capacity of soy protein films produced by compression molding was greater than that of solution casted films, indicating the impact of different film preparation techniques (Asgher et al., 2020). In addition, food packaging films containing EO, such as clove (Chen et al., 2018), oregano (Lee, Garcia, Shin, & Kim, 2019), cinnamon (Fasihi et al., 2019), thyme (Ocak, 2020) and many others, exhibited antioxidant properties. The antioxidant activity of EO-containing food packaging is primarily determined by the availability of bioactive compounds that can quench free radicals or prevent the oxidation of organic components. For instance, sulfur-containing compounds, terpenoids, and phenolic compounds (e.g., linalool, eugenol, thymol and carvacrol) are major components of EO that exhibit a high scavenging activity (Basavegowda & Baek, 2021).

2.5.4 Effect on antimicrobial properties

Antimicrobial packaging acts as a protective barrier against microbial growth in food products, and hence, increases food shelf life, quality and safety. Such packaging has gained increased attention due to consumer demand for additive-free and minimally processed foods, as well as their ability to prevent the growth of foodborne pathogens in a controlled manner. The antimicrobial properties of food packaging films are evaluated

using various methods, such as disc diffusion test, viable cell count technique, broth dilution technique and many others (Abdollahzadeh, Nematollahi, & Hosseini, 2021). Disc diffusion test is one of the most widely used techniques for determining *in vitro* antimicrobial activity of food packaging due to its flexibility, simplicity and inexpensive nature. However, it is inadequate for evaluating the antimicrobial activity of packaging films containing deep-colored agents or having low transparency. The viable cell count technique is used to determine the antimicrobial activity of packaging films in broth media or food models. A selective medium can be used to evaluate the count of viable bacteria. The broth dilution technique is an optical density-based method used to evaluate the antimicrobial activity of packaging films. In this technique, the optical density at 600 nm is measured using a UV spectrophotometer or a microplate reader to determine bacterial growth in a culture medium. However, this technique is unsuitable for determining the antimicrobial properties of packaging films containing a high level of colored agents and having poor tensile properties and moisture barrier and high solubility in water.

In recent years, researchers have reported the antimicrobial activity of packaging materials incorporated with synthetic antimicrobial agents, such as weak organic acid and its salts, chelating agents and many others. For instance, zein films incorporated with organic acids, such as lactic acid and malic acid, were very active against *E. coli*, *Klebsiella pneumoniae*, *Listeria innocua* and *S. aureus* (Sözbilen et al., 2022). Other weak organic acids, such as citric acid (Priyadarshi, Kumar, & Negi, 2018) and tartaric acid (Suganthi, Vignesh, Sundar, & Raj, 2020), have also been added into food packaging materials to improve their antimicrobial activity. Also, pullulan/gelatin films incorporated with potassium sorbate had strong inhibitory effects against *E. coli*, *Kloeckera apiculata* and

Botrytis cinerea (Kowalczyk et al., 2020). Because EDTA has a poor antimicrobial activity against Gram-negative bacteria, it is often combined with other active agents such as bacteriocins, enzymes, metal and metal oxide nanoparticles, and many others, to broaden its spectrum of antimicrobial activity. Salts of parabens, such as sodium salts of methyl, ethyl and propyl parabens, were incorporated into hydroxypropyl methylcellulose (HPMC)-lipid edible films and coatings to improve their antifungal activity against molds, such as *Penicillium digitatum, Penicillium italicum, B. cinerea* and *Alternaria alternata*, on fresh fruits and vegetables (Fagundes, Pérez-Gago, Monteiro, & Palou, 2013; Valencia-Chamorro, Palou, Del Rio, & Perez-Gago, 2008).

In addition, the incorporation of natural antimicrobial agents, such as EO, plant extracts, bacteriocins, enzymes and bacteriophages, into packaging materials improves their antimicrobial activity against a wide range of bacteria, molds and yeasts. Plant-derived EO contain various bioactive compounds that can function as antimicrobial agents. Hence, the incorporation of EO into packaging materials can significantly enhance its antimicrobial activity. For example, packaging films containing various types of EO, exhibited antimicrobial activity against Gram-negative (e.g., *E. coli, Salmonella* Typhi and *Yersinia enterocolitica*) and Gram-positive bacteria (e.g., *B. subtilis, Bacillus cereus, L. monocytogenes, S. aureus*, *Enterococcus faecalis* and *Staphylococcus carnosus*) and molds (e.g., *P. digitatum*, *Candida albicans* and *Aspergillus brasiliensis*) (Anuar et al., 2017; de Andrade et al., 2020; Fasihi et al., 2019; Hammoudi, Ziani Cherif, Borsali, Benmansour, & Meghezzi, 2020; Han Lyn & Nur Hanani, 2020; Llana-Ruiz-Cabello et al., 2016).

Grapefruit seed extract (GFSE) is one of the most studied plant extracts due to its broad spectrum of antimicrobial activity against both Gram-negative and Gram-positive

bacteria. Due to its great thermal stability, it can be extruded with various packaging materials. For instance, incorporation of grapefruit seed extract into PCL/chitosan films improved the films' antimicrobial activity against *E. coli* and *P. aeruginosa* and extended the shelf life of salmon and bread (Wang, Lim, Tong, & San Thian, 2019). Other plant extracts, including green tea extracts (Amankwaah, Li, Lee, & Pascall, 2020), olive leaf extract (Martiny, Raghavan, Moraes, Rosa, & Dotto, 2020), chicory root extract (Jaśkiewicz, Budryn, Nowak, & Efenberger-Szmechtyk, 2020) and many others, were also incorporated into biopolymers for the development of antimicrobial food packaging.

Food packaging containing antimicrobial enzymes, such as lysozyme, lactoferrin, lactoperoxidase, glucose oxidase and invertase, are developed by various techniques, that include grafting, physical blending and chemical binding in packaging materials (Becerril et al., 2020). For instance, the antimicrobial activity of PVOH films containing surface-immobilized lysozyme is greater than that of bulk-bound films (Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile, 2006). Also, Mecitoğlu et al. (2006) reported that zein films incorporated with lysozyme exhibited antimicrobial activity against *B. subtilis* and *Lactobacillus plantarum*. Disodium EDTA was added to the films, which made them effective against *E. coli*. A blend of lysozyme and other enzymes were incorporated into biopolymers to improve the antimicrobial activity against a wider spectrum of foodborne bacteria (Min, Harris, & Krochta, 2005; Moreno, Atarés, & Chiralt, 2015).

A food packaging can be also developed by incorporating bacteriocin-producing bacteria, or adding purified or partially purified bacteriocin or bacteriocin-like substances (Becerril et al., 2020). For instance, biopolymer films incorporated with bacteriocin-producing bacteria (e.g., *Lactobacillus curvatus*) (La Storia et al., 2020) and bacteriocin

extracts (Salvucci et al., 2019) were found to be effective against *L. innocua* in cooked ham and cheese samples, respectively. Nisin has been successfully incorporated into single or composite biopolymers, such as corn zein, gelatin (Ku & Song, 2007), PLA (Jin & Zhang, 2008), sodium caseinate (Cao-Hoang, Chaine, Grégoire, & Waché, 2010), PVOH/seed gum (Monjazeb Marvdashti et al., 2019) and CS/PLA (Wang et al., 2015). Bacteriocins are used in combination with other antimicrobials in food packaging because they are less effective against Gram-negative bacteria (Becerril et al., 2020). Studies showed that the incorporation of bacteriocins in combination with other antimicrobials, such as EDTA (Abarca et al., 2022), EO (Wang, Guo, Liu, Han, & Niu, 2021), plant extract (Sivarooban et al., 2008) and enzymes (Murillo-Martínez, Tello-Solís, García-Sánchez, & Ponce-Alquicira, 2013) improved the antimicrobial properties of food packaging materials.

In addition, bacteriophages have been incorporated into packaging materials, such as PCL (Choi, Chang, Kim, & Han, 2021), gelatin (Weng et al., 2021), sodium alginate (Alves et al., 2019) and whey protein isolate (Vonasek, Le, & Nitin, 2014). Incorporation of phage cocktails further improved the antimicrobial spectrum of the packaging materials (López de Dicastillo, Settier-Ramírez, Gavara, Hernández-Muñoz, & López Carballo, 2021; Tomat et al., 2019). Alves, Cerqueira, Pastrana, and Sillankorva (2020) reported the synergistic antimicrobial activity of sodium alginate emulsion-based films incorporated with phage cocktail and cinnamaldehyde against *E. coli* and *Salmonella* Enteritidis. To enhance the phage stability in the acidic conditions, bacteriophages were encapsulated using liposomes before incorporating into chitosan films (Cui, Yuan, & Lin, 2017).

Metal nanoparticles, such as Ag NP, have been incorporated into biopolymers to enhance the antimicrobial activity against foodborne bacteria, fungi and yeasts, such as *E.*

coli O157:H7, *S. aureus, L. innocua, A. niger, Penicillium expansum* and *C. albicans* (Cano et al., 2016; Mohamed & Madian, 2020; Youssef, Abdel-Aziz, & El-Sayed, 2014; Yu, Wang, et al., 2019). Gram-negative bacteria are more vulnerable to Ag NP compared to Gram-positive bacteria due to their negatively-charged cell membrane and thin layer of peptidoglycan (Hoseinnejad et al., 2018). Also, Au NP have been incorporated into biopolymers to improve the antimicrobial and antifungal activities against *E. coli, S. aureus, P. aeruginosa, C. albicans* and *A. niger* as well as the shelf life of packaged food products (Chowdhury et al., 2020; Pagno et al., 2015; Youssef et al., 2014).

Metal oxide nanoparticles, such as TiO_2 NP, are utilized as additives to improve the antimicrobial properties of films. Siripatrawan and Kaewklin (2018) concluded that chitosan films containing 1% TiO_2 NP showed ethylene photodegradation and antimicrobial activity against foodborne bacteria and fungi, such as *E. coli, P. aeruginosa, S.* Typhimurium, *S. aureus*, *Aspergillus* sp. and *Penicillium* sp., under UV illumination. The results showed that the inhibition effect of TiO_2 NP is greater under the radiation of UV light. Also, PLA films incorporated with the nanoparticles exhibited improved antimicrobial activity against *E. coli* and *L. monocytogenes* (Li et al., 2017; Segura González, Olmos, Lorente, Vélaz, & González-Benito, 2018). In addition, ZnO NP are utilized in the food packaging industry because they are cheaper and less toxic than other nanoparticles, such as Ag NP (Fu & Dudley, 2021). These nanoparticles have been incorporated into various biopolymers to improve the physiochemical and antimicrobial properties of the food packaging films. Several recent publications include ZnO NP-incorporated CS/gelatin films (Kumar et al., 2020), ZnO NP-incorporated gelatin/starch

films (Lee, Said, & Sarbon, 2021), ZnO NP-incorporated PLA films (Shankar, Wang, & Rhim, 2018) and ZnO NP-incorporated seaweed-based films (Baek & Song, 2018).

2.6 Applications of antimicrobial packaging in foods

Antimicrobial polymers have a broad range of applications in the food industry, including food packaging. They can be used to improve shelf life and safety of packaged foods by inhibiting or delaying the growth of foodborne microorganisms. Antimicrobial packaging also minimizes the risk of recontamination in processed food products and eliminates some of the food processing methods required to sterilize and sanitize food products (Appendini & Hotchkiss, 2002). Apart from antimicrobial activity, active food packaging must also meet certain film characteristics, such as tensile, barrier, optical and antioxidant properties. In recent decades, biodegradable polymer materials containing antimicrobial agents have been used in the packaging of different food products, such as meat, poultry, fruit, vegetables and many others (Cheng et al., 2021).

In recent years, antimicrobial packaging has advanced rapidly in response to a relentless outbreaks of foodborne contamination associated with meat and poultry products, that have raised consumer concerns about the food safety. Meat is an excellent source of nutrients for the growth of microorganisms. These products deteriorate rapidly due to microbial contamination, particularly by foodborne pathogens, such as *E. coli* O157:H7 and *L. monocytogenes* (Véronique, 2008). *E. coli* often grows in fresh and frozen meats, and *L. monocytogenes* can survive in a high salt concentration. Although the shelf life of meat products can be prolonged by storing them at refrigerated temperatures or using vacuum and modified atmosphere packaging, there is an increasing concern about the

survival and growth of psychrophilic (e.g., *Aeromonas hydrophila* and *Y. enterolitica*) and microaerophilic microorganisms (e.g., *Campylobacter jejuni* and *Lactobacillus*). Hence, research and development of new packaging technology is important to the meat industry.

In general, antimicrobial packaging is used to delay or inhibit microbial growth on the surface of meat products, thereby extending the shelf life of perishable products and improving the safety of packaged meat products (Véronique, 2008). The important aspects that should be considered for antimicrobial meat packaging include the selection of an antimicrobial agent and its delivery strategy, as well as the minimal influence on the organoleptic properties of packaged products (Fang et al., 2017). For example, protein-based films containing 1.50% (w/v) sorbic and benzoic acid were effective in inhibiting the growth of *E. coli* O157:H7 and *L. monocytogenes* on refrigerated beef samples (da Rocha, Loiko, Tondo, & Prentice, 2014). Incorporation of EDTA and nisin into TPS/PBAT films reduced the total viable count and lactic acid bacteria count on fresh pork meat, indicating their efficiency to inhibit microbial growth (Leelaphiwat et al., 2022). The redness of meat was stabilized by the antimicrobial films, suggesting stabilization of meat myoglobin during storage.

In addition, chitosan/starch films incorporated with pomegranate peel extract and *Thymus kotschyanus* EO effectively reduced *L. monocytogenes* count in beef samples stored in the refrigerator for 12 days (Mehdizadeh, Tajik, Langroodi, Molaei, & Mahmoudian, 2020). The total viable count, lactic acid bacteria count and *Pseudomonas* count of packaged beef samples were reduced during refrigerated storage, indicating an increase in the food shelf life. Packaging films containing EO, such as apricot kernel (D. Wang et al., 2020), oregano (Pavli et al., 2019), cinnamon (Ahmed, Hiremath, & Jacob,

2016), *Juniperus communis* and *Satureja montana* (Vasilijević et al., 2019), have also been demonstrated to exhibit antimicrobial activity against *L. monocytogenes* on meat and to extend the shelf life of foods. Starch-based films containing grape pomace extracts significantly reduced the *L. monocytogenes* count on chicken deli meats under refrigerated storage for 10 days (Xu et al., 2018). Compression molded PLA-based film containing Ag-Cu nanoparticles and cinnamon EO effectively reduced the counts of *S.* Typhimurium, *L. monocytogenes* and *C. jejuni*, in chicken meat samples during 3 weeks of refrigerated storage conditions (Ahmed et al., 2018). Choi et al. (2021) demonstrated the inhibitory effect of PCL films containing phage T4 against *E. coli* O157:H7 on raw beef samples during 5 days of storage at 10 °C. Whey protein concentrate edible films incorporated with phage cocktail significantly reduced the viable counts of *E. coli* and STEC on meat samples (Tomat et al., 2019). Sodium-alginate films loaded with ϕIBB-PF7A phage still exhibited antimicrobial activity against *Pseudomonas fluorescens* on chicken breast fillets after 5 days of storage at refrigeration temperatures (Alves et al., 2019).

Fresh produces, such as fruits and vegetables, have excellent nutritional value with high contents of vitamins, minerals and fibers. However, they are susceptible to rapid deterioration due to their high moisture concentrations, and therefore, higher water activity (Chawla et al., 2021). The primary cause of fresh produce degradation is moisture loss, which results in poor organoleptic properties, such as unpleasant appearance and firmness, as well as weight loss (Basumatary et al., 2020). Fresh produce are vulnerable to microbial contamination, enzymatic browning and formation of undesirable flavor and texture because they contain living tissues. Lack of appropriate packaging is one of the primary causes of fresh produce contamination. Due to the increased global demand for fresh

produce, an innovative technology is required to increase food shelf life, maintain food quality and reduce food waste.

Due to consumer demand for chemical-free additive products, biodegradable packaging materials loaded with natural antimicrobial agents can be used to reduce the spoilage of fresh produce and to inhibit the microbial growth. For instance, N-succinyl chitosan films incorporated with nisin and *Perilla frutescense* EO showed antimicrobial activity against *S. aureus*, *E. coli*, *S. enteritidis* and *Pseudomonas tolaasii* (Wang et al., 2021). Edible films containing nisin and 1.5% (v/v) of EO extended the shelf life of strawberry for up to 7 days at room temperature. The incorporation of nanoparticles in packaging materials for extending food shelf life has also gained increased attention due to their improved reinforcement effects and antimicrobial properties. For instance, chitosan/cellulose acetate phthalate films containing 5% (w/w) of ZnO NP exhibited antimicrobial activity against *S. aureus* and *E. coli*, and increased the shelf life of black grape fruits by up to 9 days (Indumathi, Sarojini, & Rajarajeswari, 2019). According to the disc diffusion study, chitosan films incorporated with CuO NP showed inhibitory effect against *Enterobacter cloacae*, *Salmonella, S. aureus* and *Campylobacter*, whereas the films containing ZnO NP inhibits *Salmonella* only (Kalia et al., 2021). However, both films did not cause spoilage of guava fruits after 14 days of storage at 6 °C. Furthermore, the incorporation of Ag NP, TiO_2 NP and bergamot EO into PLA films resulted in a significant reduction in total bacterial count of packaged mangoes after 9 days of storage at 20 °C, indicating improved antimicrobial activity of the films (Chi et al., 2019). Other biodegradable films incorporated with EO and plant extracts were also used to extend the shelf life of fresh produce, such as fresh-cut cucumber (Hashemi & Jafarpour, 2020),

chilies (Perdana, Ruamcharoen, Panphon, & Leelakriangsak, 2021), red grapes (Bangar et al., 2022) and strawberry (Rodrigues, Patil, Dhakane‐Lad, Nadanathangam, & Mahapatra, 2022).

Biodegradable packaging materials containing natural antimicrobial agents are also used to improve the shelf life of fish and seafood. For example, the incorporation of *Amaranthus* leaf extract into PVOH/gelatin films significantly reduced the counts of total bacteria, *S. aureus*, and fecal coliforms on *Scoliodon laticaudus* fish fillets stored at chilled temperatures (2-4 °C) for up to 12 days (Kanatt, 2020). PLA/sawdust particle films incorporated with bacteriocin were effective against foodborne bacteria, such as *L. monocytogenes*, *S. aureus*, *Aeromonas hydrophilia*, *P. aeruginosa*, *S.* Typhimurium and *E. coli* on pangasius fish fillet during chilled storage (Woraprayote et al., 2018). The addition of ZnO NP and clove EO into bovine skin gelatin films enhanced the antimicrobial activity against *S.* Typhimurium and *L. monocytogenes* in peeled shrimp samples stored at 4 °C for 20 days (Ejaz, Arfat, Mulla, & Ahmed, 2018).

Chapter 3 Chitosan/Acetylated Starch Composite Films Incorporated with Essential Oils: Physiochemical and Antimicrobial Properties

3.1 Introduction

A food packaging film increases the shelf-life of food products by reducing or avoiding food degradation caused by various environmental factors, such as temperature, light, and moisture. Chitosan (CS) is a linear polysaccharide of 1,4 linked 2-amino deoxy β-D glucan produced from chitin. As a "generally recognized as safe" (GRAS) polymer, CS is non-toxic, renewable, biodegradable, and biocompatible, giving it potential for food industry applications. Transparent packaging films produced from CS are more elastic and exhibit good antimicrobial properties that were shown to enhance food quality and safety (Wang, Lian, Wang, Jin, & Liu, 2012).

Starch has outstanding functional properties, especially in the formulation of biodegradable materials and foods. As a cheap, biodegradable and renewable polymer, starch can be used as an alternative for conventional plastic materials (Tarique et al., 2021). Films produced from starch have reduced permeability to moisture and gas molecules (Shah, Naqash, Gani, & Masoodi, 2016). However, due to its hydrophilic property, starch has several undesired characteristics, such as poor tensile properties, low stability and high solubility (Shah et al., 2016). The introduction of functional chemical groups can modify the physiochemical characteristics of starch (X. Wang et al., 2020). Sun, Zhang, and Ma (2016) reported that modified native starch showed several improvements that included increased stability during thawing and freezing cycles, reduced gelatinization temperature, and enhanced resistance to retrogradation. Acetylation is a common chemical modification technique used to incorporate acetyl functional groups into native starch (El Halal et al.,

2017). Films produced from acetylated starch (ACS) exhibit enhanced tensile strength and hydrophobicity, as compared to native starches (Chandra & Rustgi, 1998). ACS films are more resistant due to hydrogen bond interactions within the polymeric matrix (López, Zaritzky, Grossmann, & García, 2013). ACS-based films exhibited improved physiochemical characteristics compared to native starch-based films (Katerinopoulou, Giannakas, Grigoriadi, Barkoula, & Ladavos, 2014; Larotonda, Matsui, Soldi, & Laurindo, 2004; Nasseri et al., 2020; Perez Sira & Dufour, 2017). The physiochemical characteristics of the films can also be enhanced by blending two or more polymers. For example, films produced from the blend of starch and polybuthylene succinate could be used for food packaging due to their enhanced film elasticity and flexibility (Diyana et al., 2021). Also, CS/starch films exhibited lower permeability to moisture because CS has a lower permeability to oxygen and higher hydrophobicity than native starch (Pelissari, Grossmann, Yamashita, & Pineda, 2009). It is also reported that films made from a mixture of CS and ACS had excellent mechanical, barrier and antimicrobial activities against *Listeria* (Escamilla-García et al., 2017). However, despite the excellent functional properties exhibited by CS/ACS films, their lack of antimicrobial and barrier properties is still the main restriction for their wide food packaging application.

Over the last few decades, the safety of synthetic additives that are commonly added as food preservatives has been questioned because of potential toxicity, carcinogenicity and teratogenicity effects (Ju et al., 2019). Thus, natural active materials could be an alternative as reinforcing agents in packaging films because their performance may be enhanced by preventing microbial food spoilage and, hence, improve food quality and shelf-life (Shaaban, Ali, Bareh, Al-khalifa, & Amer, 2017). Essential oils (EO),

including cinnamon and clove, have been shown to have many biological activities and antimicrobial properties (Souza, Goto, Mainardi, Coelho, & Tadini, 2013). The main components of cinnamon (*Cinnamomim cassia*) and clove (*Eugenia caryophyllata*) EO, cinnamaldehyde and eugenol, respectively, contribute to their antimicrobial properties.

Edible coatings have been widely used in the preservation of solid food samples, such as meats, fruits and vegetables (Abbasi, Aminzare, Hassanzad Azar, & Rostamizadeh, 2021; Araújo, de Siqueira, Blank, Narain, & de Aquino Santana, 2018; Frazão, Blank, & de Aquino Santana, 2017). Nevertheless, the application of edible coatings is still limited because of the poor diffusion rates of the antimicrobial agents within the foods. Active food packaging is one of the novel techniques used to reduce detrimental environmental effects on food organoleptic characteristics while improving the effective utilization rate and retaining the biological activity of EO during food storage (Carpena, Nuñez-Estevez, Soria-Lopez, Garcia-Oliveira, & Prieto, 2021). In recent years, several EO, such as *Thymus kotschyanus* (Mehdizadeh et al., 2020), oregano (Pelissari et al., 2009), lemon (Bof, Jiménez, Locaso, Garcia, & Chiralt, 2016) and lemongrass (Perdana et al., 2021) were incorporated in CS/starch film for food packaging applications. When EO are incorporated into active packaging films, their stability and contact area with food products are improved as they can easily diffuse into and interact with microorganisms in the packaged foods (Anis, Pal, & Al-Zahrani, 2021). The concept of active packaging has gained increased attention as it can prolong food quality and shelf-life under certain packaging conditions (Yu et al., 2017).

In this study, the antimicrobial activity of various types of EO, such as cinnamon, clove, pine needle, balsam fir, cypress, garlic, ginger, rosemary and nutmeg, were

evaluated against *E. coli* O157:H7 using a disc diffusion test. However, only clove and cinnamon EO were chosen for subsequent analysis due to their inhibitory effects against the bacterial pathogens. The objective of this study was to develop a solution cast CS/ACS packaging film supplemented with cinnamon and clove EO, and study the physical and antimicrobial properties of the film.

3.2 Materials and methods

3.2.1 Chemicals and materials

Chitosan (190–310 kDa; degree of deacetylation 75–85%), potato starch (ACS reagent), glycerol (≥99.5%), Tween 80, acetic anhydride (≥98.0%), NaOH, KOH, HCl (36.5–38.0%), anhydrous $CaCl_2$, glacial acetic acid (≥99.7%), phenolphthalein and $Na_2S_2O_3$ (0.1 N) were procured from Sigma-Aldrich (St. Louis, MO, USA). Pure cinnamon cassia (100%) and clove EO were purchased from Now Foods (Bloomingdale, IL, USA). A cellulose nanofiber (CNF) suspension was made from the powder form, purchased from the University of Maine, that was produced using bleached wood pulp. Fresh raw bottom round beef was from Schnucks Markets, Inc., (Columbia, MO, USA). Pure corn oil was purchased from ACH Food Companies, Inc. (Oakbrook Terrace, IL, USA). MacConkey sorbitol agar, tryptic soy agar (TSA), and tryptic soy broth (TSB) were obtained from Difco Laboratories (Detroit, MI, USA). *Escherichia coli* O157:H7 (strains 3178–85, C7927, 505B, EDL-933 and MF-1847) were part of the Food Microbiology Laboratory, University of Missouri (Columbia, MO, USA) culture collection.

3.2.2 Preparation of ACS

The method reported by Zamudio-Flores, Torres, Salgado-Delgado, and Bello-Pérez (2010) was modified and used to synthesize ACS. Distilled water (225 mL) was added to a beaker containing 100 g of starch powder, and the suspension was stirred for 30 min. The pH was adjusted to 8.0 by adding 1 M NaOH solution. Acetic anhydride (7.3 mL, 8% w/w, on a dry starch basis) was added dropwise for 15–20 min with constant stirring while maintaining a pH between 8.0 and 8.4 with 1 M NaOH solution. Ten minutes later, 1.0 N HCl was added to adjust the pH to 5.5. ACS was recovered using a 50 mL Eppendorf tube by centrifugation at 3000×*g* (4852 rpm in a F-34-6-38 rotor, Model 5810R centrifuge, Eppendorf Co., Hamburg, Germany) at 25 °C for 10 min, and washing with 500 mL of distilled water, under the same centrifugation conditions, for a total of four times. The material was collected after drying overnight in an oven at 40 °C.

3.2.3 Measurement of acetylation percentage and degree of substitution (DS)

A modification of the titration technique was used to determine the acetylation percentage and DS, as reported by (Colussi et al., 2015). Briefly, 1 g of samples (unmodified native starch as blank) and 50 mL of aqueous ethanol (75%) were added to a flask, followed by stirring and heating at 50 °C for 30 min. Then, 40 mL of 0.5 M KOH was added after the slurry was cooled to 25 °C. The slurry was stirred at 200 rpm for three days. HCl (0.5 M) solution was used to back-titrate the excess alkali, and phenolphthalein was used as an indicator. The leached alkali was titrated once again after letting it sit for another 2 h. The acetylation percentage was calculated as follows:

$$Acetyl\ (\%) = \frac{\left(Volume_{blank} - Volume_{sample}\right) \times Nomality\ of\ HCl \times 100 \times 0.043}{Weight\ of\ sample}$$

where the volumes of titration utilized for the blank and each sample are represented by $Volume_{blank}$ and $Volume_{sample}$, respectively.

The DS was calculated as follows:

$$DS = \frac{162 \times Acetyl(\%)}{4300 - 42 \times Acetyl(\%)}$$

3.2.4 Preparation of composite films

To prepare the ACS suspension (3% w/w), ACS powder was added into distilled water and heated at 90 °C for 30 min. To prepare the CS suspension (1% w/v), CS was added in acetic acid solution (1% v/v) and heated at 80 °C for 60 min. Glycerol was added in a 1:1 ratio to the dry weight of CS, and continuously stirred for 10 min. The film-forming solution (FFS) of CS and ACS was prepared by blending in a 1:1 (w/w) ratio for 5 min. To improve the film's mechanical properties, 5% CNF (w/w) was added into the FFS based on the dry weight of CS. Cinnamon and clove EO, which improve the antimicrobial and water barrier properties (Xu et al., 2019) in a 3:1 (v/v) ratio, respectively, at 1.00%, 1.25%, 1.50%, 2.00% and 2.50% (v/v), and the same concentrations of Tween 80, were added into the FFS with continuous stirring at 25 °C for 10 min. The composite films were synthesized by solution-casting the FFS into sterile petri dishes and drying at 55 °C for 48 h. A film sample without the incorporation of the mixture of EO and Tween 80 was used as the control.

3.2.5 Film characterization

3.2.5.1 Color

The film color was determined using a CR-410 Minolta colorimeter (Konica Minolta Sensing, Inc., Japan). Each film was placed on a white calibration plate. The L^*, a^*, b^* and total color difference (ΔE) of the films was measured based on an earlier method (Zhang, Zhao, & Shi, 2016).

3.2.5.2 Moisture content

The MC of the films was measured based on that of P. Zhang et al. (2016) The films, with a dimension of 2.5 cm^2, were weighed (m_1) and dried at 105 °C for 24 h. After drying, the weights of the films (m_2) were measured. The MC of the films was calculated as follows:

$$MC(\%) = \frac{(m_1 - m_2)}{m_1} \times 100$$

3.2.5.3 Water solubility (WS)

The WS of the films was determined based on an earlier method (P. Zhang et al., 2016). First, the films, with a dimension of 2.5 cm^2, were dried at 105 °C overnight to obtain the initial weight (m_1). The films were then placed in distilled water for 24 h, and dried overnight at 105 °C before measuring the final dry weight (m_2). The WS of the films was evaluated as:

$$WS(\%) = \frac{(m_1 - m_2)}{m_1} \times 100$$

3.2.5.4 Tensile properties

For the measurement of tensile properties, including tensile strength (TS) and elongation at break (%EB), a TA-XT2i texture analyzer (Texture Technologies Corp., Hamilton, MA, USA) was used. First, the specimens were cut into rectangular strips (1 cm × 5 cm). The initial grip distance and grip separation speed were set at 25 mm and 0.5 mm/s, respectively. The film samples were placed on the grips and the upper grips were stretched until the films broke, using a load cell of 100 kg. A digital micrometer (Model 35-025; iGAGING, San Clemente, CA, USA) was used to measure the film thickness.

3.2.5.5 Light transmittance

The percentage light transmittance of the films was determined using a Cary 50 Bio UV-VIS spectrophotometer (Varian, Inc., Palo Alto, CA, USA). Each film was cut into a rectangular strip (0.8 cm × 3.5 cm) before being placed in a quartz cuvette. The light transmittance of each sample was measured by scanning through wavelengths from 200 to 800 nm. The film opacity was calculated by determining the O value as follows:

$$O\ value = \frac{A_{600}}{K}$$

where A_{600} is the light absorbance value at 600 nm and K is the film thickness (mm).

3.2.5.6 Water vapor permeability (WVP)

The WVP of the films was evaluated according to an earlier technique (Fitch-Vargas et al., 2016). Briefly, the films were sealed onto beakers containing 15 g of anhydrous $CaCl_2$ before placing them in a desiccator containing saturated NaCl solution.

The weight difference of the beakers was measured at 1, 2, 4, 6, 8, 10, and 12 h. The WVP of the films was calculated as follows:

$$WVP = \frac{(K \times M)}{(A \times t \times \Delta p)}$$

where K is the film thickness (m), M is the weight change, A is the film permeation area (m^2), t is the time (s), and Δp is the water vapor pressure difference across the film (Pa).

3.2.5.7 Oxygen permeability (OP)

To measure the OP of the films, a corn oil model was utilized following a previous method (Du et al., 2016). Briefly, the films were sealed onto beakers consisting of 25 mL pure corn oil before incubating them at 70 °C for one week. According to our previous technique (Yu et al., 2017), the peroxide value (POV) of each film was determined by titrating the corn oil using $Na_2S_2O_3$. The POV of the films was calculated as follows:

$$POV(meq/kg) = \frac{(V \times N \times 1000)}{W}$$

where V is the titration volume of $Na_2S_2O_3$ (mL), N is the normality of $Na_2S_2O_3$, and W is the weight of oil sample (g).

3.2.5.8 Fourier transform infrared spectroscopy

The FTIR of the films was evaluated using a Nicolet 380 attenuated total reflectance-FTIR spectrometer (Thermo Electron Corp., USA). Each IR spectrum was determined between 400-4000 cm^{-1} with 64 scans at a resolution of 4 cm^{-1}.

3.2.5.9 Surface morphology

The microstructure properties of the films were studied using environmental SEM (Quanta 600 FEG; Thermo Fisher Scientific, Waltham MA, USA). The films were placed on aluminum stubs. Then, the mounted samples were coated with gold before observing using SEM.

3.2.5.10 Antimicrobial activity on beef spoilage bacteria

Based on our previously published method (Yu et al., 2017), the antimicrobial activity of the films against spoilage bacteria was determined using fresh raw beef as a food model. Beef samples were cut into 1.5 cm × 1.5 cm × 2 cm pieces and wrapped with the films (6 × 6 cm). The wrapped beef samples were placed in sterile petri dishes and stored at 4 °C and 25% RH. Negative controls were beef samples not covered with films. After 0, 4, 7, 10 and 14 days, total bacterial counts were determined and expressed as $\log_{10}$ CFU/g. On each sampling day, the films were removed from the beef samples before placing them in sterile stomacher bags containing 99 mL of 0.1% peptone water. The samples were then homogenized using a Stomacher 400 Circulator (Seward Ltd., UK) for 120 s. The homogenates were serially diluted with peptone water and plated in TSA. Samples were incubated at 37 °C for 24 h before enumerating colonies.

3.2.5.11 Antimicrobial effect on *Escherichia coli* O157:H7 on beef products

The antimicrobial activity of the films on *E. coli* O157:H7 (3178-85, EDL-933, C7927, 505B and MF-1847) cocktail strains was evaluated using fresh raw bottom round beef as a food model. *E. coli* O157:H7 strains were transferred to TSB and incubated at

37 °C for 24 h. All strains were added in a 1:1:1:1:1 CFU/mL ratio to obtain the bacterial cocktail. The culture cocktail was then centrifuged at 12,745 ×*g* (10,000 rpm) for 5 min and washed with peptone water. The similar procedure was repeated for two additional times. Beef samples were cut into 1.5 cm × 1.5 cm × 2.0 cm pieces and dipped in the cocktail for 2 min. The inoculated beef samples were drip-dried under a laminar hood for 15 min. The dried beef samples were covered with the films and stored at 4 °C and 25% RH. Negative controls were the beef samples without the films. After 0, 4, 7, 10 and 14 days, total counts of viable *E. coli* O157:H7 were measured and expressed in $\log_{10}$ CFU/g values. On each sampling day, the films were removed and the beef samples were transferred into sterile stomacher bags and processed as described above. Diluted homogenates were spread-plated on MacConkey sorbitol agar. Samples were incubated at 37 °C for 24 h.

3.2.5.12 Observation of bacterial morphology

Ten milliliters of the five-strain *E. coli* O157:H7 cocktail were prepared, as described in the previous section. Each film was separately transferred into the bacterial cocktail solution and incubated at 37 °C for 24 h. A fixative (2% glutaraldehyde and 2% paraformaldehyde in 100 mM sodium cacodylate buffer; pH = 7.35) was used for fixation of treated and untreated cell samples. Concentrated cell pellets were produced by centrifuging each sample at 13,000×*g* (10,099 rpm) before suspending back in the primary fixative. Next, a carbon-coated copper grid was used to dry the samples before scanning by environmental SEM (Quanta 600 FEG; FEI Company, USA).

3.2.6 Statistical analyses

Data were collected from experiments that were conducted in duplicates at least thrice. Data analysis was carried out through a one-way analysis of variance (ANOVA). Tukey's multiple range test was performed to determine significant differences among average values, using SPSS software (Version 13). Compared values were considered significantly different when $P \leq 0.05$.

3.3 Results and discussion

3.3.1 Measurement of the acetylation percentage and DS

The acetylation percentage of ACS was 2.21% (Table 3.1), which was lower than the maximum percentage (2.5 g/100 g) of acetyl groups recommended by the FDA (Colussi et al., 2015). Thus, the modified starch can be used for the development of food packaging films. During the film formulation, the functional groups of modified starches may combine with other components in the polymer matrix, which may affect their barrier and tensile properties (Escamilla-García et al., 2017). The acetylation percentage and DS can be impacted by a few important factors, such as the type, size, characteristics and structure of the native starch used, temperature, pH, concentration, reaction time and presence of catalysts (Golachowski et al., 2015).

Table 3.1 The acetylation percentage and degree of substitution (DS) of acetylated starch. The values are shown as means ± standard deviation (n = 3).

	Acetylation (%)	**DS**
Average	2.21 ± 0.19	0.850 ± 0.008

3.3.2 Color

The films exhibited a light-yellow color, and as more EO were introduced, the ΔE of the films displayed an increasing trend (Table 3.2). Studies showed that the cinnamon EO increased the ΔE more than did the clove EO (Hosseini, Razavi, & Mousavi, 2009). As compared to the control films without the incorporation of EO, the films incorporated with EO assumed negative ΔL^*, positive Δa^* and positive Δb^*, which indicate darker, redder, and yellower color, respectively. Similar color changes were observed using CS-based films supplemented with cinnamon EO (Peng & Li, 2014; Xu et al., 2019). The improved compatibility was indicated by the yellowish appearance of the film, which is due to a Schiff-base reaction between CS and cinnamon EO in the polymer matrix (Chen et al., 2016; Pereda, Amica, & Marcovich, 2012; Wang et al., 2011). (Du et al., 2009) also reported that clove bud EO did not significantly change the L^* and a^* values but increased the b^* value of the films, as compared to that of a control film without the incorporation of EO, which correlated with the results shown in Table 3.2.

Table 3.2 The influence of incorporated EO on film color.

Concentration of EO % (v/v)	**L^***	**a^***	**b^***	**ΔE^x**
0.0 (Control)	84.95 ± 0.57^a	-5.46 ± 0.92^b	30.77 ± 1.41^c	32.05 ± 1.22^c
1.25	80.08 ± 0.03^b	-2.37 ± 0.29^a	34.07 ± 0.48^b	36.77 ± 0.57^b
1.5	80.56 ± 0.19^b	-2.85 ± 0.62^a	35.54 ± 0.67^b	37.88 ± 0.66^b
2.0	80.10 ± 0.08^b	-2.80 ± 0.18^a	39.04 ± 0.32^a	41.20 ± 0.30^a

Values with the same superscripts in each column were not significantly different ($P > 0.05$).

*The values are given as means ± standard deviation (n = 3).

[x]ΔE: Color change.

3.3 MC and WS

The MC, which defines the water molecules that filled the void volume in the polymer matrix (Xu, Kim, Hanna, & Nag, 2005), was significantly reduced ($P \leq 0.05$) when the EO were incorporated into the films (Figure 3.1A). For instance, the MC decreased by 22.7% when 2.5% of the EO was incorporated into the films. This could be due to covalent bond interactions between cinnamon EO and the functional groups of the polymeric chains, which led to reduced amounts of free hydroxyl and amino groups. Thus, this decreases the hydrogen bonding interactions between the water molecules and polymer matrix, suggesting a lower overall MC value of the films (Park & Zhao, 2004). The addition of EO can also disrupt the microstructure of the films and result in the formation of a new microstructure with higher hydrophobicity (Dashipour, Khaksar, Hosseini, Shojaee-Aliabadi, & Kiandokht, 2014). Therefore, the films had reduced water trapping ability, resulting in decreased MC values.

The control film exhibited low WS of about 21.4% (Figure 3.1B). The addition of 2.0% and 2.5% of the EO into the films decreased their solubility by 19.2% and 21.6%, respectively. A similar decreasing trend was also found in CS-based films supplemented with cinnamon, thyme and clove EO (Hosseini et al., 2009; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Peng & Li, 2014). The interaction between the EO components and

hydroxyl groups resulted in reduced WS, suggesting an increased hydrophobicity of the films. The cross-linking effect in the polymer matrix resulted in decreased WS and the formation of more water-resistant films due to their reduced affinity toward water (Möller, Grelier, Pardon, & Coma, 2004). Our results showed a similar trend in MC as both characteristics are primarily influenced by the availability of interactions of water molecules and the polymer matrix of the films.

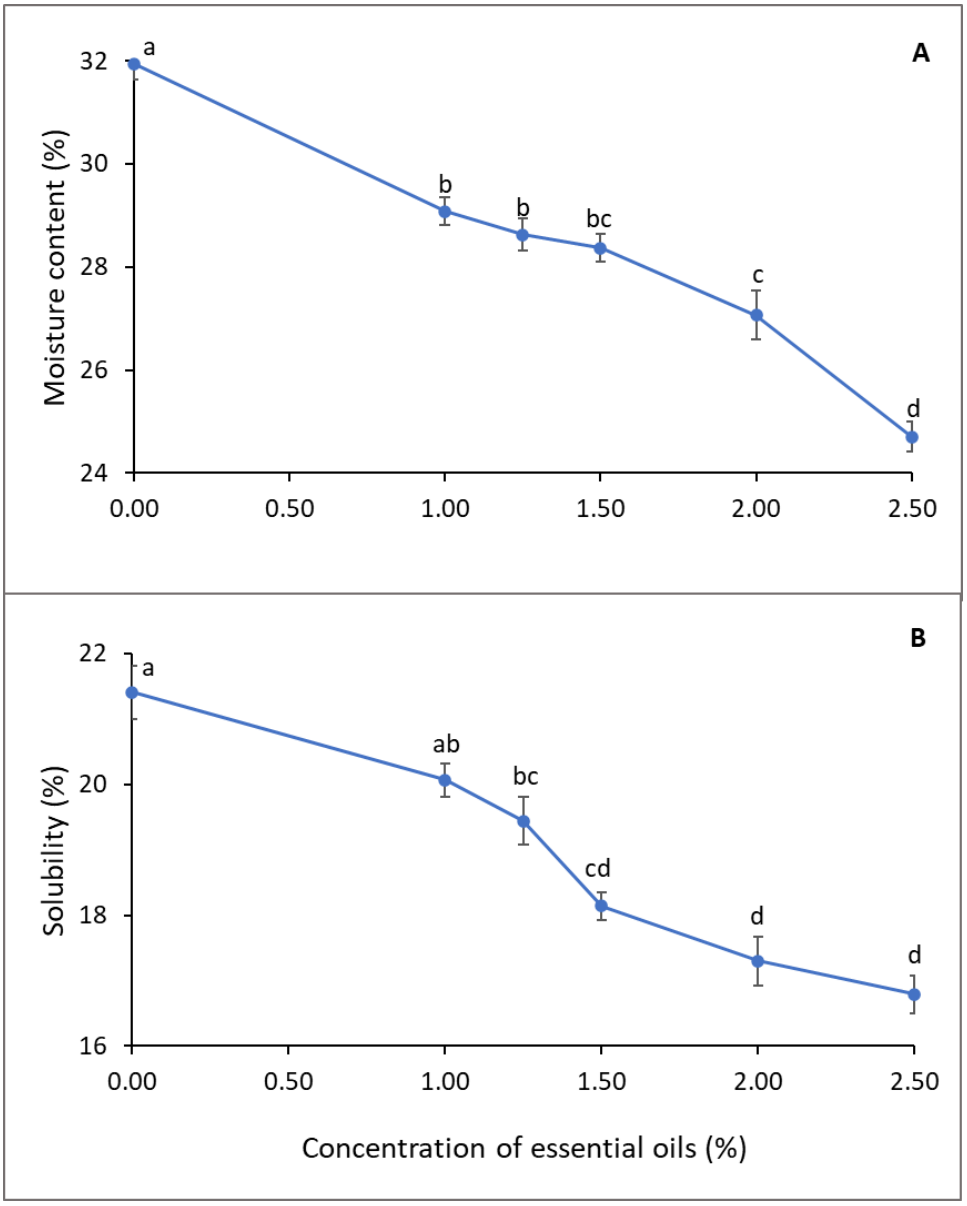


Figure 3.1 Effects of EO on moisture content (A) and solubility (B) of the films. Values are presented as means ± standard error. Values with same superscripts indicate no significant difference ($P > 0.05$).

3.3.4 Tensile properties

The TS of the films was reduced when the EO concentration increased (Figure 3.2A). As compared to the control film, the TS had the maximum reduction of 25.6% when 2.0% of EO were incorporated into the films. Wang et al. (2011) reported that the TS was reduced when EO, especially clove bud EO, were introduced into the film. They claimed that the microstructure fracture of the film was due to the incorporated EO in the films that may reduce their TS. A similar scenario was also observed when EO were incorporated into edible apple puree films (Du et al., 2009). The reduced TS could mainly be due to the weaker oil-polymer interactions that partially replace the stronger interactions between the polymers and intermolecular bonding of the polymers in the film matrix. (Xu et al., 2019). The formation of a rougher film surface, as observed in the SEM micrographs (Figure 3.6), can be correlated to the reduced TS of the films.

Overall, the %EB decreased when the EO were incorporated into the films (Figure 3.2B). The reduction of %EB could be caused by the stiff network structure, leading to a restricted chain mobility of the polymer matrix (Wang et al., 2012). Further, the hydrophilic surfactant, Tween 80, could also interact with water molecules or polysaccharides, which weakens the intermolecular hydrogen bonding, leading to a sudden reduce in %EB (Song et al., 2018). Although there was a slight increase in %EB upon the incorporation of 1.25% EO, the %EB for all the EO-incorporated films was still lower than that of the control films.

Hosseini et al. (2009) reported that the %EB increased when clove EO were incorporated into CS films. Due to the plasticizing effect of the essential oils, the %EB in our study similarly increased when 1.25% of the EO was added. A similar trend in mechanical properties was found by More, Sen, and Das (2017). The reduction of %EB may be attributed to cinnamon EO, as a higher concentration of cinnamon EO was added, compared to clove EO. A cross-linking effect may occur due to a strong interaction between the polymer matrix and the cinnamon EO. The free volume of the matrix was reduced, which led to the limited molecular mobility of the polymer. The differences between the results obtained, particularly in the mechanical properties, may be caused by the compositions of CS and ACS, the amount of plasticizer and the technique of film preparation used (Hosseini et al., 2009). The results of mechanical properties may also vary with the grip separation speed.

3.3.5 Light transmittance

As more EO were incorporated into the films, the %T displayed a declining trend between the wavelengths of 200 and 800 nm (Figure 3.3A). The photo-oxidation or lipid peroxidation of foods is primarily caused by UV-A light (315-400 nm) (Bhattacharyya, Majhi, Saha, & Mukherjee, 2014). The %T of the films was reduced to less than 1% within the UV-A region, upon the incorporation of EO. This clearly shows that the films have an excellent ability to block UV light. Ahmed, Mulla, et al. (2016) reported similar results using polylactic acid-based composite films supplemented with polyethylene glycol and cinnamon EO. In the visible region (400-800 nm), the %T of the films was also reduced with an increased concentration of EO loading. This could be due to the light scattering by

EO droplets dispersed throughout the polymer matrix. Mulla et al. (2017) reported that the incorporation of clove EO reduced the %T of linear low-density polyethylene (LLDPE)-based films at wavelengths of 280 and 600 nm.

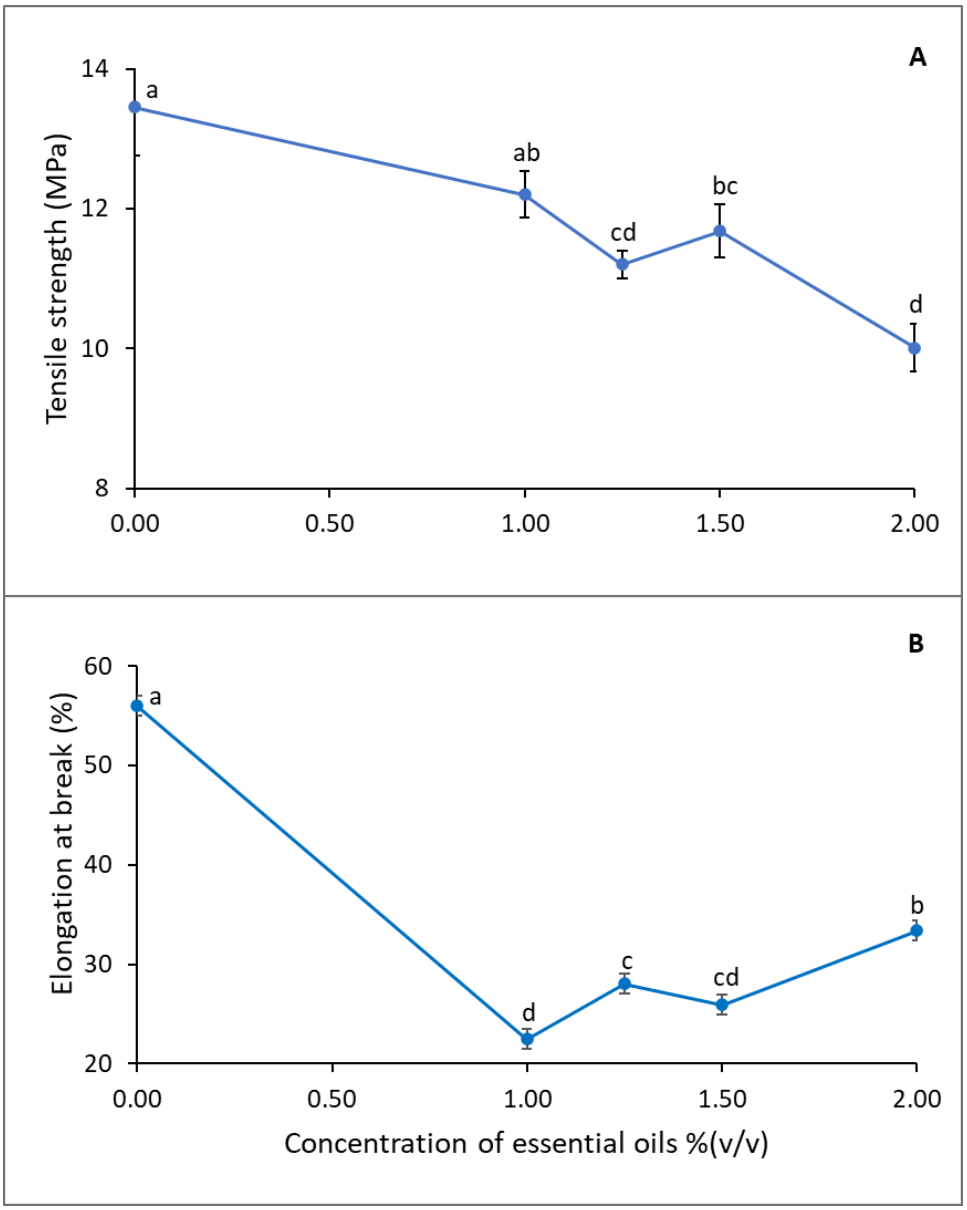


Figure 3.2 Effects of EO on tensile strength (A) and elongation at break (B) of the films. Values are presented as means ± standard error. Values with the same superscripts indicate no significant difference ($P > 0.05$).

Moreover, the addition of EO significantly affected the film opacity (Figure 3.3B). The control film appeared to be clear and transparent. The films containing EO had significantly greater opacity than the control films. The decrease in the %T was correlated with the increase in film opacity. The increase in film opacity could be due to the high color intensity of cinnamon EO and reduced intensity of light scattering, which limited the %T through the films. An edible starch-CS film containing thyme EO was developed by Mehdizadeh, Tajik, Rohani, and Oromiehie (2012), and a similar trend of results was reported. The film opacity is correlated with the ΔE, which could be associated with the formation of film microstructure and the condition of the oil droplets in the drying process (Villalobos, Chanona, Hernández, Gutiérrez, & Chiralt, 2005).

3.3.6 WVP and OP

As shown in Figure 3.4A, the WVP decreased when the EO were incorporated into the films, indicating enhanced water barrier properties. This could be due to an increased hydrophobic characteristic of the films (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009). The high concentrations of EO incorporated into the films could enhance the degree of discontinuities of the structure. Due to the dispersion of oil phase throughout polymer matrix, the pathway of water transfer in the films was blocked, which led to a greater distance for water molecules to pass through the films (Peng & Li, 2014). Studies also showed that the incorporation of a cinnamon and clove EO mixture reduced the WVP of films (Xu et al., 2019). This could be due to a reduced size of oil droplets, as reported by Peng and Li (2014), which improved the interfacial area between the EO and

the polymer and, thus, reduced hydrogen bonding interactions of water molecules and the polymer matrix.

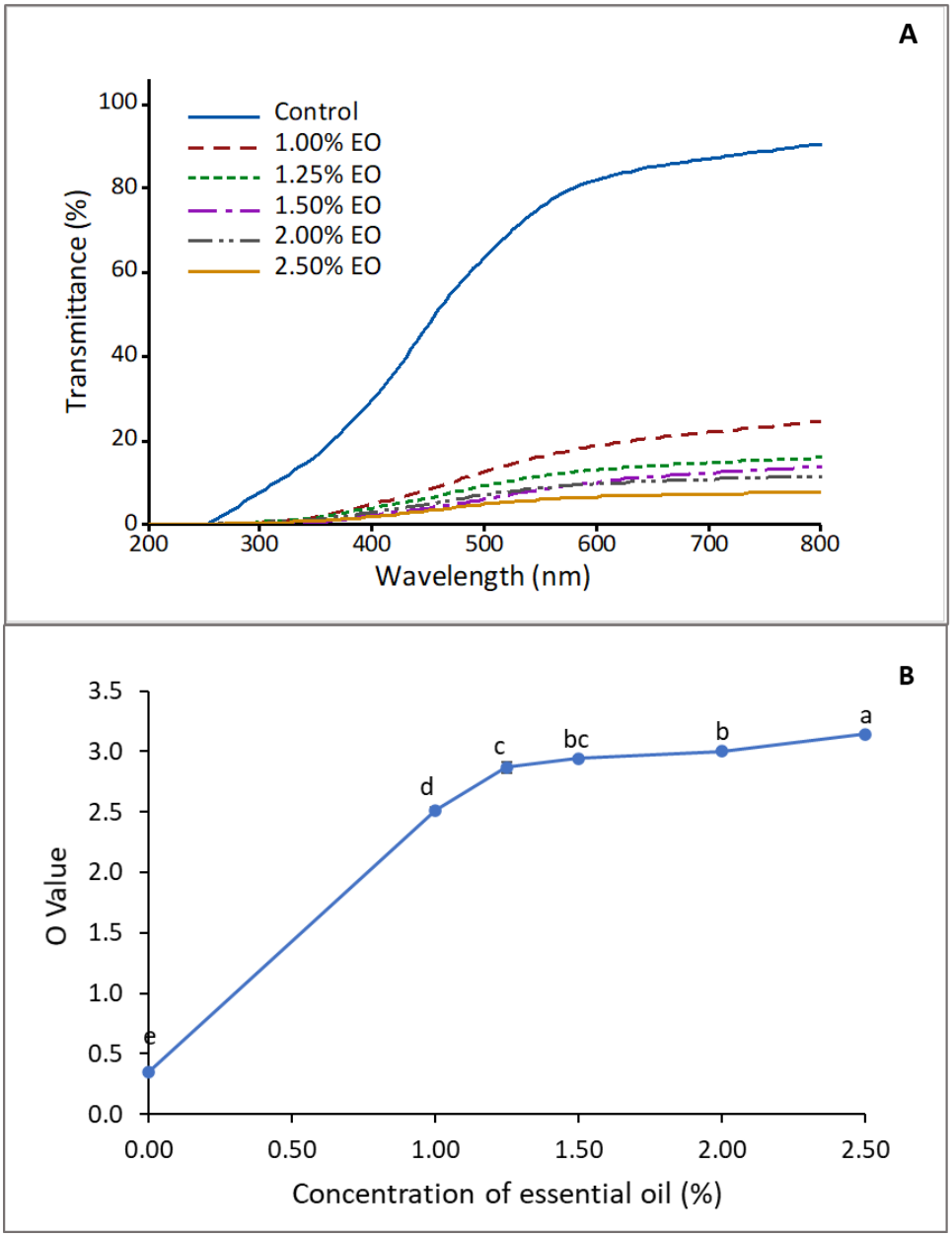


Figure 3.3 Effects of EO on percentage of light transmittance (A) and opacity value (B) of the films. Values are presented as means ± standard error. Values with the same superscripts indicate no significant difference ($P > 0.05$).

Packaging films with low OP can enhance food quality and shelf-life (Brody, Bugusu, Han, Sand, & Mchugh, 2008). The OP value was significantly reduced when EO were incorporated into the films (Figure 3.4B). The OP of starch-gelatin films was reduced upon the incorporation of EO, such as clove, cinnamon, and oregano (Acosta et al., 2016). This could be due to the oxygen scavenging role of EO because of a reduced oxygen solubility in these polar oils that improved their oxygen barrier properties. The reduced OP of low-density polyethylene-based active films containing rosemary and cinnamon EO was also reported and correlated with the effective introduction of the oil phase into the polymer matrix (Dong, Xu, Ahmed, Li, & Lin, 2018). The films incorporated with 1.50% of the EO and Tween 80 displayed a sudden drop in POV, which could be due the emulsification effect of the surfactant, resulting in greater compatibility between the EO and the film matrix. Thus, less non-polar oxygen molecules could pass through the films. The increased surfactant concentration may reduce the exposure of non-polar groups of cinnamon EO in the films. Thus, the interaction of non-polar oxygen molecules with EO may be hindered in the polymer matrix that reduced the OP of the films (Yoo & Krochta, 2011). Similar results had also been presented by Han et al. (2018) that cinnamon EO could be easily incorporated into the FFS when the surfactant concentration increased, resulting in reduced exposure of non-polar groups in a sodium alginate/carboxymethyl cellulose film matrix.

3.3.7 Fourier transform infrared spectroscopy

From the FTIR spectrum of the control film, a strong broad band was found at 3289 cm^{-1}, associated to the -OH stretching of CS and ACS and the hydrogen bonding interaction

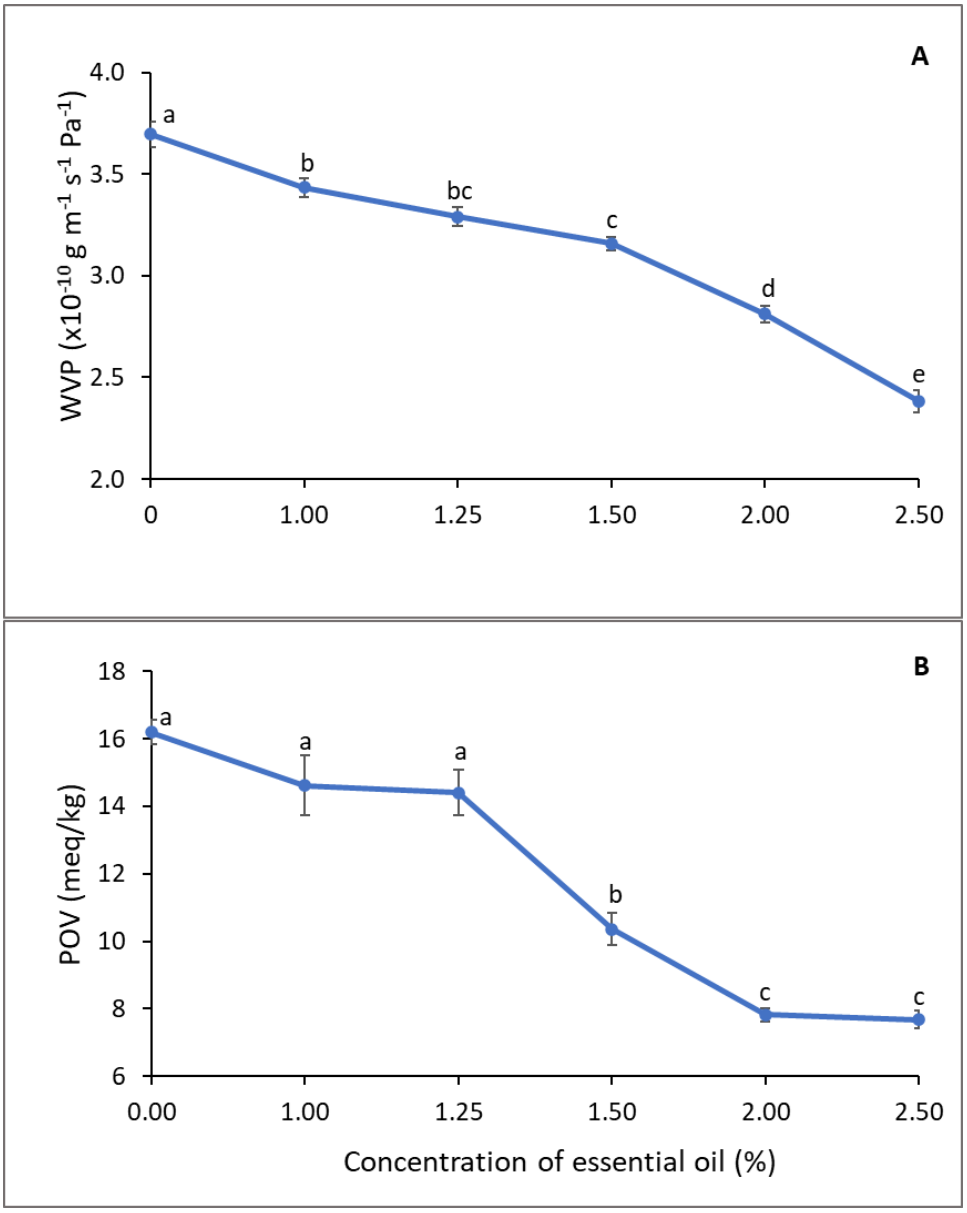


Figure 3.4 Effects of EO on water vapor permeability (A) and oxygen permeability (B) of the films. Values are presented as means ± standard error. Values with the same superscripts indicate no significant difference ($P > 0.05$).

with solvents (Figure 3.5). The broad band overlapped the peak that corresponds to the -NH stretching of CS (Xu et al., 2005). The amide I and amide II bands were observed at 1634 cm^{-1} and 1559 cm^{-1}, respectively. Cinnamaldehyde had a characteristic peak at 1666 cm^{-1}, attributed to the C=O stretching vibration of the aldehyde conjugated with a double bond that has the appearance at 1624 cm^{-1} (Wang et al., 2011). The aromatic C=C stretching at 1510 cm^{-1} is the characteristic peak of eugenol. The angular deformation of the C-H group which is located outside the plane of aromatic rings is represented by the bands ranging from 908 cm^{-1} and 795 cm^{-1} (Pereira dos Santos et al., 2019). After the incorporation of 2.50% of EO into the films, the band attributed to the -OH stretching vibration shifted to 3351 cm^{-1} and remained as high intensity and broad, which indicated a strong hydrogen bonding interaction between the EO and the polymer matrix. The carbonyl stretching peak shifted to a higher wavenumber, *viz*. 1733 cm^{-1}, which also suggested a strong hydrogen bonding interaction between the aldehyde groups in cinnamaldehyde and the polymer matrix. The stretching vibration of -CH in the aromatic ring may be represented by the new peak at 1452 cm^{-1} (Chen et al., 2016), where the intensity increased as more EO were incorporated. Several new peaks at 2370-2340 cm^{-1} may be attributed to the absorption peaks of EO (Zhang et al., 2018). The peak at 1559 cm^{-1} may be ascribed to the C=C vibration from aromatic rings in aromatic compounds, where the intensity decreases upon the addition of clove EO (Xu et al., 2019). The results indicated that cinnamon and clove EO added into the films could lead to some oil-polymer interactions. In addition, the films produced a combined spectrum of both cinnamon and clove EO.

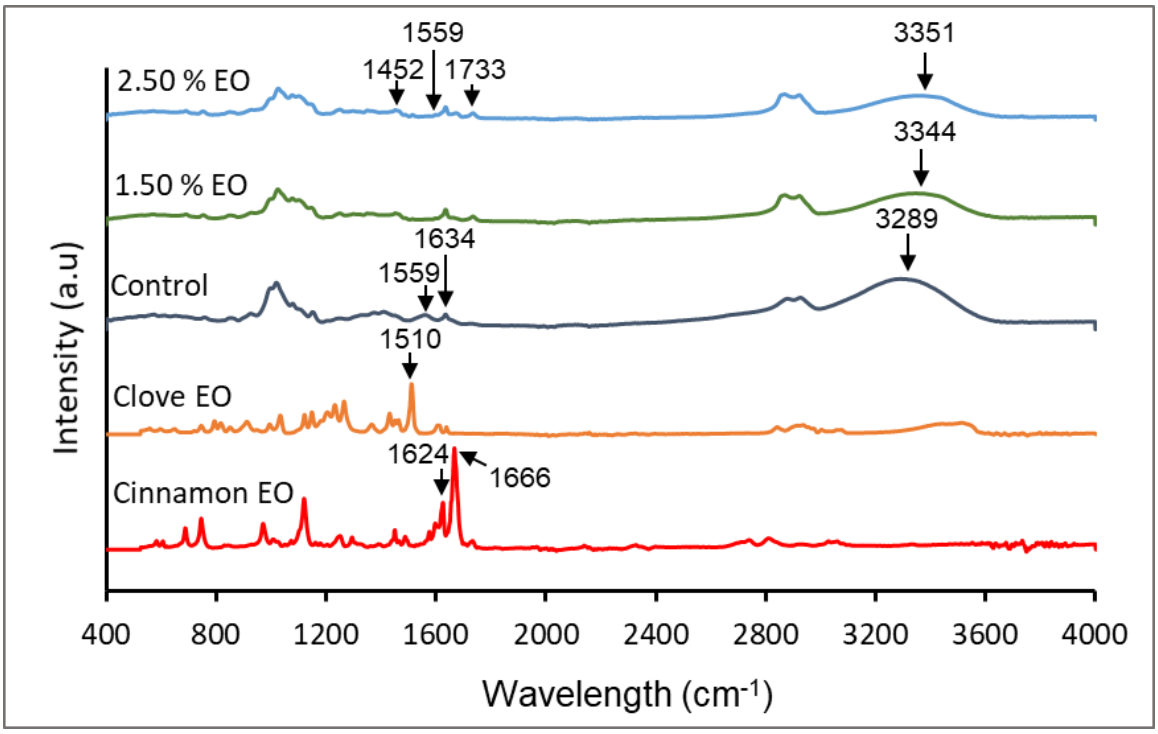


Figure 3.5 FTIR spectra of the cinnamon and clove EO composite films with 0, 1.50 and 2.50% of EO.

3.3.8 Surface morphology

The films were homogeneous and compact without phase separation, and the film surface was smooth without cracks or pores (Figure 3.6A). When the EO were incorporated into the films, a denser structure was formed which indicated that the oil droplets were uniformly incorporated in the polymer matrix (Figure 3.6B). An almost homogeneous and smooth surface with the presence of few streaks or ribbed lines was observed. Pores (shown by the red arrows in tile B) were also observed when the EO were incorporated into the films. This could be due to the coalescence and flocculation of small drops of EO when the films were drying, as reported by López-Mata et al. (2013), López-Mata et al. (2015), and Peng and Li (2014). The stability of EO could also be improved by adding a surfactant,

including Tween 80, into the films, resulting in a more homogeneous and smoother surface owing to a reduced surface tension between water and oil (Peng, Yin, & Li, 2013). As more EO were incorporated into the films, the presence of streaks or ribbed lines was more obvious, and the film surface was rougher (Figure 3.6C). Spherical microcapsules of approximately 1 μm in diameter were found embedded on the film surfaces. The production of microcapsules may be attributed to the addition of the surfactant. Similar results have been reported by Klinkesorn and Namatsila (2009), and López-Mata et al. (2013) that Tween 80 was used to stabilize tuna oil because the surfactant could cover the hydrophobic surface of oil droplets to produce a negatively-charged surface and combine with positively-charged CS. The microstructure of CS/ACS incorporated with EO could be associated with the reduced TS of the films, as discussed in Section 3.3.4 above.

3.3.9 Antimicrobial activity on spoilage bacteria on raw beef

The bacterial count of uncovered beef samples increased from approximately 5.8 log CFU/g to 8.7 log CFU/g after 4 days of storage, and remained constant at about 8.3 log CFU/g at day 14 (Figure 3.7A). Except for the control films, the bacterial count of the raw beef wrapped with the films was significantly lowered, as expected ($P \leq 0.05$). Counts showed a decreasing trend when more EO were added into the films. A microbial load of around 6 to 7 log CFU/g commonly indicates meat spoilage. Thus, beef samples wrapped with films incorporated with less than 1.0% of EO spoiled on day 4. Samples covered with films incorporated with 0%, 1.0% and 1.5% of EO spoiled on day 7. On day 10, the beef samples wrapped with films with 2.0% EO spoiled, while those covered by films with 2.5% of EO had a bacterial count of about 5.7 log CFU/g after 14 days of storage. These

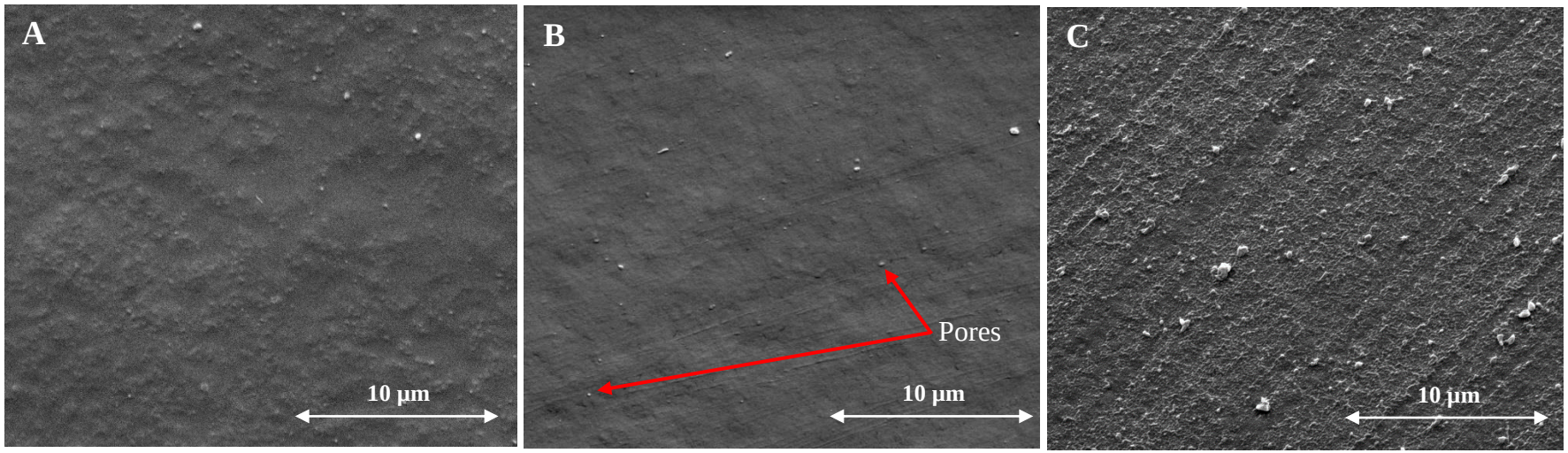


Figure 3.6 SEM observations of the surfaces (A: 0% EO, B: 1.25% EO and C: 2.00% EO) of the films. Scale bar: 10 µm. Magnification: 10,000×.

results demonstrated that the growth rate of spoilage bacteria on the beef samples could be reduced when the beef were wrapped with the films incorporated with a high concentration of EO (2.5%). Packaging films incorporated with EO were found to be more efficient in controlling bacterial growth on foods, such as meat, compared to other techniques, such as by direct addition of EO onto foods. The direct addition of EO is not favorable because the microbial quality could not be improved and some of them may negatively affect the meat quality (Fernández-Pan, Carrión-Granda, & Maté, 2014). The insoluble films had the ability to control the release of EO on the surface of food products, which led to a higher microbiological quality of beef products. Thus, the CS-based films incorporated with cinnamon and clove EO can potentially be utilized as an alternative, particularly for the preservation of poultry and meat products (Radha Krishnan et al., 2015). A similar result was also reported by Saad et al. (2019) that the use of clove oil-incorporated films significantly decreased the number of natural microflora, using minced chicken meat as food model.

3.3.10 Antimicrobial activity on *Escherichia coli* O157:H7 on raw beef

Numbers of *E. coli* O157:H7 on unwrapped beef samples increased by approximately 1.3 log CFU/g after 4 storage days and remained constant from day 4 to day 14 (about 7.5 log CFU/g) (Figure 3.7B). As compared to the negative controls, the *E. coli* O157:H7 count of beef samples covered with films was significantly reduced by about 1.5 – 2 log CFU/g ($P \leq 0.05$) at day 4, and remained constant at approximately 5 - 6 log CFU/g after 10 days of storage. On day 14, the beef samples wrapped with films containing 2.0%

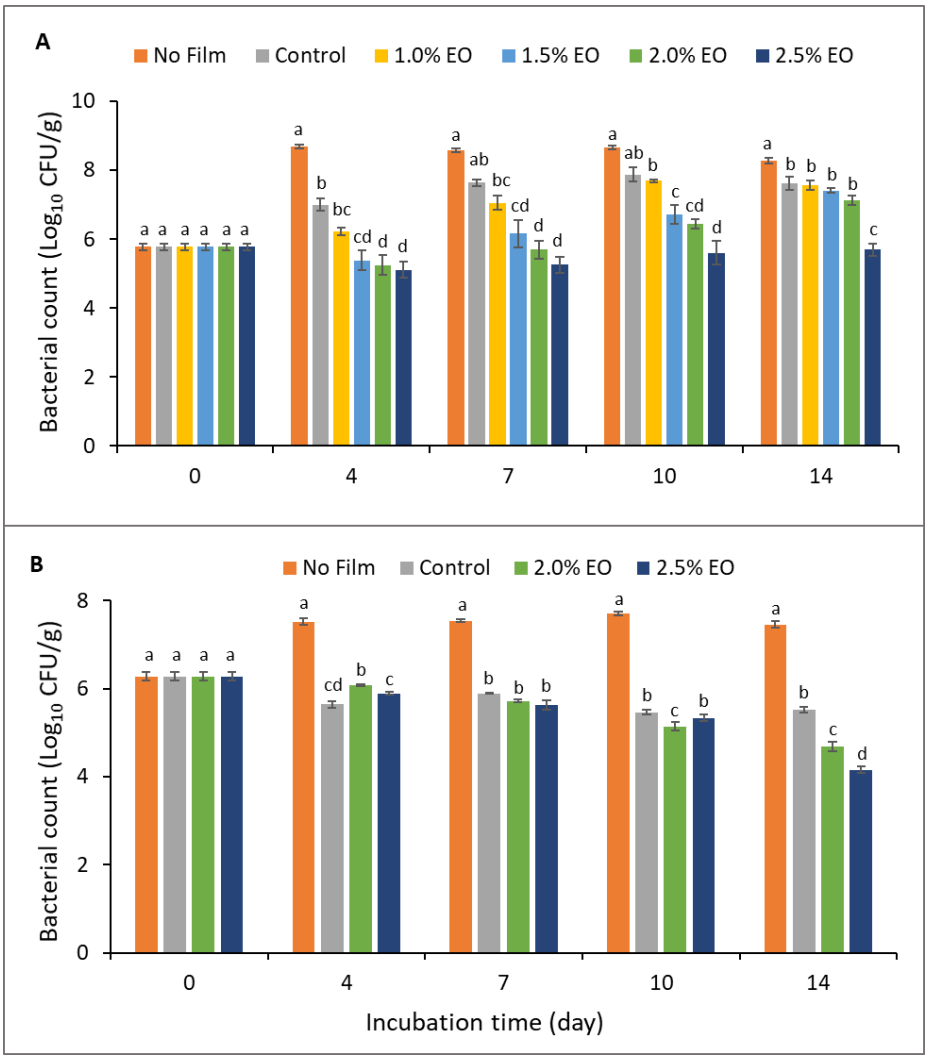


Figure 3.7 Antimicrobial activity of the films incorporated with EO on spoilage bacteria (A) and *Escherichia coli* O157:H7 (B) on a fresh beef model. Values are presented as means ± standard error. The same lowercase letters within the same group indicate that no significant differences ($P > 0.05$).

and 2.5% of EO had significantly lower *E. coli* O157:H7 counts than those of both the positive and negative control films ($P \leq 0.05$). These results showed that the growth of *E. coli* O157:H7 on raw beef could be controlled by the packaging films containing a high concentration (2.5%) of both cinnamon and clove EO. The inhibition trend of *E. coli*

O157:H7 was different from that of natural microflora found in the beef shelf-life test. Studies showed that the antibacterial activity of corn starch films was improved against *E. coli* with increased concentrations of clove and cinnamon EO (Radha Krishnan et al., 2015). The antimicrobial activity of CS-gum Arabic films supplemented with cinnamon and clove EO was evaluated against *E. coli* by a liquid culture test. A single film incorporated with both cinnamon and clove EO showed a higher antimicrobial activity against the bacterium (Xu et al., 2019). A similar study also showed that edible apple films exhibited an effective antimicrobial activity against *E. coli* O157:H7 when cinnamon and clove bud EO were incorporated (Du et al., 2009).

3.3.11 Observation of bacterial cell morphology

SEM micrographs of cells treated with CS/ACS films, with and without the EO, are given in Figure 3.8. The cell morphology of *E. coli* O157:H7 in a normal condition is shown in Figure 3.8A. Treatment with the films containing EO resulted in cell shrinkage which led to the formation of irregular cell shapes, and enhanced the cell permeability (Figure 3.8B, C). The changes in bacterial morphology may be affected by membrane lysis and transformation because cinnamon EO could greatly affect the integrity and permeability of the cell membrane (Zhang, Liu, Wang, Jiang, & Quek, 2016). When the pathogens were treated with films containing a higher concentration of EO, the bacterial shape was completely distorted, which resulted in leakage of cellular materials and eventually cell death (Figure 3.8D). This observation was similarly reported by Oussalah et al. (2006) where the bacterial cell treated with EO appeared depleted, indicating that the cell walls of the bacteria were negatively affected by the antimicrobial components. The

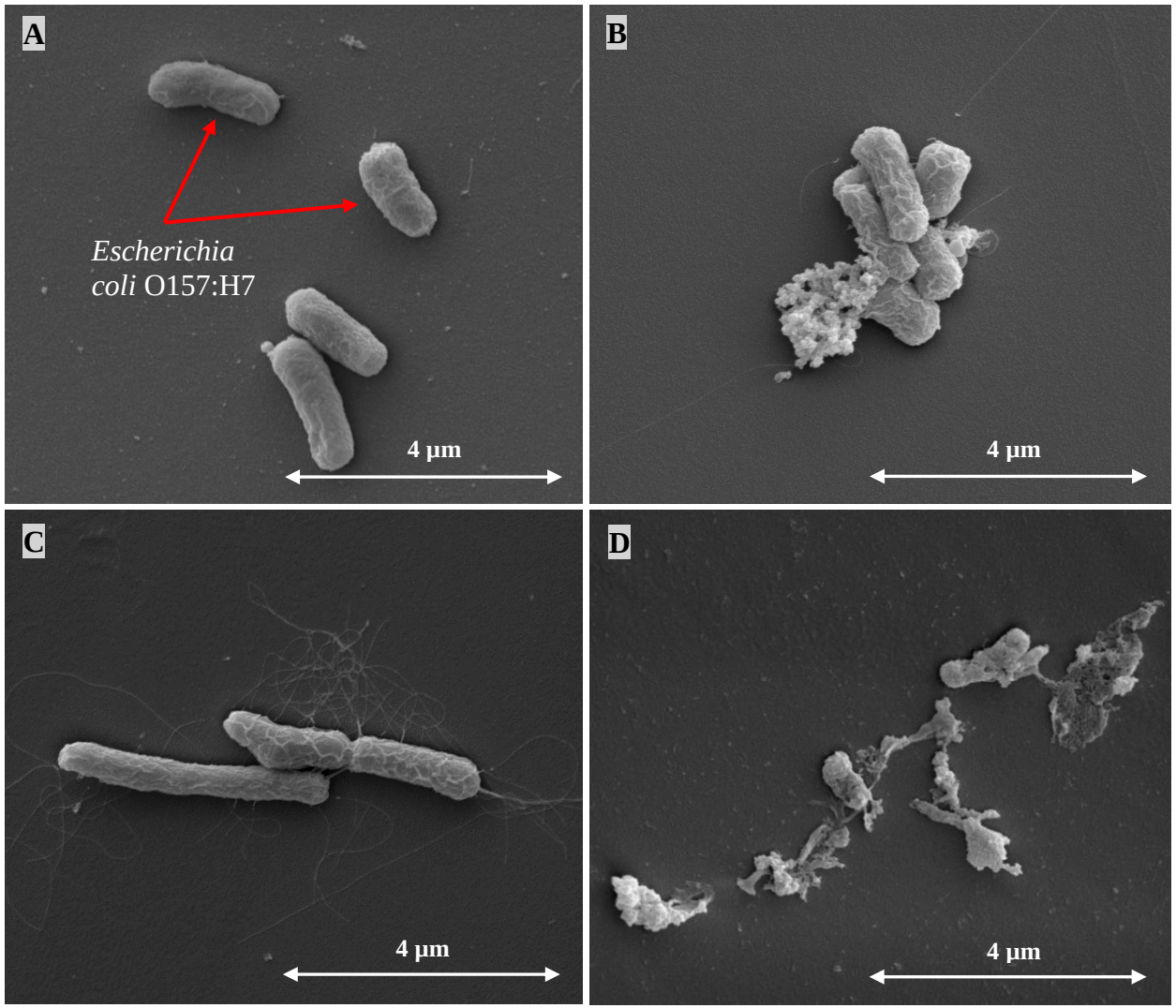


Figure 3.8 Morphologies of *Escherichia coli* O157:H7 after treatment with the films (A: 0% EO, B: 1.00% EO, C: 1.50% EO and D: 2.50% EO). Scale bar: 4 µm. Magnification: 30,000×.

significant antimicrobial activity of the films on *E. coli* O157:H7 may mostly contribute to the observed damage of bacterial cell wall. Phenolics, such as cinnamaldehyde and eugenol, may contribute to the antimicrobial activities of the cinnamon and clove EO, respectively, in the films. Hydrophobic natural phenolics could damage different bacterial cell components, that include the cell membrane, cell wall, membrane-bound enzymes, and membrane proteins, which may finally cause cell death (Shan, Cai, Brooks, & Corke, 2007; Shan, Cai, Sun, & Corke, 2005). EO may also affect bacterial respiration and improve the permeability of cell membranes, which eventually led to cell death after large amounts of ion leakage (Figure 3.8D).

3.4 Conclusions

The incorporation of cinnamon and clove EO was shown to exhibit excellent effects on the characteristics of CS/ACS films. The films incorporated with EO exhibited lower MC, less WS, and reduced TS and %EB. The incorporation of EO in the films led to a significant improvement in protection against light, water vapor and oxygen, which could be due to stronger bonding interactions with the CS/ACS matrix. With the incorporation of higher concentrations of EO, the films showed stronger antimicrobial properties against spoilage bacteria and a Gram-negative bacterial pathogen (*E. coli* O157:H7) in a beef model, which was due to the increased concentrations of antimicrobial components and enhanced barrier to oxygen. These results suggest that the EO-incorporated CS/ACS films have a high potential to be utilized as antimicrobial packaging materials in the food sector.

Chapter 4 Development of Polyvinyl Alcohol/Chitosan/Modified Bacterial Nanocellulose Films Incorporated with 4-hexylresorcinol for Food Packaging Applications

4.1 Introduction

Active packaging, whereby incorporated active components are released in a controlled manner, can serve as an effective way for increasing the safety and shelf life of food products. Food packaging produced from natural resources, such as cellulose, chitosan, gelatin, pectin, and soy protein, has been developed as alternatives to conventional packaging materials due to their biodegradability and low toxicity (Mangaraj, Yadav, et al., 2019; Yu et al., 2018). However, these biopolymers often exhibit reduced mechanical strength and low environmental stability. These limitations could be improved upon by using different approaches, including polymer blending, addition of nanofillers, chemical modifications, and incorporation of natural plasticizers (Arrieta, de Dicastillo, Garrido, Roa, & Galotto, 2018; Garavand, Rouhi, Razavi, Cacciotti, & Mohammadi, 2017; Jayakumar et al., 2019).

Chitosan (CS) can be utilized for active packaging applications due to its excellent biological and film-forming properties (Choo et al., 2016). However, CS films suffer from certain drawbacks, including low tensile strength and water barrier properties. The thermal degradation of CS at above 200-220 °C also indicates a limited thermal stability of the biopolymer, which could restrict its packaging applications (Grząbka-Zasadzińska, Amietszajew, & Borysiak, 2017). Polymer blending of CS and polyvinyl alcohol (PVOH) was reported to enhance the physical, thermal and barrier properties, while preserving the intrinsic characteristics of CS films (Narasagoudr, Hegde, Vanjeri, Chougale, & Masti,

2020). PVOH is a synthetic semicrystalline polymer, which has a great film-forming ability and excellent characteristics, including high tensile and barrier properties, biocompatibility, and strong protection against many alkaline and acidic solvents (Haghighi et al., 2020). PVOH is potentially miscible with hydrophilic polymers, particularly CS, due to its abundance of hydroxyl groups. Film production using PVOH and CS is an effective method to gain desired properties, particularly as related to tensile properties and cost performance ratio (Bonilla, Fortunati, Atarés, Chiralt, & Kenny, 2014; Srinivasa, Ramesh, Kumar, & Tharanathan, 2003; Tripathi, Mehrotra, & Dutta, 2009).

Bacterial nanocellulose (BNC), an abundant polysaccharide, has a similar structure to celluloses from plants and vegetables. However, BNC is more effective as a reinforcing agent for the development of composite films due to its stronger reinforcing effects, such as higher tensile strength and degree of polymerization (Salari, Khiabani, Mokarram, Ghanbarzadeh, & Kafil, 2018). For instance, polylactic acid (PLA) composite films incorporated with as low as 0.5 to 1.0% (w/w) of BNC showed higher mechanical properties and stronger water vapor barrier properties than pure PLA and PLA/cellulose nanofibrils films (Gitari, Chang, Misra, Navabi, & Mohanty, 2019). To further enhance the overall properties of the composite films, the surface properties of BNC can be modified to improve the interfacial adhesion between the filler and the polymer matrix. Thus, improvements can be made through various surface modification techniques, including oxidation, silane grafting, acylation and incorporation of nanoparticles (Frone et al., 2018; Somord et al., 2018; Spaic, Small, Cook, & Wan, 2014; W. Wang et al., 2020). By introducing functional groups into BNC, its properties can be modified and, thus, improving the compatibility to the polymer matrix. The incorporation of silane-modified

nanocellulose to PLA composite films improved the tensile properties, heat stability, light barrier property, and air permeability of the films (Jin, Tang, Zhu, & Zhou, 2020; Qian, Sheng, Yu, Xu, & Lopez, 2018). Also, Chanda et al. (2021) reported the enhanced dispersion, thermal and mechanical properties of polyethylene oxide films by incorporating silane-modified cellulose nanocrystals. The previous results showed that silane modification of nanocellulose allows them to be compatible with hydrophobic as wells as hydrophilic polymer matrices. However, due to the lack of antimicrobial and antioxidant activities, additional functional materials must be added for the development of active packaging films.

Food additives have been incorporated in composite films for many purposes, including to improve the food texture, color, and flavor, as well as to control the growth of microorganisms during storage. Examples of these food additives are nisin, ethyl lauryl arginate, essential oils (EO), organic acids and their salts, natural antioxidants, and antimicrobials (Haghighi et al., 2020; Jin, Liu, Zhang, & Hicks, 2009; Valencia-Chamorro et al., 2008; Zhao, Xiong, Zhou, & Xiao, 2019). Among them, 4-hexylresorcinol (4-HR) has been commonly used for prevention of melanosis in shrimps and other crustaceans (Gonçalves & de Oliveira, 2016). This antioxidant has been approved as a food additive in the EU and many countries, such as Canada, Brazil, and Australia for specified use in crustaceans (Selçuk & Özden, 2017). It was reported that poly(lactic-co-glycolic acid) films incorporated with a low concentration of 0.4% (w/w) 4-HR showed excellent antimicrobial activity (Kemme & Heinzel-Wieland, 2018). However, little research has been conducted to produce composite films incorporated with the active compound as a novel method to enhance food quality and safety.

The goals of this study were to produce antioxidative PVOH/CS composite films incorporated with aminosilane-modified BNC (mBNC) and 4-HR using a solution casting technique. The morphological, physical, mechanical, and barrier properties of the films were studied using different mechanical and analytical techniques. The filling effects of 4-HR on the antioxidant properties of the films were determined using ABTS and DPPH techniques. The effects of the composite films on the shelf-life of vacuum-packaged refrigerated raw beef were also investigated.

4.2 Materials and methods

4.2.1 Materials

Medium molecular weight CS (degree of deacetylation 75-85%), citric acid, dimethyl sulfoxide (DMSO), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma Aldrich (St. Louis, MO). PVOH (average MW 85,000-120,000, 88% hydrolyzed), n-2-aminoethyl-3-aminopropyltrimethoxysilane (AAPS), anhydrous $CaCl_2$, and potassium persulfate were from ACROS Organics™ (Fair Lawn, NJ) and 4-HR was purchased from Alfa Aesar™ (Tewksbury, MA). Glucose, Na_2HPO_4, NaOH, NaCl and methanol were purchased from Fisher Scientific (Waltham, MA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical was supplied by TCI America (Portland, OR).

4.2.2 Production of modified bacterial nanocellulose

BNC was produced using Hestrin and Schramm (HS) medium based on our previous method (W. Wang et al., 2020). In 500 mL of deionized water, 10 g glucose, 2.5

g yeast extract, 2.5 g peptone, 1.35 g Na_2HPO_4, and 0.75 g citric acid were mixed to make the culture medium. BNC was produced using a static incubation method of *Gluconobacter xylinus* ATCC 53582 in HS medium at 25 °C. After one week of cultivation, a BNC layer was formed on the surface of the medium. The BNC was removed and added into deionized water at 70 °C for 3 h, followed by treatment with NaOH solution (0.1 M) at 70 °C for 1.5 h. To remove unwanted materials, the BNC was washed with deionized water until the pH adjusted to 7.0.

The modified BNC (mBNC) was produced via polycondensation of alkoxysilane using AAPS based on a previous technique with slight modifications (Shao et al., 2017). Briefly, the BNC was cut into square 2.5 cm^2 pieces and soaked in a 10% (w/w) AAPS in ethanol solution. The mixture solution was slowly stirred at room temperature for 4 h. To remove impurities and unreacted AAPS, the mBNC was washed with ethanol. The mBNC slurry was prepared by blending at high speed (120 V, 60 Hz and 3.0 amps) for 0.5 h using a Waring commercial blender (Model 51BL31, New Hartford, CT, USA).

4.2.3 Preparation of composite films

PVOH crystals were dissolved in distilled water at 80 °C with constant stirring to make a 5% (w/w) PVOH solution. A 1% (w/w) CS solution was prepared by dissolving CS powder in 1% (w/w) acetic acid solution at 80 °C for 1.5 h under constant stirring. Both solutions were cooled to room temperature, followed by mixing in a 1:1 ratio until the development of a homogeneous suspension. 4-HR was dissolved in DMSO to obtain a 50 mg/g solution before adding into the film-forming solution (FFS), and the mixture stirred for 10 min. While stirring, 4% (w/w) of mBNC was added into the FFS. Five types of

composite suspensions were prepared: PVOH/CS/mBNC without the incorporation of 4-HR (PCB) and PVOH/CS/mBNC incorporated with various concentrations of 4-HR, *viz*., 2.5 wt.% (PCBH-2.5), 5.0 wt.% (PCBH-5.0), 7.5 wt.% (PCBH-7.5), and 10.0 wt.% (PCBH-10.0). PVOH/CS films without the incorporation of mBNC or 4-HR (PC) were used as the control. Next, 75 g of FFS were transferred onto sterile square-shaped 12 cm^2 petri dish and left overnight at room temperature before drying in an oven at 45 °C for 1-2 days.

4.2.4 Film characterization

4.2.4.1 Scanning electron microscopy

An environmental SEM (Quanta 600 FEG; Thermo Fisher Scientific, Massachusetts, USA) was used to investigate the morphological properties of the PVOH/CS composite films. The films (1 cm^2) were mounted on aluminum stubs, followed by sputter-coating with a 10 nm gold layer before SEM observation. The film samples were scanned using an Everhart-Thornley secondary electron detector under a high-vacuum mode at 5.0 kV (surface morphology of films) or 10.0 kV (cross-sections of films).

4.2.4.2 Fourier transform infrared spectroscopy

The films were studied using an ATR-FTIR spectrometer (Nicolet 380, Thermo, USA). FTIR spectra were obtained by scanning in the wavelength range of 4000 to 400 cm^{-1} with 4.0 cm^{-1} spectral resolution.

4.2.4.3 Color, light transmittance, and opacity

The film color was measured using a colorimeter (CR-410, Konica Minolta Sensing, Inc., Japan). The total color difference (ΔE) of the films was determined based on the L*, a*, and b* values measured. Light transmittance of the films was measured using an UV-Vis spectrophotometer (Cary 50, Varian, Inc., USA). The transmittance of 0.8 cm × 3.5 cm films at 600 nm was recorded. A test cell without the films was used as the blank. The film opacity was determined based on an earlier method (Yu et al., 2019) using the following equation:

$$Opacity = \frac{-\log(Trans_{600})}{K}$$

where $Trans_{600}$ is the transmittance of the films at 600 nm and K is the film thickness (mm) determined at five different locations using a digital electronic caliper.

4.2.4.4 Moisture content and water solubility

MC of the films was evaluated as the weight loss percentage after drying, based on a previous method (Valizadeh, Naseri, Babaei, Hosseini, & Imani, 2019). The films were cut into square 25 mm^2 pieces, followed by oven-drying at 105 °C for 24 h. The initial and final weights of the films were recorded for the measurement of MC as shown below:

$$MC(\%) = \frac{(Initial\ film\ weight - Final\ film\ weight)}{Initial\ film\ weight} \times 100$$

WS of the film samples was evaluated according to a previous method with slight modifications (Valizadeh et al., 2019). The oven-dried films were placed in 50 mL distilled water for 24 h. The films were then collected and oven-dried at 105 °C for 24 h. The initial

and final weights of the dried films were recorded for the measurement of WS, using the following formula:

$$WS(\%) = \frac{(Initial\ dried\ film\ weight - Final\ dried\ film\ weight)}{Initial\ dried\ film\ weight} \times 100$$

4.2.4.5 Tensile properties

Tensile studies were conducted using a texture analyzer (TA-XT2i, Texture Technologies Corp., USA). Film samples were cut into 1 cm × 5 cm rectangular strips before subjecting them to the tensile measurement. The crosshead speed and initial grip separation were set at 0.5 mm/s and 25 mm, respectively.

4.2.4.6 Water vapor permeability

WVP of the film samples was studied based on an earlier method (Wu, Deng, Luo, & Deng, 2019). First, 15 g of anhydrous $CaCl_2$ were added into a beaker, which was covered by the film samples and sealed using parafilm. The wrapped beaker was placed in a humid (75 % RH at 25 °C) desiccator that contained saturated NaCl solution. For up to 12 h, the weight of the beaker was monitored at various intervals. WVP was measured based on the equation:

$$WVP = (\Delta M \times K)/(\Delta P \times t \times S)$$

where ΔM is the weight variation (g), K is the film thickness (m), ΔP is the vapor pressure difference across the film (Pa), t is the time, and S is the vapor exposed area under test (m^2).

4.2.4.7 Antioxidant properties

The antioxidant properties of the films were measured based on an earlier method with slight modifications (Yu, Dhital, et al., 2019). First, the films, with a dimension of 10 mm × 50 mm were incubated in 5 mL of 70% and 95% methanol solutions for 24 h. Then, the film extract solution was subjected to DPPH and ABTS radical scavenging assays.

For the DPPH assay, the film extract solution (2 mL) was mixed with methanolic DPPH solution (2 mL, 0.1 mM) before keeping it in the dark for 30 min. The absorbance was measured at 517 nm using an UV-Vis spectrophotometer. For the ABTS assay, ABTS (7 mM) was first added in a potassium persulfate solution (2.45 mM) before keeping in the dark for 16 h. Methanol was added to make a diluted solution with an absorbance value of about 0.7 at 734 nm. Then, the diluted solution (3.96 mL) was mixed with the film extract solution (40 µL) and the mixture kept in the dark for 6 min. The absorbance value was measured at 734 nm using a UV-Vis spectrophotometer. The following equation was used to determine the DPPH and ABTS radical scavenging activity:

$$Scavenging\ activity(\%) = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

where $Abs_{control}$ and Abs_{sample} are the absorbances of the control and sample, respectively.

4.2.4.8 Application study on vacuum-packaged beef samples

Beef samples, cut to a dimension of 15 mm^3, were wrapped with the films before packaging them in vacuum-seal bags using a vacuum sealer (Model FoodSaver BagVac™, Tilia, Inc., USA). All the vacuum-packaged beef (PB) samples were kept at 4 °C 25% RH for a maximum of two weeks. Unwrapped beef samples with and without vacuum-

packaging were used as the controls. The beef samples were pulled out from the packages at predetermined periods, *viz*., 4, 7, 10, and 14 days after storage. Then, each sample was placed in sterile stomacher bags that contained 99 mL of 0.1% peptone water (pH=7.2). A Stomacher^{TM} Circulator (Model 400, Seward^{TM}, UK) was used to homogenize the samples. The homogenates were diluted with peptone water before pour-plating with tryptic soy agar (TSA) on sterile petri dishes. All the plates were then placed in an incubator at 37 °C for 24 h and bacterial colonies on each plate were enumerated.

4.2.5 Statistical analysis

All experiments were conducted at least thrice with duplicate (beef studies) or triplicate (all other studies) samples. The collected data were expressed as means ± standard deviations. Data analyses were performed through one-way ANOVA and Tukey's test was conducted by Minitab software (Version 19). A significant difference between mean values was represented by $P \leq 0.05$.

4.3 Results and discussion

4.3.1 Film microstructure

The PC film's surface was homogeneous and smooth without phase separation, indicating that the PVOH and CS were homogenously mixed (Fig. 4.1A). Compared to the surface morphology of the PC film, the PCB film showed a rougher surface with the presence of streaks or ribbed lines (Fig. 4.1B). The incorporation of mBNCs in the film resulted in a slight aggregation of these compounds that appeared as white dots and streaks (Fig. 4.1B). By incorporating 4-HR into the PCB film, the surface became rougher, as

shown in Fig. 4.1C. As more 4-HR was added, the level of ridges and ripples in the film increased due to aggregation of the organic compound in the polymer matrix (Fig. 4.1D). In Fig. 4.1E, the cross-sectional morphology of the PC film showed a homogenous surface without pores and cracks. When mBNCs were added into the film, these compounds aggregated and appeared as white dots or streaks (Fig. 4.1F). Also, pores were formed in the microstructure of the PCB film, which could be attributed to air trapped in the film during drying (Jahed, Khaledabad, Almasi, & Hasanzadeh, 2017). By adding 4-HR into the film, the aggregation of mBNCs was reduced in these areas as fewer white streaks or spots were observed in the SEM micrographs (Fig. 4.1G-H). This could be due to the emulsifying effect of DMSO, which resulted in better dispersion of 4-HR and mBNCs in the film (Ortiz, Salgado, Dufresne, & Mauri, 2018). Compared to PCBH-5.0, the transverse section of PCBH-10.0 film (Fig. 4.1H) showed more wrinkles and ripples, which could be due to an increased density of crack deflection sites (Tian, Yan, Lu, & Jiang, 2017).

4.3.2 Fourier transform infrared spectroscopy

The FTIR spectrum of the PC film showed a broad and strong absorption band at 3277 cm^{-1}, which was attributed to overlapping of both O-H and N-H stretching vibrations, indicating the formation of a strong hydrogen bonding between PVOH and CS (Fig. 4.2). Also, the FTIR spectrum showed C-H stretching (2916 cm^{-1}), C=O ester stretching (1714 cm^{-1}), amide I (1647 cm^{-1}), amide II (1566 cm^{-1}), CH and CH_2 bending (1248 cm^{-1}), C-O stretching (1090 cm^{-1} and 1031 cm^{-1}), and C-C stretching (847 cm^{-1}) (Choo et al., 2016; Lan et al., 2020). In the spectrum of the PCB film, the absorption bands at 4000-3300 cm^{-1} were strongly fluctuated, indicating the coupling reaction between the hydroxyl groups

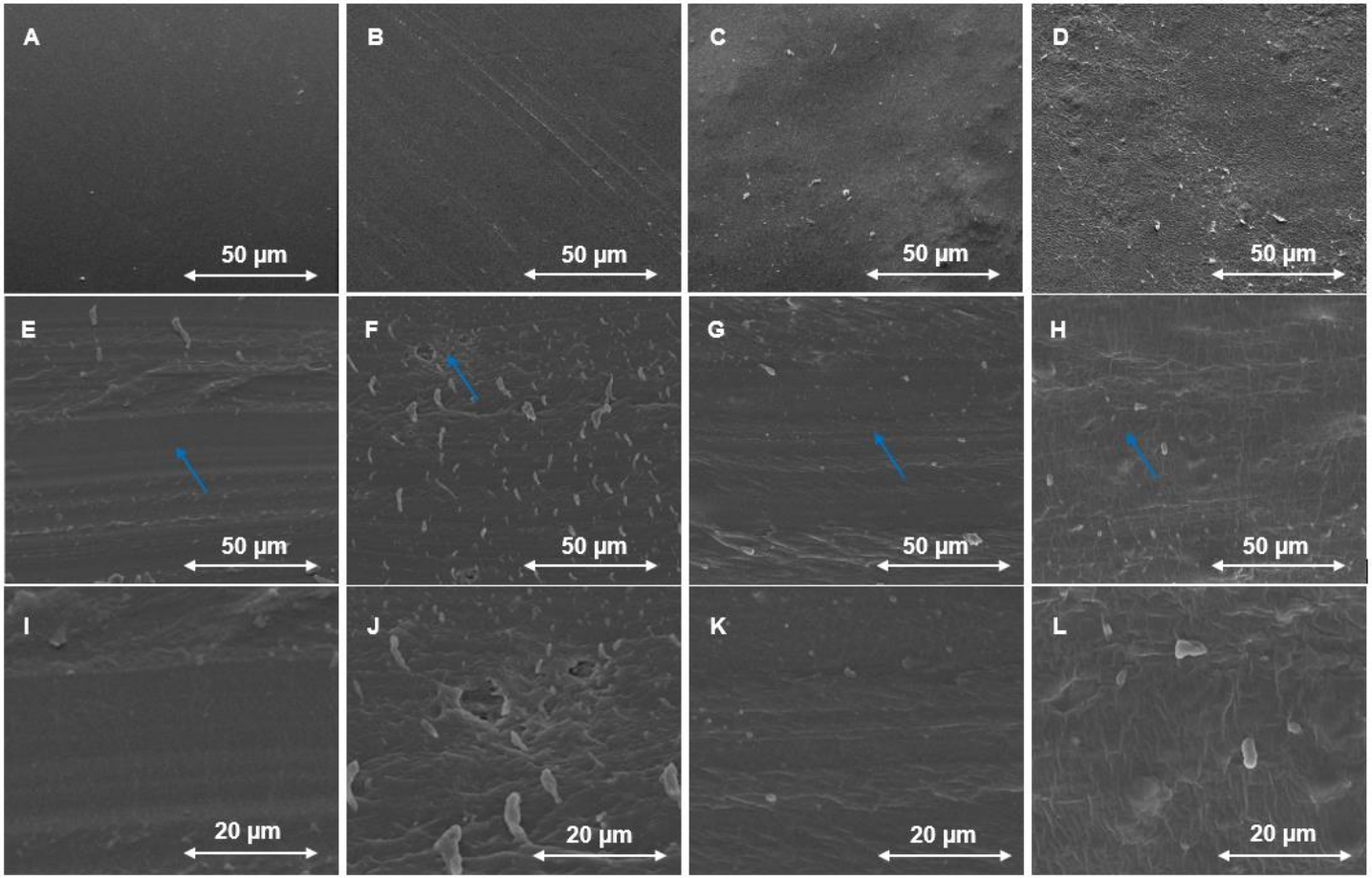


Figure 4.1 SEM micrographs of surface (A-D) and cross-sectional (E-L) morphology of (A, E, I) PC, (B, F, J) PCB, (C, G, K) PCBH-5.0, and (D, H, L) PCBH-10.0 films. The regions pointed by blue arrows were enlarged and shown in I-L. Magnification = A-H, 1,000×; I-L, 2,500×.

of mBNCs and AAPS, that resulted in the production of a hydroxyl association peak (Jin et al., 2020). Compared to the PC film, the O-H stretching band of the PCB film was broader due to the increased formation of intermolecular hydrogen bonding between PVOH, CS and mBNCs. The vibration of Si-O-C was attributed to a new absorption peak at 1143 cm^{-1}, suggesting a condensation reaction between silanol and hydroxyl groups of BNC (Qian & Sheng, 2017). Due to a weaker intensity of these peaks, they can be overlapped by other strong absorption bands, such as a C-O stretching band, resulting in difficult peak analysis. Upon the addition of 4-HR into the film, several new peaks were formed in the spectra of PCBH-5.0 and PCBH-10.0. For instance, the aromatic C-H stretching peak was observed at 3008 cm^{-1}. The characteristic peaks at 1615 cm^{-1} and 1522 cm^{-1} were attributed to the aromatic ring stretching vibration. The aromatic C-H out-of-plane bending was assigned to the band at 705 cm^{-1}. Compared to the PC and PCB, the peak at 1012 cm^{-1} was more intense due to the C-O vibration of 4-HR (Jo et al., 2017). The results suggested that mBNCs and 4-HR were successfully incorporated into the film. The FTIR results were in agreement with those of Yang, Ha, Kim, and Chae (2016).

4.3.3 Color and opacity

The incorporation of mBNCs had no significant effect on the redness of the PC film (Table 4.1). Compared to the PC film, the PCB film showed an increase in yellowness and a slight decrease in lightness, which could be due to the incorporation of mBNCs that are white yellowish in color. It was reported that rice starch films with increased yellowness and reduced lightness were formed upon the incorporation of silane-modified palm pressed fiber (PPF) compared to unmodified PPF (Phattaraporn, Waranyou, & Thawien, 2011).

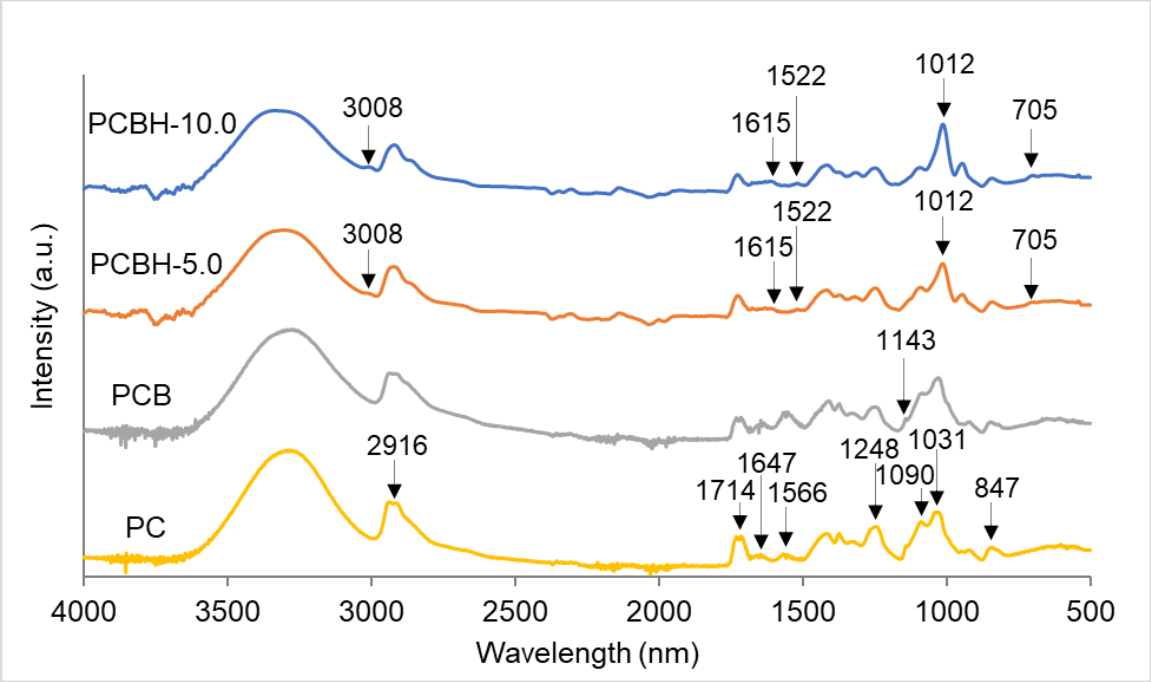


Figure 4.2 FTIR spectra of composite films.

Also, the addition of silane-modified cellulose nanofibers produced a PLA composite that was yellower, as reported by Robles, Urruzola, Labidi, and Serrano (2015). When 4-HR was added, the films showed an increase in redness (higher a* value) and a slight decrease in lightness and yellowness. The increase in redness could be due to the addition of the pale red colored 4-HR solution into the films. The incorporation of mBNCs and 4-HR increased the ΔE values of the films. This behavior might be attributed to the decrease in the L* value and increase in film thickness, and a* and b* values.

Without the incorporation of mBNCs and 4-HR, the PC film was visually clear and transparent. The film opacity increased after the addition of mBNCs and 4-HR, indicating a lower film transparency than the PC film (Table 4.1). The opacity values might be affected by the film thickness. It was observed that the film opacity increased with increasing film thickness. A similar behavior was also found by Galdeano, Wilhelm, Mali,

and Grossmann (2013) using the plasticized oat starch films. When a higher concentration of 4-HR was added, the film opacity slightly increased, which could be due to the incorporation of the hydrophobic material into the film. It might be due to a higher degree of crosslinking in the polymer matrix, reducing the interchain spacing in the polymer that, in turn, decreases the intensity of light passing through the film (Bierhalz, da Silva, & Kieckbusch, 2012). The opacity difference between the PCB films, with and without incorporation of 4-HR, is negligible. It could be due to only a small amount of 4-HR that was added into the film. Due to the color change and lower transparency of the films, subsequently packaged foods may have higher UV and visible light barrier properties, resulting in better protection of their flavor, color and nutrients.

4.3.4 Moisture content (MC) and water solubility (WS)

The MC of the films is defined by the total amount of water that occupies the empty volume of a film's microstructure (Haghighi et al., 2019). Upon the addition of mBNCs, the MC of the PC film decreased by 13.4% (Table 4.2). This could be due to a higher degree of physical crosslinking and hydrogen bonding interaction between PVOH, CS and mBNCs. The findings were similar to those of S. Zhang et al. (2016). The MC of the films incorporated with 4-HR was significantly higher ($P \leq 0.05$) compared to those of the PC and PCB films. As expected, the MC of the films significantly increased ($P \leq 0.05$) as a higher concentration of 4-HR was added. This could be due to the amphiphilic properties of 4-HR, that allowed the hydrophilic moieties to interact with water. The film thickness is an important parameter for evaluating the MC of the films. The MC of the films were increased with increasing film thickness. The increase in film thickness may allow more

Table 4.1 DMSO concentration, thickness, color and opacity values of composite films.

Sample	DMSO (wt%)	Thickness (mm)	L*	a*	b*	ΔE	Opacity
PC	0	0.176 ± 0.005^{e}	92.43 ± 0.15^{a}	-1.45 ± 0.13^{b}	11.96 ± 1.01^{c}	8.03 ± 1.01^{d}	0.78 ± 0.03^{c}
PCB	0	0.188 ± 0.004^{de}	87.88 ± 0.18^{b}	-1.22 ± 0.04^{b}	30.86 ± 0.54^{a}	27.29 ± 0.57^{b}	0.93 ± 0.03^{b}
PCBH-2.5	2.38	0.198 ± 0.004^{cd}	83.44 ± 0.24^{d}	7.63 ± 0.28^{a}	31.42 ± 0.33^{a}	29.97 ± 0.41^{a}	0.93 ± 0.02^{b}
PCBH-5.0	4.75	0.210 ± 0.007^{bc}	84.13 ± 0.26^{cd}	7.44 ± 0.25^{a}	24.77 ± 0.61^{b}	23.73 ± 0.64^{c}	1.03 ± 0.02^{a}
PCBH-7.5	7.13	0.220 ± 0.007^{b}	84.58 ± 0.16^{c}	7.20 ± 0.39^{a}	23.29 ± 0.30^{b}	22.20 ± 0.23^{c}	0.96 ± 0.07^{ab}
PCBH-10.0	9.50	0.236 ± 0.009^{a}	84.12 ± 0.44^{cd}	7.21 ± 0.11^{a}	23.55 ± 0.73^{b}	22.61 ± 0.53^{c}	0.96 ± 0.03^{ab}

Values with the same superscripts in each column indicate no significant differences ($P > 0.05$).

*The values are given as means ± SD (n =3).

hydrophilic groups of 4-HR to interact with water. A similar result was also reported by Mali, Grossmann, García, Martino, and Zaritzky (2004) using the plasticized yam starch films.

The WS of the PC film increased by 34.0% after the incorporation of mBNCs, which could be due to the presence of hydrophilic R-groups on the silane coupling agents that allowed them to interact with water (Table 4.2). Further, agglomeration of mBNCs may allow water molecules to enter and interact with the film more freely, resulting in a higher WS value (Zhang, Lu, Fan, Jiang, & Dong, 2019). In contrast, it was reported that water absorption of a soy protein isolate-based film decreased upon the incorporation of 1.23% silane-modified cellulose nanocrystals (S. Zhang et al., 2016). The WS of the composite films may greatly depend on the concentration and hydrophilic moieties of the modified nanocellulose. When 4-HR was added, the WS of the films was significantly decreased ($P \leq 0.05$). Compared to the other films, PCBH-10.0 had the lowest WS as the film hydrophilicity was decreased with the addition of 4-HR. The decrease in WS could also be due to the emulsifying effect of DMSO in the polymer matrix, which decreases the interaction between PVOH, CS, mBNCs, and 4-HR. Similarly, the WS of CS-thyme EO films was reduced with the addition of emulsifying agents (Lian, Peng, Shi, & Wang, 2019).

4.3.5 Tensile properties

The tensile strength (TS) of the PCB film was slightly improved compared to that of the PC film (Table 4.2). The TS of the film could be affected by various factors, including the crosslinking interaction between active groups on the surface of the nanofiller with the polymer matrix and the degree of agglomeration due to interactions between

nanofillers in the polymer matrix (P. Zhang et al., 2019). Phattaraporn et al. (2011) reported that upon the addition of silane-treated PPF, the TS of rice starch film gradually increased, but it decreased when the concentration of PPF was 40%. Here, the TS of the film was slightly improved, which could be due to agglomeration of mBNCs, as confirmed by the SEM results (Fig. 4.1). The elongation at break (EAB) of the film was decreased by 14.9% upon the incorporation of mBNCs (Table 4.2). The reinforcement of nanocellulose produces films with improved continuity within the polymer matrix, which reduces the chain mobility, leading to increased film hardness and stiffness (Asad et al., 2018). Lee, You, Jin, and Kwak (2020) similarly reported that the EAB of a PVOH film was reduced upon the addition of dialdehyde-modified cellulose nanocrystals. When 4-HR was added, the TS of the film was significantly reduced ($P \leq 0.05$). The TS of the PCB film significantly decreased, while the EAB of the PCB film increased significantly, indicating that the 4-HR worked as a plasticizer in the composite film. A similar result was observed in gelatin films incorporated with resorcinol (Bang, Shankar, & Rhim, 2019). Also, the PCB film had higher flexibility and lower rigidity with the addition of a plasticizer (e.g., DMSO). This could be due to the decreased formation of the intermolecular Van der Waals forces between the polymers and increased formation of the intermolecular bond between the polymers and plasticizer. It was reported that the increase in plasticizer content improved the elongation of bioplastic produced from microalgae (Gozan & Noviasari, 2018). When 4-HR was added into the PCB film, the EAB of the films was increased, which could be due to the plasticizing effects caused by the presence of residual solvents in the films. These findings were similar to that of Bochek et al. (2011).

4.3.6 Water vapor permeability (WVP)

WVP is an important parameter for barrier properties of packaging films which depends on various factors, including the ratio of hydrophilic and hydrophobic group, cracks, tortuosity, and porosity in the film. The incorporation of mBNCs considerably lowered the WVP of the PCB film by 29.1% (Table 4.2). This could be because of a reduction in water absorptivity of mBNCs due to covalent or hydrogen bonding interactions between the silane coupling agent with the free hydroxyl groups on the surface of mBNCs (Demir, Atikler, Balköse, & Tıhmınlıoğlu, 2006). As more 4-HR was added, the WVP of the film gradually increased. The increased amount of plasticizer improved the flexibility and mobility of the polymer network chains, leading to the formation of a looser network in the polymer matrix. The structural changes in the polymer matrix could lead to the increase in WVP values. Similar findings were reported when other plasticizers, such as sorbitol, glycerol, and Tween 80 were incorporated into CS films (Patricia Miranda, Garnica, Lara-Sagahon, & Cárdenas, 2004).

4.3.7 Antioxidant properties

Both the PC and PCB films showed weak ABTS and DPPH radical-scavenging activities in the tested food simulants (Fig. 4.3). The residual free amino groups on the surfaces of CS and mBNCs react with free radicals to produce stable macromolecule radicals, contributing to the film scavenging activities (Jahed et al., 2017). The PC and PCB films showed higher DPPH-radical scavenging activities than ABTS-radical scavenging activities (Fig. 4.3A). This could be due to the pH-dependent DPPH cation, which is highly unstable in acidic media. It was reported that the presence of acidic solvents

Table 4.2 Physical and tensile properties of films.

Sample	MC (%)	WS (%)	TS (MPa)	EAB (%)	WVP ($\times 10^{-10}$ g m^{-1} s^{-1} Pa^{-1})
PC	26.43 ± 1.21^{d}	28.36 ± 1.18^{b}	20.49 ± 0.42^{a}	223.89 ± 7.50^{b}	0.86 ± 0.02^{d}
PCB	22.89 ± 0.44^{e}	38.01 ± 1.27^{a}	20.77 ± 1.62^{a}	190.61 ± 4.95^{c}	0.61 ± 0.02^{e}
PCBH-2.5	29.33 ± 0.58^{c}	17.63 ± 1.91^{c}	14.04 ± 0.50^{b}	224.13 ± 14.32^{b}	1.87 ± 0.11^{c}
PCBH-5.0	37.33 ± 0.35^{b}	14.07 ± 0.63^{d}	10.11 ± 1.17^{c}	231.97 ± 8.24^{b}	2.92 ± 0.07^{b}
PCBH-7.5	38.02 ± 0.94^{b}	12.28 ± 0.17^{d}	9.99 ± 0.40^{c}	278.72 ± 7.78^{a}	2.98 ± 0.06^{b}
PCBH-10.0	44.11 ± 1.03^{a}	9.48 ± 0.59^{e}	8.12 ± 0.49^{d}	281.10 ± 24.69^{a}	4.06 ± 0.20^{a}

Values with the same superscripts in each column indicate no significant differences ($P > 0.05$).

*The values are given as means ± SD (n =3).

may result in false-positive results in a DPPH radical-scavenging assay (Ferri, Gianotti, & Tassoni, 2013). After the addition of 4-HR into the films, the scavenging activities of the films were enhanced against ABTS and DPPH radicals. In both food stimulants, PCBH-2.5 film showed strong ABTS and DPPH radical-scavenging activities, even when a low concentration of 4-HR was added. When more 4-HR was added, the DPPH and ABTS radical-scavenging activities of the films (e.g., PCBH-5.0, PCBH-7.5, and PCBH-10.0) remained at similar levels. These strong scavenging activities could be due to the ability of 4-HR to donate a proton by phenolic hydrogen to free radicals. Because of the amphiphilic properties of 4-HR, the oxidation reaction of proteins and lipids could also be reduced, resulting in a higher quality of packaged food products (Fidalgo, Deglesne, Arroya, Ranneva, & Deprez, 2018). Thus, the high concentration of phenolic constituents in 4-HR greatly contributed to the strong antioxidant properties of the films.

4.3.8 Application study on vacuum-packaged beef samples

Raw beef samples without vacuum packaging film were used as the control. Compared to the control, all the PB samples had lower bacterial counts after two weeks of storage (Fig. 4.4). At day 4, the bacterial counts of the control and PB sample without film were 7.7 and 6.5 log CFU/g, respectively. Compared to those films, the microbial load of PB samples wrapped with PC film was reduced by about 2-3 log CFU/g during storage. At days 4 and 14, the PCB film significantly decreased the bacterial loads of PB samples ($P \leq 0.05$). Also, the bacterial loads of PB samples wrapped with all the 4-HR-incorporated PCB films were below the limit of detection, which was 1 log CFU/g. However, the bacterial load of PB samples with PCBH-2.5 increased above the limit of detection to 4.8

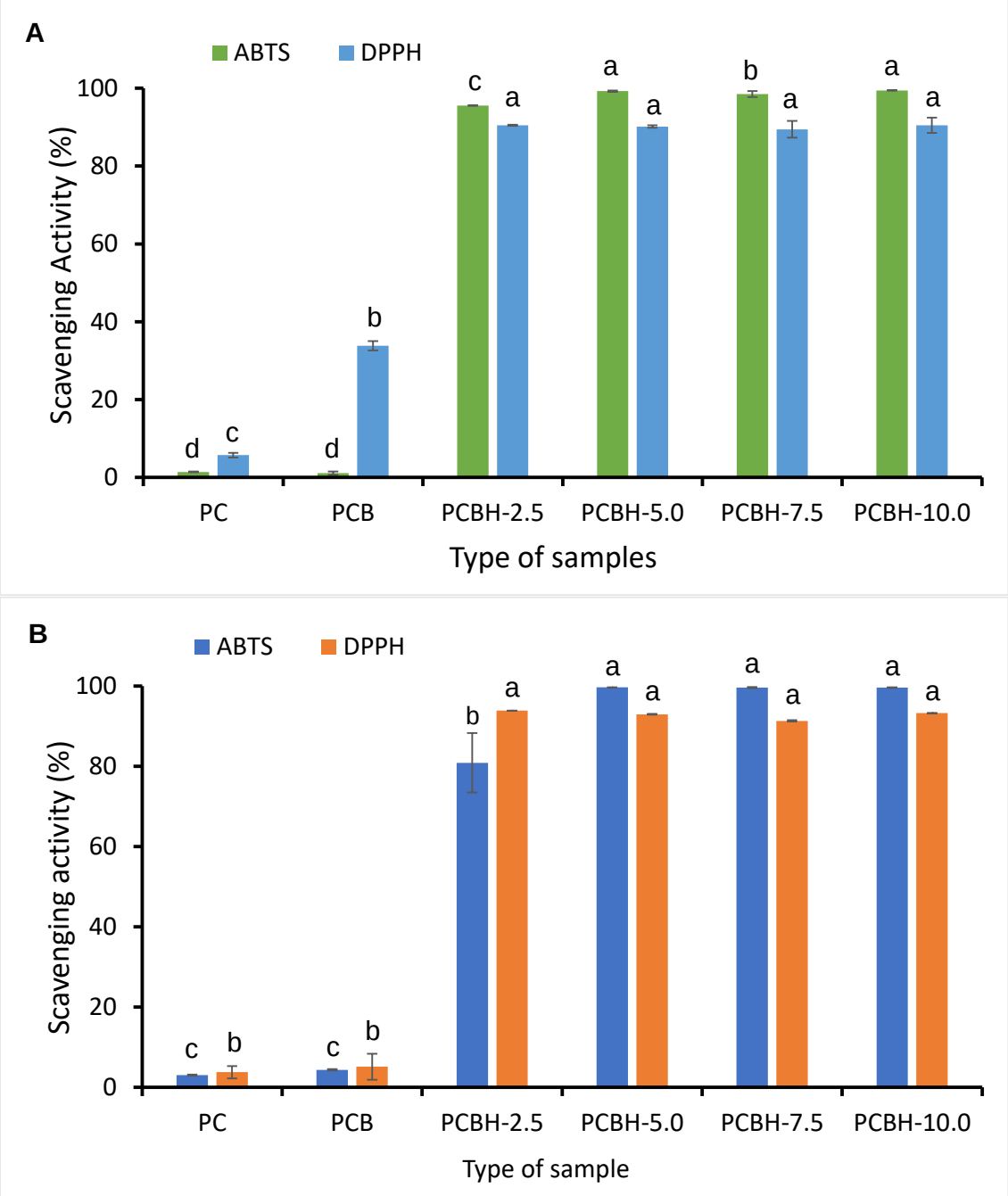


Figure 4.3 Scavenging activities of composite films on ABTS and DPPH radicals in (A) 70% and (B) 95% methanol.

log CFU/g at day 14. Further, the bacterial loads of PB samples without film were maintained at 6.5-6.7 log CFU/g during storage. All the bacterial loads of beef samples at

days 7, 10, and 14 followed the decreasing trend of bacterial loads at day 4 (Fig. 4.4). The bacterial loads of PB samples wrapped with all the tested films were below those of the PB samples without film, suggesting that all the composite films were effective in inhibiting and retarding bacterial growth under vacuum packaging conditions. Similarly, other researchers reported the applications of vacuum packaging and CS coating to improve the shelf-life of refrigerated beef (Duran & Kahve, 2020) and grilled pork samples (Yingyuad et al., 2006) at refrigeration temperature. In this work, an anaerobic environment was achieved by applying vacuum packaging to the food products. Vacuum packaging inhibits the growth of aerobic spoilage microorganisms, resulting in a lower rate of food spoilage. Also, the positively-charged amino groups on the surfaces of CS and mBNCs can interact with the negatively-charged cell membranes of the spoilage bacteria, resulting in cell damage and leakage of cellular materials (Fernandes et al., 2013; Yingyuad et al., 2006). Upon the addition of 4-HR, the antimicrobial activities of the films were significantly improved ($p \leq 0.05$). This could be due to the capability of 4-HR to penetrate bacterial cell membranes and interact with the cell nucleus and proteins, as confirmed by the SEM results (Fig. 4.1).

4.4 Conclusions

In this study, mBNCs were produced by grafting aminosilane groups onto the surface of BNCs through a polycondensation reaction. Using a solution-casting method, PC-based composite films were developed with the addition of mBNCs and 4-HR. SEM and FTIR results confirmed that the morphological properties and chemical structure of the films were affected by the incorporation of mBNCs and 4-HR. The addition of mBNCs

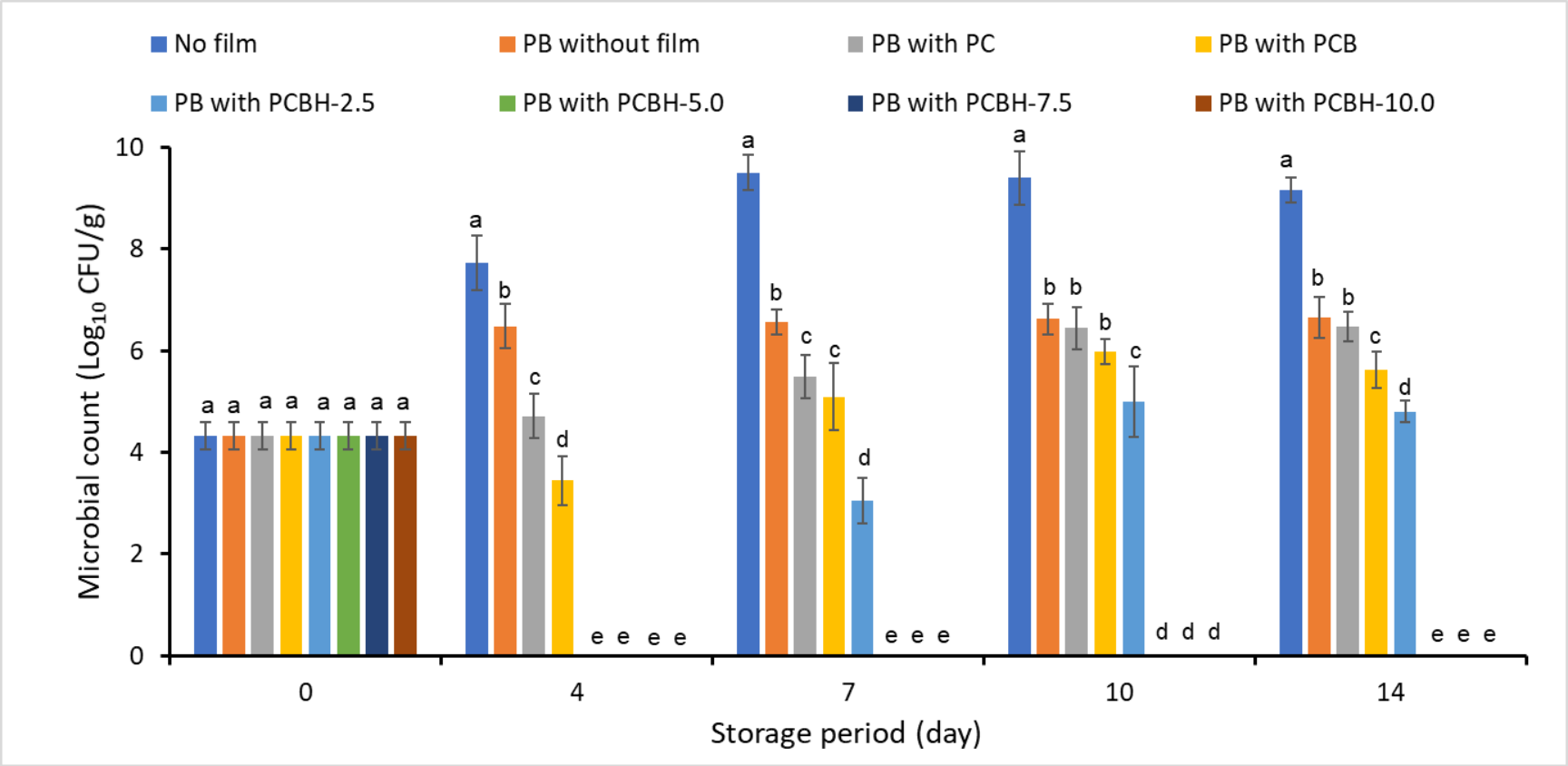


Figure 4.4 Antimicrobial activity of composite films on spoilage bacteria on a raw beef model.

and 4-HR improved the redness, yellowness, opacity, and MC of the films. However, the brightness and WS of the PC films were decreased upon the addition of mBNCs and 4-HR. The mechanical test and WVP results showed that the addition of mBNCs and 4-HR produced films with higher flexibility, less rigidity, and lower water vapor barrier properties compared to the pure PC film, due to the plasticizing effect. The antioxidant activity of the films was improved with the incorporation of 4-HR due to the presence of phenolic hydrogens that could interact with the free radicals. Using vacuum packaging, the growth of bacteria on refrigerated raw beef was inhibited and food spoilage was retarded, resulting in an increased shelf-life of raw beef samples. Consequently, the results showed that the PC/mBNCs/4-HR film has a great potential to be utilized as an active food packaging material due to the enhanced film flexibility with excellent antioxidant and antimicrobial properties of CS, mBNCs, and 4-HR (Agulló, Rodríguez, Ramos, & Albertengo, 2003).

Chapter 5 CAM-21, A Novel Lytic Phage with High Specificity Towards *Escherichia coli* O157:H7 in Food Products

5.1 Introduction

Escherichia coli O157:H7, a serogroup member of Shiga toxin-producing *E. coli* (STEC) can produce harmful Shiga-like toxins, that result in foodborne outbreaks and clinical illness, ranging from mild to life-threatening symptoms (Fan et al., 2021). Based on the Centers for Disease Control and Prevention (CDC), about 265,000 cases of foodborne illness caused by *E. coli* O157:H7 and six other non-O157 serotypes are estimated annually in the United States (Bertoldi, Richardson, Schneider, Kurdmongkolthan, & Schneider, 2018). STEC infections can arise because of human transmission, direct contact with infected animals and their surroundings, or intake of contaminated food products (Mulchandani et al., 2021). The major causes of STEC outbreaks include contaminated foods, such as fresh produce and fruit (Bottichio et al., 2020; Tack et al., 2021), raw or undercooked beef (CDC, 2010), dairy products (Farrokh et al., 2013) and unpasteurized fruit juice (Cody et al., 1999). Thus, an effective control measure in foods is important.

To decrease STEC transmission in foods, the food industry uses different control strategies that include physical (thermal processing, high-pressure treatment, pulsed-electric field processing, ultrasound, irradiations and UV light), and chemical approaches (chlorine dioxide, ozone, hydrogen peroxide and chlorine) (Erickson & Doyle, 2007). However, these methods have their limitations. For instance, thermal processing may result in protein denaturation, changes in color, aroma and taste, and loss of nutrients in food products (Vinnikova, Synytsia, & Kyshenia, 2019). Although irradiation has a higher

efficiency of microbial inactivation than thermal treatment, the physical intervention may also result in changes in food texture and aroma, loss of nutritional values and lipid oxidation in foods (Duc et al., 2020; Indiarto, Pratama, Sari, & Theodora, 2020). The use of chloride-based disinfectants may result in the production of carcinogens, including semicarbazide, trihalomethanes and others, which develop substantial health risks to humans (Chen, Zhang, & Yang, 2020). Further, extensive uses of chemical disinfectants are insufficient to assure the food safety and quality because some pathogens can develop resistance (Wirtanen & Salo, 2003).

In addition to conventional methods, antibiotic-based control methods have also been utilized to decrease and control STEC, particularly *E. coli* O157:H7 (Mir & Kudva, 2019). Nevertheless, the inappropriate uses of antibiotics have resulted in the development of antibiotic-resistance genes in the pathogens, leading to human health risks (Mir & Kudva, 2019; Yan Zhou et al., 2021). Thus, all the current physical-, chemical- and antibiotic-based control strategies are still insufficient, as evidenced by the relentless foodborne infection outbreaks and recalls of contaminated foods.

The potential use of bacteriophages as an alternative to antibiotics has gained attention because of the formation of antibiotic- or drug-resistant bacteria (Ghasemian, Bavand, & Moradpour, 2017). Lytic bacteriophages are capable to infect and kill pathogenic bacterial cells by lysis. They are stable under harsh conditions, including extreme pHs and temperatures. Naturally existing bacteriophages can be isolated in the environment, for example from water, sewage, feces, soil, and produce and animal farms (Petsong, Benjakul, Chaturongakul, Switt, & Vongkamjan, 2019). Due to their unique host-specificity, phages can infect highly targeted bacterial species (Islam et al., 2020).

Phages are safe for human and animal consumption, and are harmless to beneficial microorganisms that exist in the gastrointestinal tract and foods (Duc et al., 2020). Due to these advantages, phages can potentially be used as an alternative tool to control foodborne pathogens. ListShield™, EcoShield™, and SalmoFresh™ are among the FDA-approved commercial phage products that can be applied in foods to control *Listeria monocytogenes*, *Salmonella*, and *E. coli* O157:H7, respectively (Carter et al., 2012; Yang, Sadekuzzaman, & Ha, 2017; X. Zhang et al., 2019).

Numerous studies have reported the biocontrol effect of bacteriophages to combat foodborne pathogens, such as STEC (Tomat, Casabonne, Aquili, Balagué, & Quiberoni, 2018), *Salmonella* (Islam et al., 2020), *L. monocytogenes* (Stone et al., 2020) and *Campylobacter* (Chinivasagam et al., 2020) in various food products. Also, bacteriophages have been used to kill drug-resistant foodborne pathogens (Elbahnasawy, ElSayed, & Azzam, 2021; Z. Li et al., 2020; Peng et al., 2020; Safwat Mohamed, Farouk Ahmed, Mohamed Mahmoud, Abd El-Baky, & John, 2018). Since most *E. coli* O157:H7 infections result from contaminated food products, biocontrol of the pathogen in foods is critical. Several studies have used bacteriophages for biocontrol of this pathogen in various foods, such as whole beef (Hudson et al., 2013), burger patties (Park & Park, 2021), lettuce (Carter et al., 2012), milk (Li et al., 2021), fruit (L. Wang et al., 2017) and ground beef (Abuladze et al., 2008).

There are certain criteria for bacteriophages to be used as a food biocontrol agent against pathogens. For instance, the phages should not possess any virulence factor or toxin-encoding and antimicrobial resistance genes. Thus, a thorough genomic analysis of the bacteriophages is necessary to ensure the safety of the biocontrol agent for use in foods

(Guo et al., 2021). Bacteriophages are promising biocontrol agents if they are not able to promote horizontal gene transfers (Cong et al., 2021). This study aimed to determine the morphological, biological, and genetic characteristics of lytic bacteriophages isolated from environmental samples, and to evaluate their biocontrol abilities on *E. coli* O157:H7 in pasteurized milk, ground beef and spinach.

5.2 Materials and methods

5.2.1 Bacterial strains

Ninety bacterial strains, obtained from the University of Missouri, Food Microbiology Lab culture collection (Table 5.1) were cultured in Tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) for 24 h at 37 °C. All bacterial strains were utilized for the phage host range study, and *E. coli* O157:H7 strain C7927 was utilized as the host bacteria for phage isolation and purification, and production of a high titer phage stock. This bacterial strain was also used to evaluate the inhibition effect of isolated phages in foods.

5.2.2 Isolation and purification of CAM-21

Twenty lagoon slurry and fecal samples were collected from Foremost Dairy Research Center (Columbia, MO, USA) for phage isolation. Briefly, 5 g of sample were mixed with saline magnesium (SM) buffer (0.1 M NaCl, 0.008 M $MgSO_4$, 0.05 M Tris-HCl, 0.01% gelatin, pH 7.5) and centrifuged at 10,000 × g for 10 min at 4 °C. Then, the mixture was filtered through a polyether sulfone (PES) filter membrane (pore size = 0.20 µm; Nalgene, Rochester, NJ, USA). The double-layer agar technique was performed by

adding 100 µL of diluted filtrate and 100 µL of log-phase host strain in a test tube containing 5 mL of melted top agar (TSB, 1.25 mM $CaCl_2$, and 0.5% agar). The mixture was then overlaid on a pre-poured tryptic soy agar (TSA) (Difco Laboratories) plate. The melted agar was allowed to solidify before incubation of the sample for 24 h at 37 °C. To purify the phages, each plaque was picked from the agar plate and added into 300 µL of SM buffer. The phage suspension was stored overnight at 4 °C. The double-layer agar technique, as previously mentioned, was performed using the overnight suspension (100 µL). Then, the melted agar was allowed to solidify before incubating for 24 h at 37 °C. The purification step was repeated three times. The purified phage was then stored in SM buffer at 4 °C before subsequent analyses.

5.2.3 Production of high titer phage stock

A high titer phage stock was produced using a polyethylene glycol (PEG) precipitation technique. One hundred microliters of purified phage solution were mixed with 1 mL of saturated bacterial culture (~10^9 CFU/mL) in TSB and the mixture allowed to sit for 15 min at 37 °C. The phage/bacterial suspension was added into 10 mL of TSB and cultured in a shaker-incubator with shaking at a speed of 200 rpm for 24 h at 37 °C. After centrifuging at 11,739 × g for 15 min, the suspension was filtered using a PES filter membrane (pore size = 0.22 µm; Merck Millipore, Cork, Ireland). Then, 10 wt.% of PEG 8000 was added, and the solution was left overnight at 4 °C. The phage pellet was precipitated by centrifuging at 16,904 × g for 15 min at 4 °C. After the removal of the supernatant, 1 mL of SM buffer was added to resuspend the pellet. The phage count was determined using the double-layer agar method.

5.2.4 Host range study of CAM-21

The host spectrum of CAM-21 was studied based on a reported plaque assay method (Elhalag, Nasr‐Eldin, Hussien, & Ahmad, 2018) with some modifications. Ninety strains of bacteria (Table 5.1) were selected for the study. Briefly, 100 µL of phage suspension (10^9 PFU/mL) and 100 µL of log-phase host bacteria were mixed with 5 mL of melted top agar, as mentioned in Section 5.2.2. The melted agar was poured onto pre-solidified TSA plates. The plaque formation on the agar plates was determined after incubation at 37 °C for 24 h. SM buffer was utilized as a control.

5.2.5 Lytic activity of CAM-21

The lytic activity of CAM-21 was evaluated based on a reported method with modifications (C. Huang et al., 2018). One hundred microliters of phage suspensions (10^3 – 10^9 PFU/mL) were added into 100 µL of *E. coli* O157:H7 (10^7 CFU/mL) at various multiplicity of infection (MOI) from 0.0001 to 100 in a 96-well plate before incubating at 37 °C for up to 24 h. SM buffer was utilized as a control. A microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) was used to measure the optical density at a wavelength of 600 nm (OD_{600}) at various time intervals.

5.2.6 Phage morphology

The phage morphology was investigated using a transmission electron microscopy (TEM) (JEM-1400, JEOL Ltd., Peabody, MA). Phage particles were concentrated by centrifuging at 46,378 × g at 4 °C for 2 h (Model Optima L-90K, Beckman Coulter, Inc., Brea, CA, USA) before resuspending the pellet in SM buffer. A negative staining technique

was performed for phage preparation. Five microliters of phage suspension were deposited on copper/carbon grids for 1 min and stained with 2% phosphotungstic acid for 30 s. The grids were air-dried before TEM imaging at 80 kV. The phage size was estimated using an ImageJ software.

5.2.7 TEM imaging of bacterial lysis by CAM-21

To investigate the bacterial lysis by CAM-21, the concentrated phage suspension was first prepared according to the method described in Section 5.2.3. Before TEM imaging, the specimen was prepared based on a previous method (Yu et al., 2020). Sodium cacodylate (SC) buffer (0.1 M, pH=7.35) containing 2% paraformaldehyde and 2% glutaraldehyde was used to primarily fix the bacterial cells, followed by rinsing with 0.13 M sucrose in SC buffer (0.1 M). SC buffer containing 1% OsO_4 was used for a secondary microwave fixation for 1 min, and the sample left for 1 h at 4 °C. The specimens were rinsed with SC buffer and distilled water. Aqueous uranyl acetate (1%) was used for *en bloc* staining before storing the sample sovernight at 4 °C. The treated samples were rinsed with distilled water, followed by undergoing a series of graded dehydration. An Epon resin was used to infiltrate the dehydrated samples for 24 h before polymerization at 60 °C overnight. An ultramicrotome (Ultracut UCT, Leica Microsystems, Germany) and a diamond knife (Diatome, Hatfield, PA, USA) were utilized to prepare thin sections with a thickness of 85 nm before observing with TEM at 80 kV.

5.2.8 Optimal MOI

The optimal MOI of CAM-21 was evaluated based on an earlier method with some modifications (Y. Zhang et al., 2021). One hundred microliters of phage suspension and 100 µL of log phase *E. coli* O157:H7 (~10^8 CFU/mL) were mixed at various MOI values from 0.0001 to 1000. The mixture solutions were added into 800 µL of TSB and incubated in a shaker incubator with a shaking speed of 180 rpm for 4 h at 37 °C. The cultures were centrifuged at 9,300 × *g* for 15 min before phage titers were evaluated using the double-layer agar technique. The optimal MOI of the phage is the one with the highest phage count.

5.2.9 Adsorption study

The adsorption study of CAM-21 was performed as previously reported (Litt & Jaroni, 2017), with some modifications. One milliliter of a freshly grown (3 h) culture of *E. coli* O157:H7 (10^8 CFU/mL) was centrifuged at 9,300 × *g* for 5 min to obtain a cell pellet that was resuspended in 900 µL of 1× phosphate-buffered saline (PBS). Then, 100 µL of phage suspension (10^6 PFU/mL) was added, followed by incubation at 37 °C. At intervals of 1 min for the first 5 min and 5 min for the next 10 min, a 100 µL aliquot was collected and added into 900 µL of PBS. The suspension was centrifuged at 13,400 × *g* for 2 min. The unabsorbed phage count was determined using the double-layer agar method. The proportion of phage count to the initial phage count at each time interval was expressed as the percentage of unabsorbed phage.

5.2.10 One-step growth curve

One-step growth of CAM-21 was evaluated using an earlier method with some adjustments (Litt & Jaroni, 2017). Briefly, 1 mL of a freshly grown (3 h) culture of *E. coli* O157:H7 (10^8 CFU/mL) was centrifuged at 9,300 × *g* for 5 min to obtain a cell pellet before resuspending it in 1 mL of PBS. Next, 1 mL of phage suspension (10^5 PFU/mL) was mixed with the bacterial suspension to achieve a MOI of 0.001. The mixture solution was incubated for 15 min at 37 °C, followed by centrifugation at 9,300 × *g* for 5 min. The cell pellet was washed twice using TSB under the same centrifugation conditions, and resuspended in 10 mL of TSB. The sample was incubated in a shaker incubator while shaking at a speed of 180 rpm at 37 °C. For the measurement of phage number, 100 µL of aliquot were collected at every 10 min for 2 h and plated using the double-layer agar method. The latent time and burst size were estimated using the growth curve of CAM-21.

5.2.11 Genome sequencing of CAM-21

CAM-21 genomic DNA extraction was performed using a phage DNA isolation kit (Norgen Biotek, Corp., Thorold, ON, Canada) based on the manufacturer's instructions. Whole genome sequencing (WGS) was conducted using the MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA). The high-quality filtered reads were assembled using Unicycler. The open reading frames (ORF) were predicted and annotated using the RAST server (Aziz et al., 2008) and BLASTP analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Potential tRNA genes were predicted using tRNAscan-SE (Chan & Lowe, 2019). The potential virulence and antimicrobial resistance factors of CAM-21 were screened using VirulenceFinder-2.0 (Malberg Tetzschner,

Johnson, Johnston, Lund, & Scheutz, 2020) and ResFinder-4.1 (Bortolaia et al., 2020), respectively. Screenings for genes related to lysogenic activity and allergenicity were conducted using the databases of PHASTER (Arndt et al., 2016) and AllergenOnline (Version 21) (Goodman et al., 2016), respectively. The CGView server was used to construct a circular genome map of CAM-21 (Grant & Stothard, 2008). To construct the phylogenetic trees, 30 bacteriophages were chosen from the subfamily, *Tevenvirinae*, including CAM-21. First, Virus Classification and Tree Building Online Resource (VICTOR) was used to develop a phylogenetic tree based on the whole genome sequences of these phages (Meier-Kolthoff & Göker, 2017). The phylogenetic trees were also constructed based on the major capsid protein and terminase large subunit of the bacteriophages by the neighbor-joining technique with a bootstrap value of 1000 after the multiple protein sequence alignment was performed, using the Mega software (Version 11) (Tamura, Stecher, & Kumar, 2021). Using EasyFig (Version 2.2.5), a multiple gene alignment was performed to compare the genome of CAM-21 to its closely related phages, including *Escherichia* phage wv7, vB_EcoM_BECP11, vB_EcoM_UFV09, and T4 (Sullivan, Petty, & Beatson, 2011).

5.2.12 Phage application in foods

The inhibitory effect of CAM-21 against *E. coli* O157:H7 strain C7927 was studied using liquid (pasteurized milk) and solid (ground beef and baby spinach) food samples, which were purchased from a local supermarket. First, the bacterial culture was transferred to fresh TSB and incubated overnight at 37 °C. To obtain a bacterial culture with high

purity, the overnight culture was centrifuged at 9,300 × g for 5 min and washed with PBS for twice. Then the culture was spiked at different concentrations in the food samples.

For the liquid food study, 100 µL of the purified cultures (10^5 and 10^6 CFU/mL) were added to each tube containing 9.9 mL of pasteurized milk, followed by the addition of 100 µL of phage suspensions (10^9 PFU/mL) to give MOI values of 10,000 and 1,000, respectively. SM buffer (100 µL) was used as a control. All the samples were stored in a refrigerator at 4 °C. After 0, 3, 6, 9, 12, and 24 h of incubation, and aliquots were collected, diluted and plated on MacConkey sorbitol agar plates. After the plates were incubated for 24 h at 37 °C, total viable counts of *E. coli* O157:H7 were determined and expressed in log CFU/mL values.

For the solid food study, 10 g of ground beef and a piece of baby spinach leaf (about 1 g) were placed in duplicate respective sterile petri plates. One hundred microliters each of 10^5 and 10^6 CFU/mL bacterial cultures were added and spread on the surface of each food sample with a pipet tip. The spiked foods were left under a laminar hood for 30 min to allow for bacterial attachment. Then, a 100 µL of phage suspension (10^9 PFU/mL) was added to give MOI values of 10,000 and 1,000, respectively. SM buffer (100 µL) was used as a control. The food samples were placed in a refrigerator at 4 °C and 25% RH and collected after 0, 3, 6, 9, 12, and 24 h of incubations. On each sampling day, the ground beef and baby spinach samples were placed into sterile stomacher bags, followed by the addition of 90 mL and 9 mL of peptone water (0.1% (w/v)), respectively. The samples were homogenized using a Stomacher™ Circulator Model 400 (Seward, Ltd., UK). The serially-diluted homogenates were plated on MacConkey sorbitol agar plates and incubated

for 24 h at 37 °C. Total viable counts of the bacteria were determined and expressed in log CFU/g values.

5.2.13 Statistical analysis

Except for the phage application in foods, which was done in duplicates, all experiments were conducted in triplicate. Data analyses were performed through one-way analysis of variance (ANOVA) and Tukey's test, using Minitab software (Version 19). The collected results are presented as means ± standard deviations. A significant difference between mean values was represented by $P \leq 0.05$.

5.3 Results

5.3.1 Phage isolation

Five bacteriophages were initially isolated and purified from collected dairy farm samples, using *E. coli* O157:H7 strain C7927 as the host bacteria. Because the isolated phages demonstrated similar host range and genetic characteristics, as demonstrated by WGS, only one bacteriophage was selected for subsequent analysis. The novel lytic bacteriophage, subsequently named CAM-21, was isolated from a lagoon slurry of a dairy farm. CAM-21 produced many clear plaques (diameter = about 1 mm) on a bacterial lawn of *E. coli* O157:H7 C7927 in a double-layer agar method (Fig. 5.1).

5.3.2 Host range analysis

Using a plaque assay method, CAM-21 exhibited a broad host spectrum against STEC (Table 5.1), forming plaques with 32 out of 90 total bacterial strains tested. CAM-21 was

capable to lyse several strains of STEC serotypes, which include *E. coli* O157:H7 (10/10), O26 (5/7), O103 (9/9) and O145 (8/11). On the contrary, it did not lyse non-O157 STEC, *viz*., O45, O104, O111, and O121, non-pathogenic *E. coli*, and other bacterial genera, including *Salmonella*, *Shigella*, *Listeria*, *Staphylococcus*, and *Bacillus*.

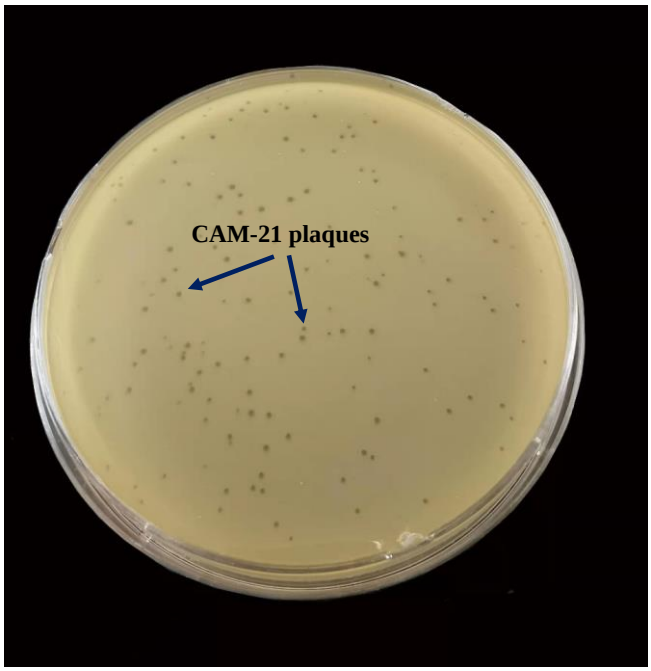


Figure 5.1 Plaques of CAM-21 against *Escherichia coli* O157:H7 C7927.

5.3.3 TEM analysis

CAM-21 has a polyhedron capsid (diameter = 92.83 ± 8.97 nm) and a contractile tail (length = 129.75 ± 1.50 nm) (Fig. 5.2A). Morphological analysis of host lysis showed that infection of target bacteria was initiated by attachment of the phage tail fibers to surface receptors on the cell wall of the bacteria (Fig. 5.2B). Progeny virions were formed in the cell following DNA replication and packaging (Fig. 5.2C). Progeny phages were eventually released through bacterial cell lysis (Fig. 5.2D).

Table 5.1 Host specificity of *Escherichia* phage CAM-21.

Bacterial species	**Source**	**Strain**	**Plaque formation**
Escherichia coli	Clinical isolate	ATCC 25922	-
	Not known	K12	-
E. coli O157:H7	Human	C7927	+
		3178-95	+
		G5101	+
		86-24	+
		93-111	+
		OK-1	+
		2886-75	+
	Beef	505B	+
		MF 1847	+
	Hamburger	EDL933	+
E. coli O26:H11	Not known	H19	+
	Human	97-3250	-
E. coli O26	Human	DEC10C	+
		TB285C	+
		VP30	+
		DEC9A	+
		DEC9F	-
E. coli O45:H2	Human	M103-19	-
		MI01-88	-
		MI05-14	-
E. coli O45:H NM	Human	DA-21	-
E. coli O45	Human	DEC11C	-
		5431-72	-
		4309-65	-
	Cow	88-4110-H	-

		D88-28058	-
	Pig	2566-58	-
E. coli O103:H2	Human	MT#80	+
E. coli O103:H6	Human	TB154A	+
E. coli O103:H N	Human	PT91-24	+
E. coli O103:H25	Human	8419	+
E. coli O103	Human	DA-41	+
		6:38	+
		DA-55	+
		87-2931	+
		GS G5550637	+
E. coli O104:H	Cow	TW 01435	-
	Human	ECOR-28	-
E. coli O104	Human	E28	-
E. coli O111:H2	Human	RD8	-
E. coli O111:H8	Human	3215-99	-
E. coli O111:H11	Human	0201 9611	-
E. coli O111:H NM	Human	3007-85	-
E. coli O111	Human	CL-37	-
		DEC8B	-
		TB226A	-
		928/91	-
		412/55	-
	Cow	DEC8C	-
E. coli O121:H19	Human	MT#2	-
E. coli O121:H [19]	Human	DA5	-
E. coli O121	Human	87-2914	-
		DA1	-
	Not known	7927+++	-
		PT91-4	-
E. coli O145:H NT	Human	IH-16	+

	Unknown	D177	+
E. coli O145:H NM	Human	75-83	-
		GS G5578620	-
E. coli O145	Human	TB269C	+
		MT#66	+
		DEC10I	-
	Cow	BCL73	+
		B6820-C1	+
	Not known	70300885	+
		6940	+
Salmonella Enteritidis	Turkey	I4-7	-
		I4-10	-
		I4-11	-
Salmonella Newport	Not known		-
Salmonella bongori	Not known		-
Salmonella Thompson	Not known		-
Salmonella Bareily	Not known		-
Salmonella Typhimurium	Chicken	ATCC 14028	-
	Turkey	I4	-
		788	-
Shigella sonnei	Not known	ATCC 9290	-
Shigella flexneri	Feces	ATCC 25929	-
Listeria innocua	Cabbage	ATCC 51742	-
Listeria monocytogenes	Rabbit	ATCC 15313	-
	Human	ATCC 7644	-
Staphylococcus aureus	Wound	ATCC 29213	-

	Pleural fluid	ATCC 12600	-
	Not known	ATCC 29737	-
Bacillus cereus	Not known		-
Bacillus coagulans	Not known		-
Bacillus megaterium	Not known		-
Bacillus sphaericus	Not known		-

(+): formation of plaques; (-): absence of plaque

5.3.4 Lytic activity

As compared to the control, CAM-21 had a strong inhibitory effect on target bacterial growth at all tested MOI values (Fig. 5.3). Without exposure to CAM-21, the bacterial cells reached the exponential growth phase after a 2-h incubation, and the OD_{600} value increased for up to 24 h. With the phage treatment at MOI $\geq$ 0.001, the bacterial growth was clearly inhibited as all the OD_{600} values were less than 0.15 after 24 h of incubation. However, at MOI = 0.0001, the OD_{600} value increased for up to 4 h, followed by a slight reduction to about 0.2, indicating limited growth. However, the OD_{600} value was still less than that of the phage-free control after 24 h, indicating the strong inhibitory effect of CAM-21 on the target bacterial growth.

5.3.5 Optimal MOI

At a MOI of 0.001, CAM-21 produced the highest phage titer, which was 8.08 log PFU/mL, indicating the optimal MOI of this phage (Fig. 5.4A). However, the phage titer was not significantly different ($P > 0.05$) from those at MOI of 0.1 and 0.01, which were 7.87 and 7.91 log PFU/mL, respectively. The findings revealed that the bacterial infection was still effective even when at a lower phage count.

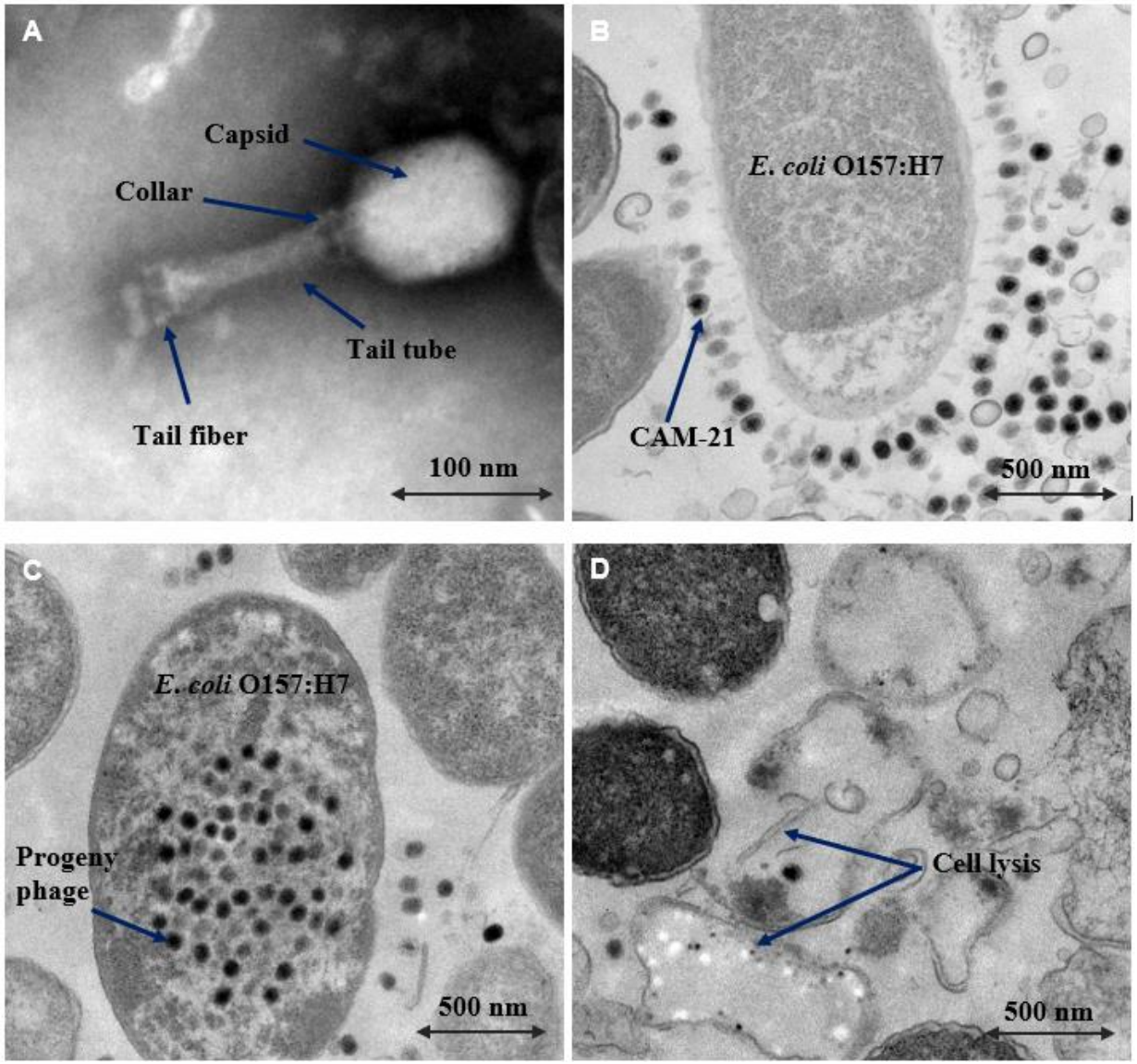


Figure 5.2 Transmission electron microscopic images showing the morphology of *Escherichia* phage CAM-21 (A) and the phage-bacteria interactions: Attachment of numerous phages on surface receptors of the bacterial cell (B); Formation of progeny phages in the bacterial cell (C); Release of progeny phages from infected cells through cell lysis (D). Scale bar = A, 100 nm; B-D, 500 nm.

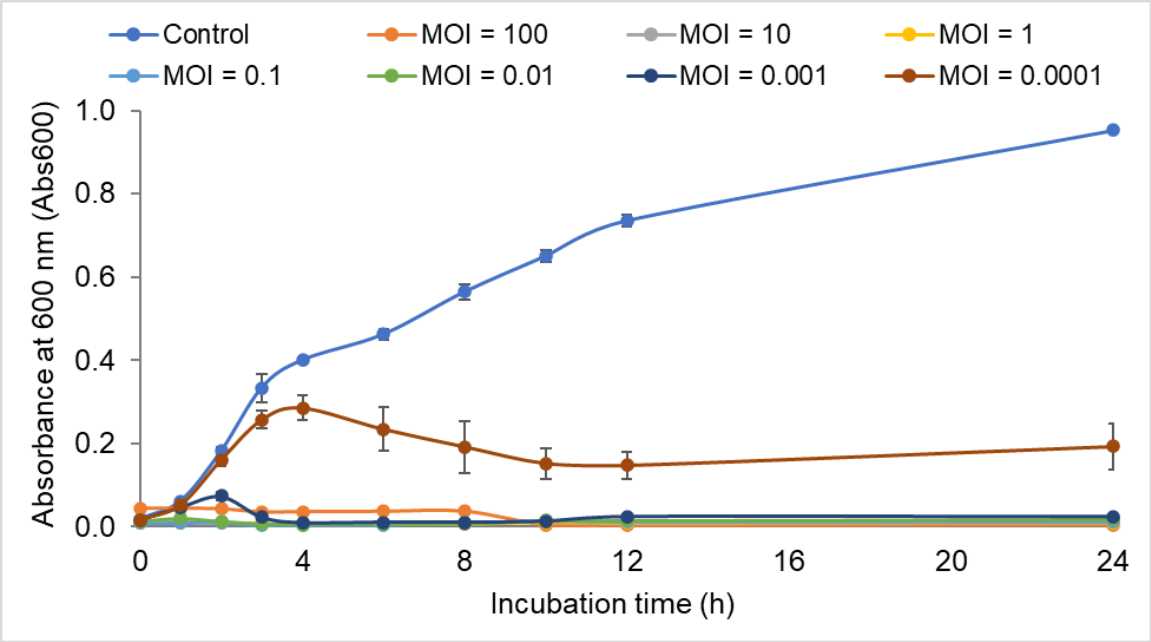
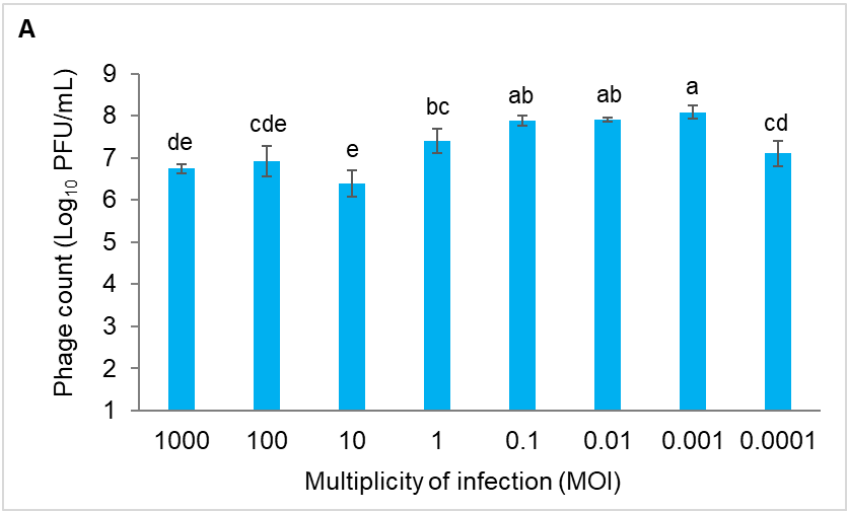
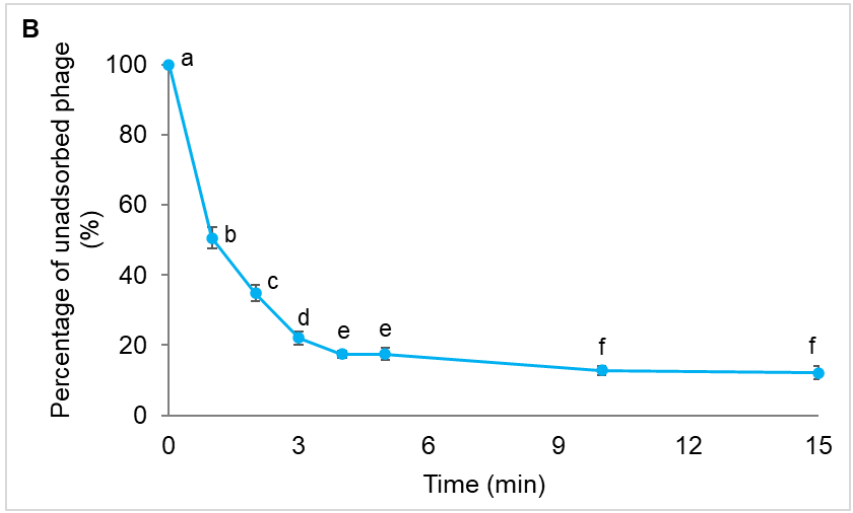


Figure 5.3 The evaluation of lytic activity of *Escherichia* phage CAM-21 using *E. coli* O157:H7 strain C7927 as the host bacteria at multiplicity of infection values ranging from 0.0001 to 1,000.

5.3.6 Phage adsorption and one-step growth

The growth characteristics of CAM-21 were evaluated using adsorption and one-step growth analysis at an optimum MOI of 0.001. After 1 min of incubation, approximately 49% of the phage had adsorbed to the host bacteria, as compared to the original population (Fig. 5.4B). As the suspension was incubated for a longer period, the percentage of unabsorbed phage was decreased. After 15 min of incubation, about 88% of the initial population was absorbed to the host cells. Based on the one-step growth curve, the burst size and latent time of CAM-21 were approximately 69 PFU/infected cell and 20 min, respectively (Fig. 5.4C).





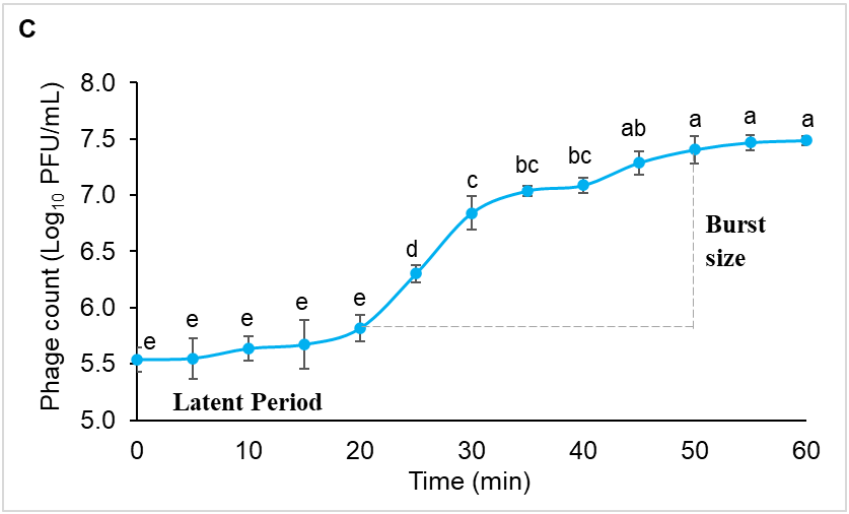


Figure 5.4 Biological evaluation of *Escherichia* phage CAM-21. Optimal multiplicity of infection (MOI) (A), phage adsorption (B), and one-step growth curve (C) of CAM-21.

5.3.7 Genomic DNA analysis

CAM-21 was found to have dsDNA with a genome size of 166,962-bp and a total GC content of 35.49%. The phage genome was predicted to encode 265 ORFs and 11 tRNAs. Based on the gene annotation results, 122 ORFs were predicted to be functional, while 143 ORFs were assumed to be hypothetical proteins with unknown functions. The putative functional ORFs were classified into four groups, such as host lysis (e.g., tail lysozyme and holin), DNA replication and nucleotide metabolism (e.g., endonuclease, exonuclease, DNA and RNA polymerase, DNA primase, DNA helicase, DNA and RNA ligase, dCMP deaminase, dUTP diphosphatase, and dCMP hydroxymethylase), phage packaging and structure (e.g., head, outer and major capsid, neck, tail fiber, tube and sheath, baseplate hub and wedge, terminase, and fibritin proteins), and additional functions (e.g., thymidylate synthase, thymidine kinase, peptidase, and glutaredoxin) (Fig. 5.5). From the similarity analysis of amino acid or nucleotide sequences using all the above-mentioned databases, the results indicated that the genome of CAM-21 did not encode toxins, virulence factors, antibiotic resistance, lysogeny and allergens.

Based on BLASTN analysis, the gene sequence of CAM-21 showed a high homology with the *Myoviridae* family phages, which include *Escherichia* phage vB_EcoM-BECP11 (accession number MW286157; 97% query coverage, and 97% identity), vB_EcoM-UFV09 (accession number MZ291552, 95% query coverage, and 97% identity), and wV7 (accession number HM997020, 96% query coverage, and 98% identity). The classification of CAM-21 was confirmed by a phylogenetic tree analysis. Based on the complete genome, CAM-21 was clustered with *Tequatrovirus* genus phages, such as RB27,

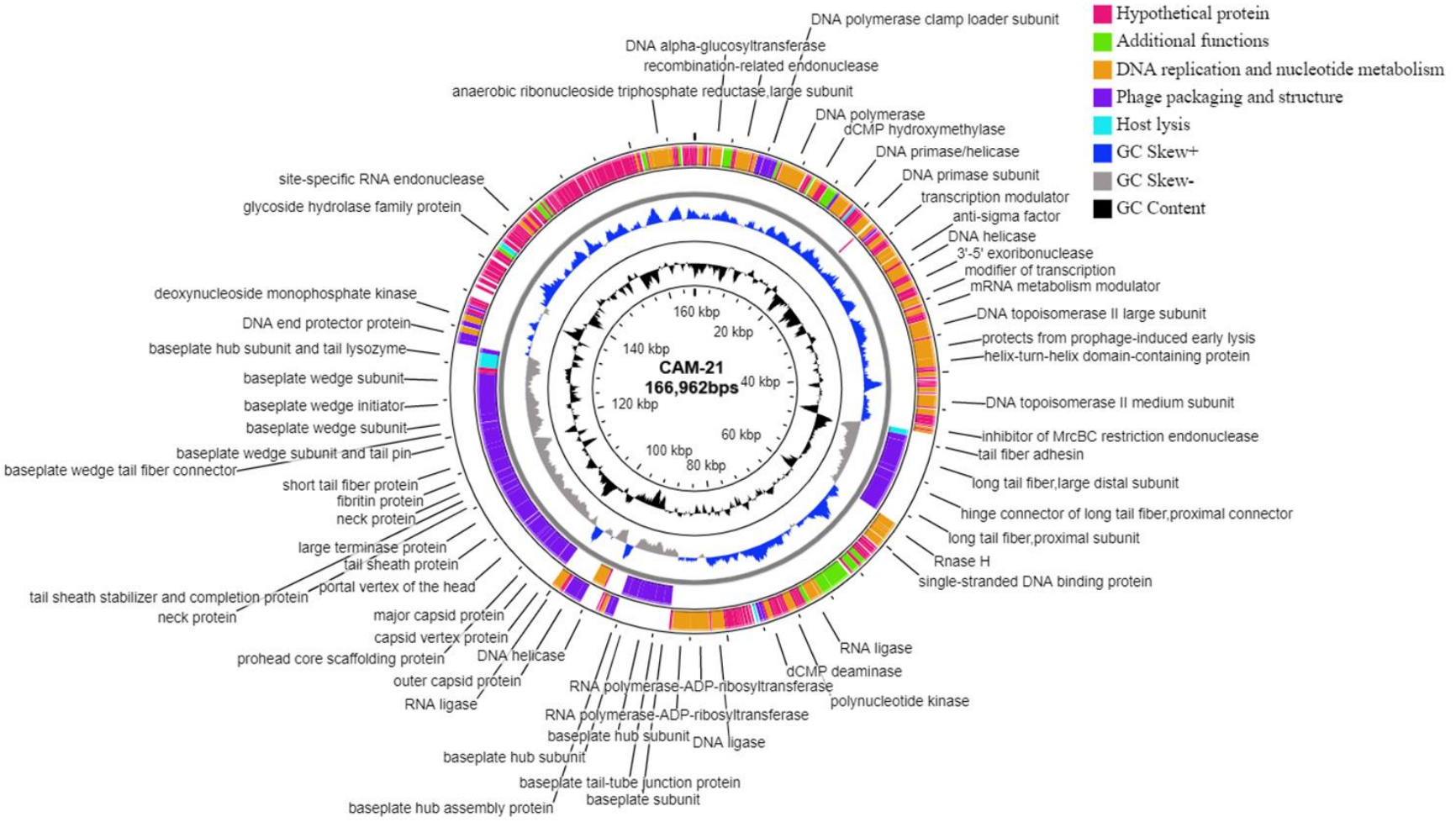


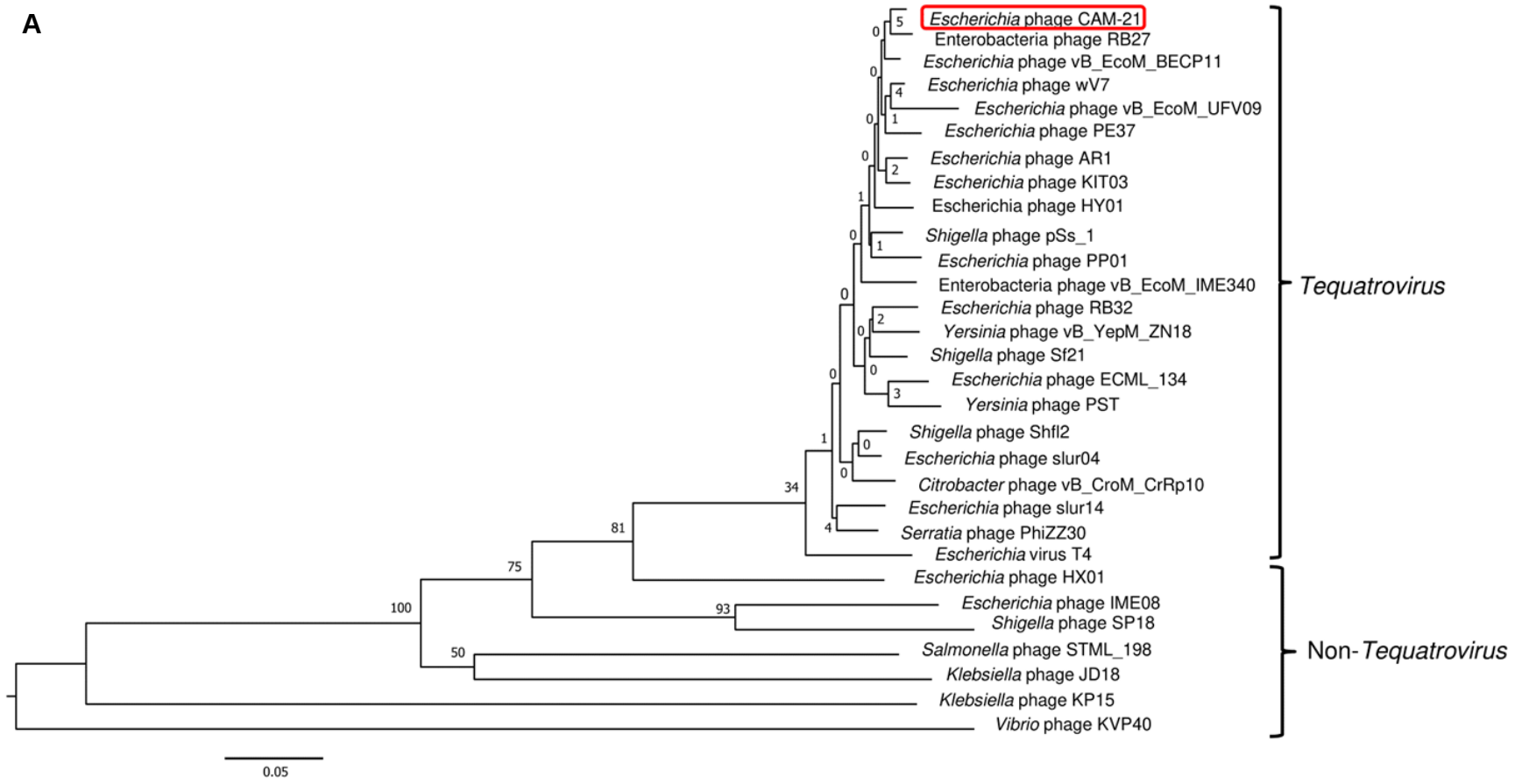
Figure 5.5 Genomic map of *Escherichia* phage CAM-21. Each color in the figure legend represents predicted open reading frames (ORF), GC skew and GC content of CAM-21.

vB_EcoM-BECP11, wV7, vB_EcoM-UFV09, and PE37 (Fig. 5.6A). Based on Fig. 5.6B, the major capsid protein of CAM-21 was closely related to those of ten *Tequatrovirus* phages. For the phylogenetic study based on the large terminase subunit, CAM-21 was clustered with eight *Tequatrovirus* phages, such as RB51, EcNP1, KIT03, wV7, AR1, vB_EcoM-BECP11, slur04, and slur02 (Fig, 5.6C), indicating that these phages have a similar DNA packaging process.

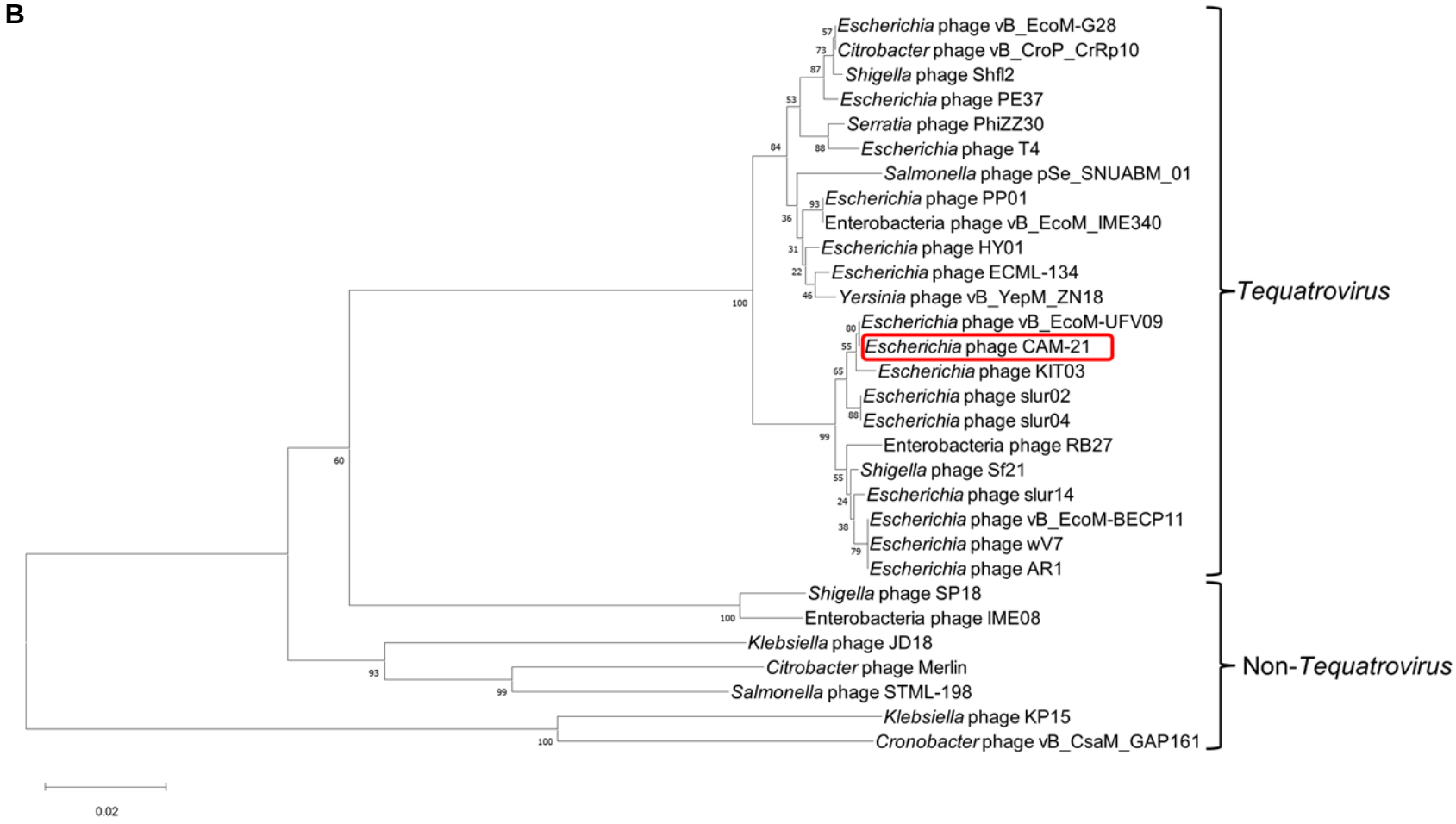
To evaluate the novelty of CAM-21, its phage DNA genome was compared to those of *Tequatrovirus* phages, such as wV7, vB_EcoM-BECP11, vB_EcoM-UFV09 and T4 (Fig. 5.7). The gene sequence of CAM-21 showed a conserved gene alignment with T4-like phages, based on the genome comparison. The gene sequences encoding the tail fiber proteins, which are responsible for host recognition were found to have high similarity (>80% identity) between CAM-21 and the selected phages, except for phage T4. As compared with other *Tequatrovirus* phages above, the long tail fiber subunit (gp37_ORF91) and the tail fiber adhesin (gp38_ORF90) of CAM-21 and vB_EcoM-BECP11 revealed the highest homology, which were 94% and 96%, respectively. Interestingly, the gp37_ORF91 of CAM-21 shared an identity of 35% while the gp38_ORF90 showed no homology, as compared to those of T4.

5.3.8 Phage applications in foods

The biocontrol effect of CAM-21 against *E. coli* O157:H7 was evaluated in milk (Figs. 5.8A-B), ground beef (Figs. 5.8C-D), and baby spinach (Figs. 5.8E-F). The food samples were first spiked with *E. coli* O157:H7 C7927 before the phage treatment. For the control group, the bacterial counts in all the foods remained almost constant after 24 h of


A
Escherichia phage CAM-21
Enterobacteria phage RB27
Escherichia phage vB_EcoM_BECP11
Escherichia phage wV7
Escherichia phage vB_EcoM_UFV09
Escherichia phage PE37
Escherichia phage AR1
Escherichia phage KIT03
Escherichia phage HY01
Shigella phage pSs_1
Escherichia phage PP01
Enterobacteria phage vB_EcoM_IME340
Escherichia phage RB32
Yersinia phage vB_YepM_ZN18
Shigella phage Sf21
Escherichia phage ECML_134
Yersinia phage PST
Shigella phage Shfl2
Escherichia phage slur04
Citrobacter phage vB_CroM_CrRp10
Escherichia phage slur14
Serratia phage PhiZZ30
Escherichia virus T4
Escherichia phage HX01
Escherichia phage IME08
Shigella phage SP18
Salmonella phage STML_198
Klebsiella phage JD18
Klebsiella phage KP15
Vibrio phage KVP40
Tequatrovirus
Non-Tequatrovirus
0.05

B


Escherichia phage vB_EcoM-G28
Citrobacter phage vB_CroP_CrRp10
Shigella phage Shfl2
Escherichia phage PE37
Serratia phage PhiZZ30
Escherichia phage T4
Salmonella phage pSe_SNUABM_01
Escherichia phage PP01
Enterobacteria phage vB_EcoM_IME340
Escherichia phage HY01
Escherichia phage ECML-134
Yersinia phage vB_YepM_ZN18
Escherichia phage vB_EcoM-UFV09
Escherichia phage CAM-21
Escherichia phage KIT03
Escherichia phage slur02
Escherichia phage slur04
Enterobacteria phage RB27
Shigella phage Sf21
Escherichia phage slur14
Escherichia phage vB_EcoM-BECP11
Escherichia phage wV7
Escherichia phage AR1
Shigella phage SP18
Enterobacteria phage IME08
Klebsiella phage JD18
Citrobacter phage Merlin
Salmonella phage STML-198
Klebsiella phage KP15
Cronobacter phage vB_CsaM_GAP161
Tequatrovirus
Non-Tequatrovirus
0.02

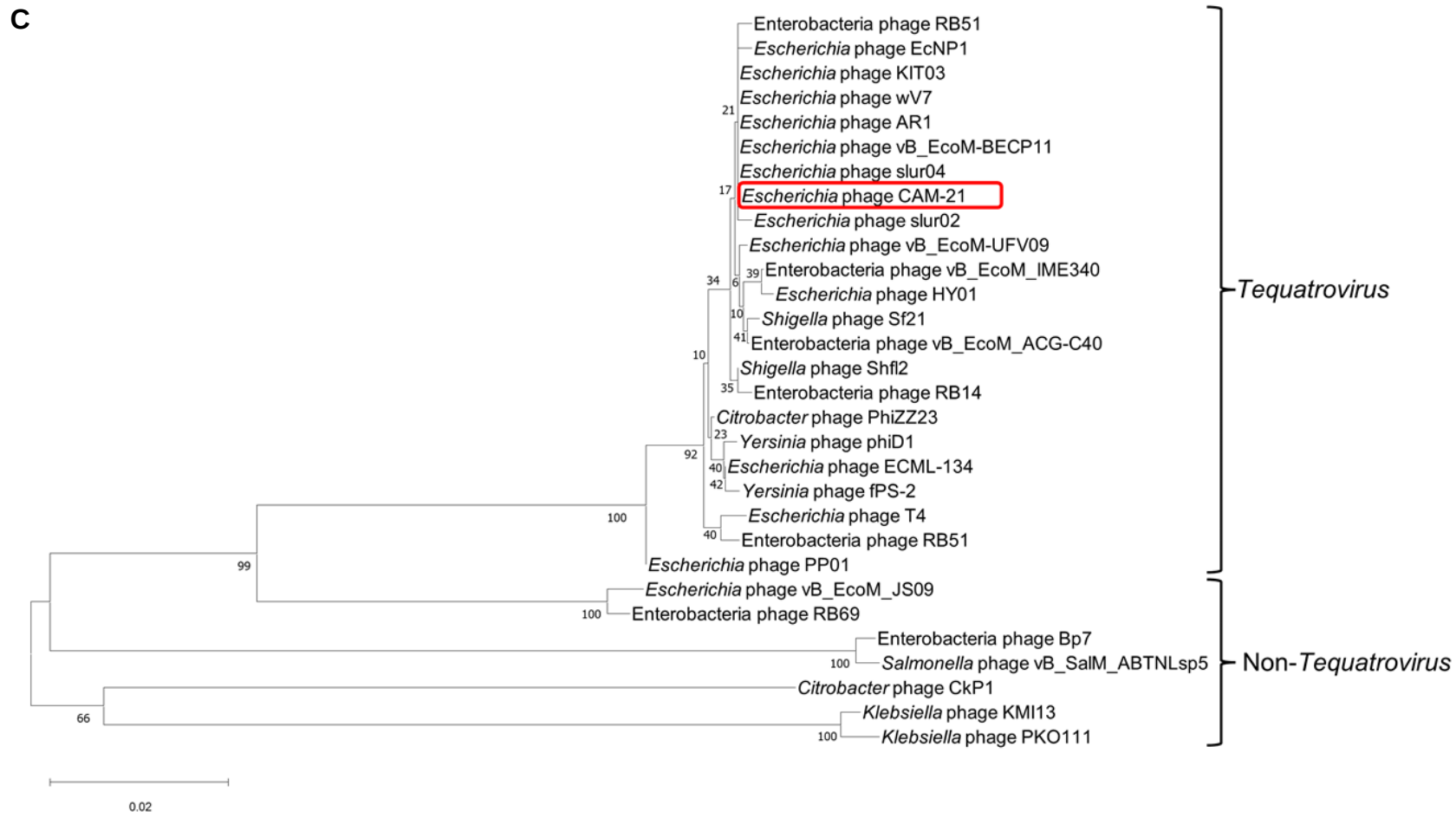


Figure 5.6 Phylogenetic tree based on complete genome sequence (A), major capsid protein (B), and terminase large subunit (C) of bacteriophages from the subfamily of *Tevenvirinae*, including *Escherichia* phage CAM-21.

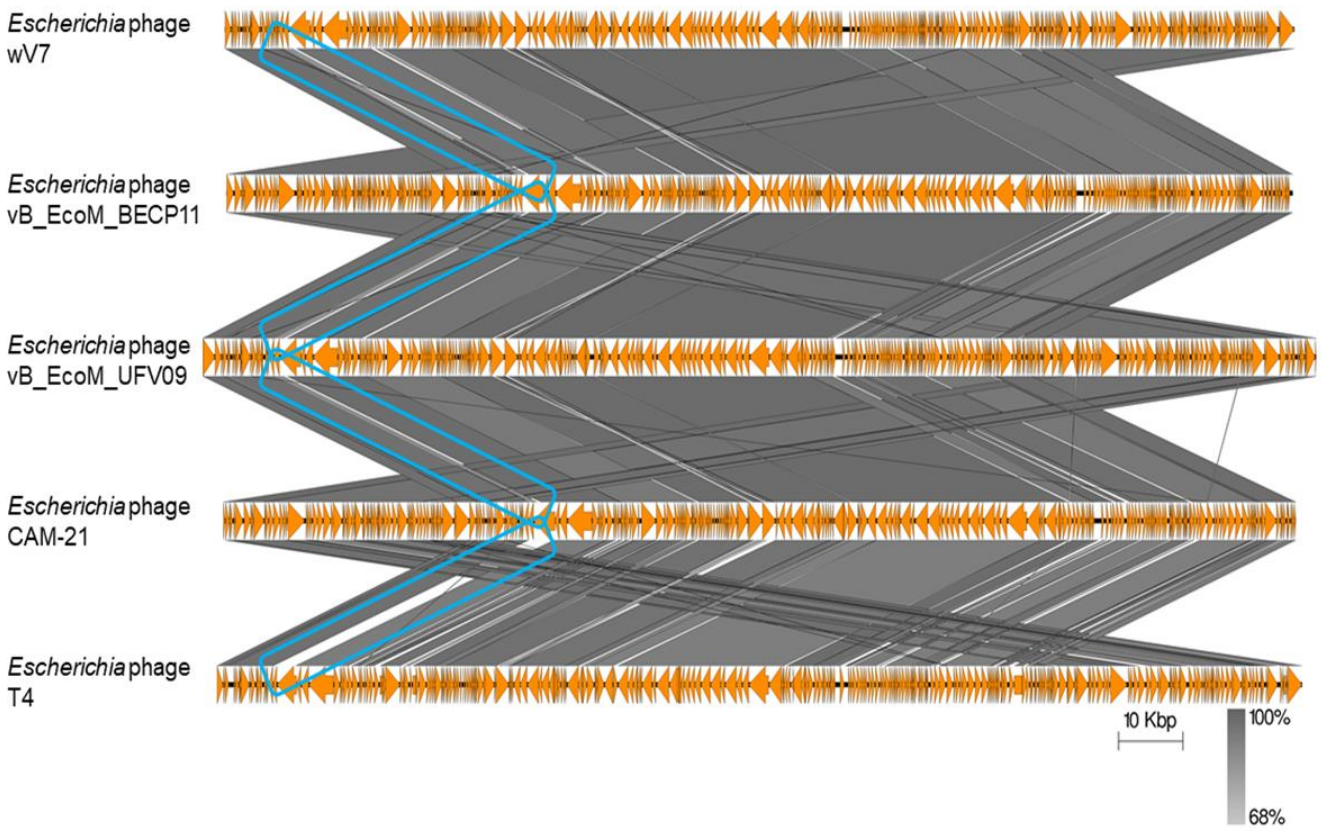


Figure 5.7 Genomic comparison of *Escherichia* phage CAM-21 with four other closely-related phages, including *Escherichia* phage wv7, vB_EcoM_BECP11, vB_EcoM_UFV09, and T4, using EasyFig. Orange-colored arrows show the open reading frames and direction of transcription. Gray shading denotes the homologous regions between the phages. Blue box indicates the homology between phage CAM-21 with other phages at the regions of gp37_ORF91 and gp38_ORF90.

incubation at 4 °C, showing only a slight reduction of less than 0.4 $\log_{10}$ CFU/mL at the tested MOIs. After 24 h of incubation, the bacterial counts in the treated milk samples were significantly decreased ($P \leq 0.05$) by 1.4 and 2.0 $\log_{10}$ CFU/mL at MOIs of 1,000 and 10,000, respectively. Also, the application of CAM-21 on ground beef and baby spinach at MOIs of 1,000 decreased the bacterial counts by 1.4 and 1.3 $\log_{10}$ CFU/g, respectively, after 24 h of storage. At a MOI of 10,000, the phage treatment significantly decreased ($P \leq 0.05$) the bacterial counts on ground beef and baby spinach by 1.3 and 1.4 $\log_{10}$ CFU/g, respectively. As shown in Fig. 5.8, treatment with CAM-21 at MOIs of 1,000 and 10,000 significantly decreased ($P \leq 0.05$) the bacterial counts in all the refrigerated food samples at all the tested time points, as compared to those of controls.

5.4 Discussion

E. coli O157:H7-related foodborne infections are a major public health problem. Various conventional control strategies, utilized during food processing, are unable to prevent foodborne outbreaks due to this pathogen and the development of antibiotic-resistant bacteria (Rubab & Oh, 2020). Due to the growing negative perceptions of chemical disinfectants among consumers, alternatives that can be found in nature are in high demand. As a result, the potential use of eco-friendly lytic bacteriophages as a safer alternative for controlling pathogenic bacterial growth in foods has received increased attentions. Despite their use in food safety and quality, bacteriophages can also be used to infect multidrug-resistant bacteria (Mangieri, Picozzi, Cocuzzi, & Foschino, 2020; Yang, Lin, Aljuffali, & Fang, 2017).

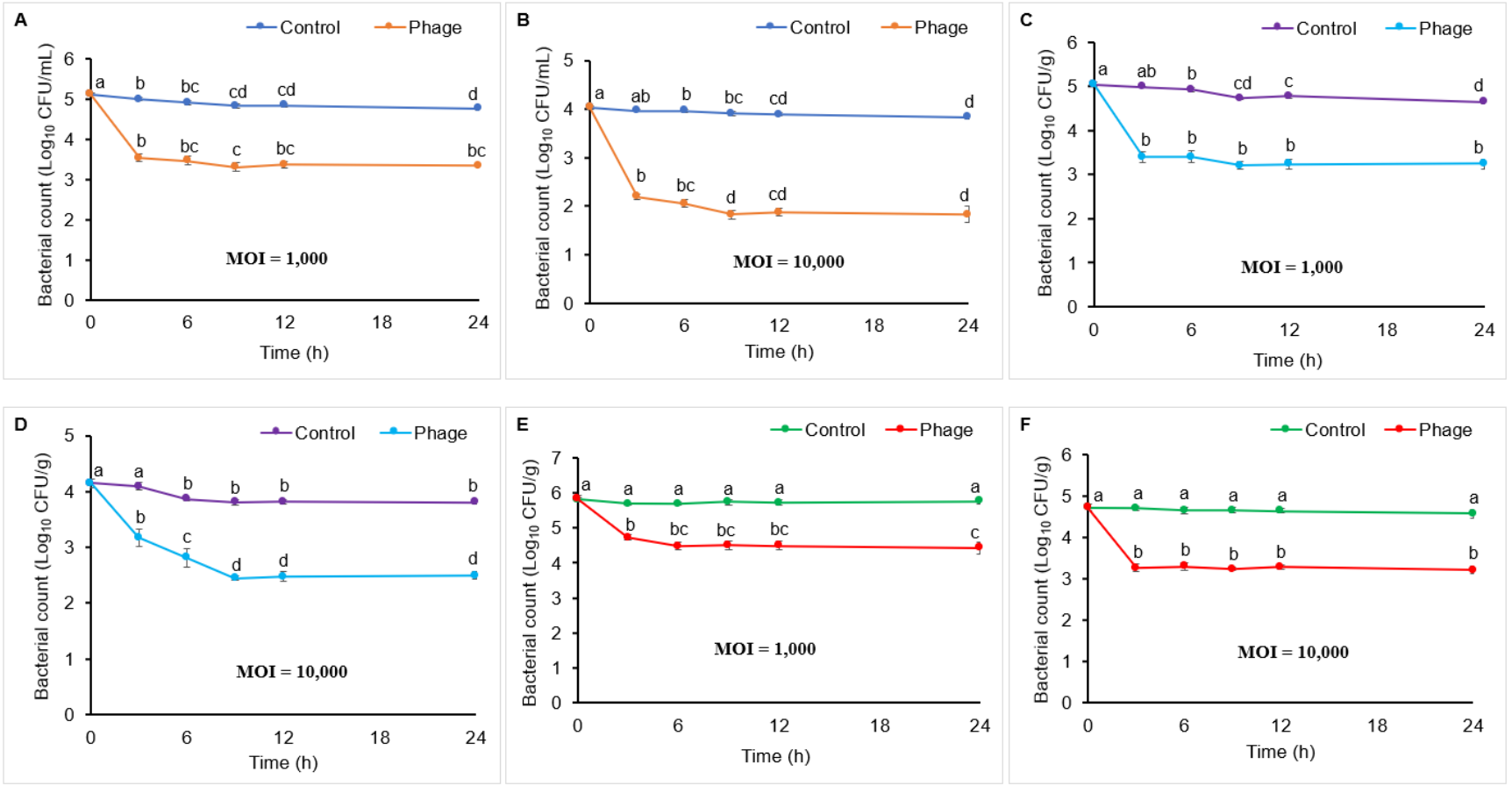


Figure 5.8 Antimicrobial effect of *Escherichia* phage CAM-21 on the survival of *E. coli* O157:H7 on refrigerated food samples, including milk (A, B), ground beef (C, D), and baby spinach (E, F), at multiplicity of infection values of 1,000 and 10,000.

In this study, a novel *Escherichia* phage, subsequently named CAM-21, was isolated, purified and characterized. CAM-21 showed high lytic activity against all tested strains of *E. coli* O157:H7, a most common cause of pediatric hemolytic uremic syndrome (Preußel, Höhle, Stark, & Werber, 2013). CAM-21 also showed lytic activities against various non-O157 STEC, such as O26, O103, and O145. The receptor binding protein of bacteriophages is essential for recognizing surface components of host bacteria, specifically the lipopolysaccharides (LPS), which are classified into the smooth and rough type (R-type). Both types of LPS contain a core and lipid A but the presence or absence of O-antigens distinguishes them, respectively. Because many Gram-negative bacteria have a well-conserved LPS core structure, T4-like bacteriophages that target the R-type LPS may have a broader host spectrum (Nobrega et al., 2018; Rakhuba, Kolomiets, Dey, & Novik, 2010). Similarly, phage vB_EcoM-ECP26 was found to have a broad host spectrum against different serogroups of STEC, including O157 strains (Park, Lim, Lee, & Park, 2020). The TEM micrograph of CAM-21 showed several distinctive morphological features, including a visible collar and a contractile tail, suggesting that this phage belongs to the *Myoviridae* family (Nobrega et al., 2018).

In addition, because only temperate phages use their lysogenic ability to form turbid plaques, the formation of clear plaques on the bacterial lawn suggested that CAM-21 is a lytic bacteriophage (Jurczak-Kurek et al., 2016). As observed by TEM, the infection mechanism of host bacteria by CAM-21 was identical to that of T4 phage. A similar infection process was also observed by Zhang et al. (2013) using a T4-like *Escherichia* phage Bp7. The bacterial lysis cycle was initiated by the attachment of phage long tail fibers to receptors on the cell surface. To allow the successful penetration of DNA into the

bacterial cell, at least three phage tail fibers must be attached to the cell to allow the conformation changes of the tail fibers (Rakhuba et al., 2010). During the DNA packaging step, the progeny phages were assembled into packages in the cell. Meanwhile, holin was expressed and accumulated in the cell membrane without affecting the membrane structure. After a threshold amount of holin was produced, its structure was altered, followed by the formation of mature holes on the cell membrane, which allows the release of progeny phages (Bavda & Jain, 2020).

To be utilized as a biocontrol agent in foods, bacteriophages should have a strong lysis capability. The effectiveness of CAM-21 was evaluated based on its lytic activity against *E. coli* O157:H7 at different MOIs. CAM-21 was able to suppress bacterial growth at a low MOI (e.g., 0.001), indicating that bacterial growth can be controlled at a reduced amount of the phage. For food applications, a lower MOI was found to be more cost-effective and beneficial (Kim, Adeyemi, & Park, 2021). However, the bacteria were found to regrow after 24 h of incubation at MOI of 0.0001. This could be associated with the formation of bacteria with bacteriophage-insensitive mutants (BIM), resulting in the transduction of antibiotic resistance or virulence genes (Park & Park, 2021). The selection for BIM can produce safety issues for phage applications (Pinto, Almeida, & Azeredo, 2020). At a MOI $\geq$ 0.001, the inhibition effect of CAM-21 against the bacteria was maintained for the entire incubation time (24 h), which was longer than that of *Myoviridae* phages, such as phage BECP10 (10 h) (Park & Park, 2021), KFS-EC (8 h) (Lee, Choi, Park, & Park, 2020), and HY01 (1 h) (Lee et al., 2016). Therefore, it can be concluded that CAM-21 may be a promising biocontrol agent, particularly for application in food products that are processed and can be preserved for a long time.

Two important factors in determining the effectiveness of bacteriophage as a biocontrol agent are the phage adsorption and one-step growth analysis. The results showed that about 80% of CAM-21 were adsorbed to the host bacteria after 3 min of incubation, indicating that the adsorption of CAM-21 occurs rapidly. The growth curve revealed that CAM-21 had a burst size of 69 PFU/infected cell, which was within the typical value (50-100/infected cell) for most *Myoviridae* phages (Litt & Jaroni, 2017). CAM-21 had a relatively shorter latent time (20 min) than that of other *Myoviridae* phages, including vB_EcoM-ECP26 (55 min) (Park et al., 2020), AR1 (40 min) (Goodridge, Gallaccio, & Griffiths, 2003), HY01 (25 min) (Lee et al., 2016), and KFS-EC (30 min) (C. Lee et al., 2020), suggesting that the phage was able to lyse the host bacteria in a limited time. The results indicated that the phage has strong lytic properties, suggesting that it could be utilized as a biocontrol agent in foods (Park et al., 2020).

Based on WGS, gene annotation and genomic comparative analysis, CAM-21 was classified into the *Tequatrovirus* genus. The classification of CAM-21 in this genus was further confirmed by the phylogenetic analysis with other genera within the *Tevenvirinae* subfamily. The size, GC content and number of ORFs of CAM-21's genome were almost identical to those of other *Tequatrovirus* phages (Pham-Khanh et al., 2019). However, The GC content of CAM-21 (35.49%) was lower than that of the host bacteria (about 50%). This could be due to the limited availability of guanine and cytosine and increased metabolic stress for the formation of guanine and cytosine than adenine and thymine (Niu et al., 2012). Genomic DNA sequencing of CAM-21 was also used to provide information about its infection mechanism and other characteristics. For instance, the genome of *Escherichia* phage vB_EcoM-BECP11 had a high similarity with that of CAM-21. The

BLASTP and genomic comparative analysis showed slight differences between their gene sequences that encode a long tail fiber subunit (gp37_ORF91) and tail fiber adhesin (gp38_ORF90). However, the gp37_ORF91 and gp38_ORF90 of CAM-21 showed lower similarity with those of T4. Therefore, these phages may infect different host bacteria due to these similarities and diversity (Duc et al., 2020). The findings were confirmed by the phage morphology (Fig. 5.2A), which showed the distinctive structures, such as the complex tail fiber and spike structures. The obtained results showed that CAM-21 is a new species in the *Tequatrovirus* genus of the *Myoviridae* family due to the high homology with other phages of this genus.

The safety of CAM-21 as a biocontrol agent were also evaluated by genomic analysis. Based on the obtained results, genes that encode toxins, virulence factors, antibiotic resistance, lysogeny (e.g., exicionase and integrase), and food allergens were not found in CAM-21's genome. For food applications, temperate phages are not desirable because they can deliver harmful genes and improve host immunity by lysogenic conversion (Park & Park, 2021). Thus, lytic bacteriophage, such as CAM-21, are more effective and safer to be used as a biocontrol agent in food products than temperate phages.

In recent decades, *E. coli* O157:H7 has been associated with numerous outbreaks in foods, such as dairy products, ground beef and fresh produce (Denny, Bhat, & Eckmann, 2008; Parker et al., 2012; Vogt & Dippold, 2005). Thus, the effectiveness of CAM-21 in controlling this pathogen in foods, such as milk, ground beef, and baby spinach was evaluated. For all the untreated food samples, the bacterial count remained constant during 24 h of incubation at 4 °C. Similar trends of bacterial count were also observed by Li et al. (2021) and Park et al. (2020) using refrigerated foods, including milk, raw beef and

romaine lettuce after 24 h of incubation. The bacteria can survive at the refrigerated temperature, but their growth can be restricted, resulting in the inhibition of the infection cycle (Duc et al., 2020). Nevertheless, phage adsorption and host lysis can happen at refrigerated temperatures. The results revealed that CAM-21 significantly ($P \leq 0.05$) decreased the bacterial count in all the refrigerated foods tested when compared with untreated samples. Li et al. (2021) also reported similar results that the *Escherichia* phage JN01 decreased the bacterial count by 1.3 $\log_{10}$ CFU/mL and 2.3 $\log_{10}$ CFU/cm^2 in refrigerated milk and beef samples, respectively, after 1 day of storage at a MOI of 10^5. The use of a phage suspension at a higher concentration (10^9 PFU/mL) may lead to a considerable decrease in microbial count due to the increased chances of interaction between phage and host bacteria (Li et al., 2021). Also, the reduction in bacterial count could be due to host lysis using the "lysis from without (LO)" mechanism with the help of a baseplate hub subunit and tail lysozyme (CAM-21_ORF187) (Duc et al., 2020). When a large number of phage particles is attached to a host's cell surface, it may cause breakage of the host cell wall, leading to cell lysis. Several studies have reported the biocontrol activity of *Escherichia* phages against the pathogenic bacteria on various refrigerated food samples using the LO mechanism (Park et al., 2020; Park & Park, 2021; Yıldirim, Sakin, Akçelik, & Akçelik, 2021). In fact, foodborne pathogens are usually present at relatively low concentration in foods, therefore host bacteria may be completely killed prior to the development of phage resistant cells in refrigerated temperatures (Duc et al., 2020).

5.5 Conclusions

In summary, a broad-spectrum *Escherichia* lytic phage, CAM-21, isolated from the natural environment, was demonstrated to possess a strong lytic activity against STEC, particularly *E. coli* O157:H7. Morphology and genome analyses indicated the classification of CAM-21 under the *Tequatrovirus* genus of the *Myoviridae* family. Genomic comparative analysis showed that CAM-21 is a novel species in this genus. Genes associated with toxin production, virulence, antibiotic resistance, lysogeny and allergens were not found in the genome of CAM-21. CAM-21 had a relatively shorter latent time, indicating its potential use as a biocontrol agent in foods. This was demonstrated when it significantly ($P \leq 0.05$) decreased the bacterial count of *E. coli* O157:H7 in the tested refrigerated foods, milk, ground beef and baby spinach. Thus, CAM-21 is a potential candidate that could be used as a biocontrol agent in foods, especially in fresh produce, meat, and dairy products.

Chapter 6 Preparation and Characterization of Soy Protein Isolate-Based Films Incorporated with Bacteriophages for Antimicrobial Food Packaging Application

6.1 Introduction

In recent decades, consumers have become increasingly concerned about food safety and quality because of relentless outbreaks of foodborne illnesses, are acquired from consumption of contaminated foods. Pathogenic bacteria can easily contaminate foods during handling, processing, distribution and storage. (Choi et al., 2021). Shiga toxin-producing *Escherichia coli* (STEC) is one of the common pathogens related to foodborne outbreaks worldwide, causing clinical illnesses, including diarrhea and hemolytic uremic syndrome (Tomat et al., 2019). STEC-associated foodborne disease outbreaks from raw and cooked meat products have been widely reported in recent years (Omer et al., 2018). Although the food industry has implemented various control measures, they are still insufficient to prevent and reduce foodborne infection outbreaks. The misuse of antibiotics in the food industry has also led to the formation of antibiotic-resistance genes in foodborne pathogens, posing a threat to human health (Mir & Kudva, 2019).

Hence, increased consumer health concern has generated a greater interest in the development of antimicrobial packaging, which can inhibit microbial growth on food surfaces, hence, improving the safety, quality and shelf life of package foods (Roy & Rhim, 2020). Several types of antimicrobial agents have been used to develop antimicrobial packaging materials. They mainly include synthetic antimicrobials, including organic acid (da Rocha et al., 2014; Sözbilen et al., 2022), nanoclays (Yahiaoui et al., 2015), metal and metal oxide nanoparticles (Cano et al., 2016; Li et al., 2017), and natural antimicrobials,

such as essential oils (EO) (Perdana et al., 2021; Priyadarshi, Kumar, Deeba, et al., 2018), plant extracts (da Rosa et al., 2020; Martins et al., 2018), bacteriocins (Shiroodi et al., 2016; Woraprayote et al., 2018) and enzymes (Min et al., 2005). Bacteriophages, a group of viruses that can infect and lyse specific bacteria, have been known as one of the most effective antimicrobials for controlling pathogenic bacteria in the food and agricultural industries (Alves et al., 2019). They have received increased attention from academic and industrial food researchers due to their unique characteristics, which include low toxicity, low cost, ease of production, wide availability and long shelf life (Alves et al., 2019). Bacteriophages can infect certain species of harmful bacteria in foods without affecting beneficial bacteria, may be responsible for the organoleptic properties of foods (López de Dicastillo et al., 2021).

Bacteriophages can be directly utilized on solid food surfaces via different methods, including brushing, dipping or spraying. They have been utilized as antimicrobial agents against STEC in a variety of food products, such as milk and meat (Li et al., 2021; Tomat et al., 2018), mung bean seeds (Liao, Zhang, Salvador, Harden, & Wu, 2022), romaine lettuce (Park et al., 2020) and burger patties (Park & Park, 2021). However, the direct addition techniques may have a negative impact on the antimicrobial activity of bacteriophages because of their low stability in food environments and uncontrolled antimicrobial release (Alves et al., 2019; Costa et al., 2021). Hence, novel solutions are required to overcome these issues. For instance, bacteriophages can be incorporated into food packaging materials for an enhanced stability and a more controlled release.

Soy protein isolate (SPI) has been widely used for the development of biodegradable food packaging materials because of their wide availability, low cost and toxicity, biodegradability, renewability, excellent gas barrier properties and high protein level (Yu et al., 2018). Because of the inherent hydrophilicity and strong molecular interaction of natural protein, native SPI films do not exhibit sufficient tensile and water vapor barrier characteristics for commercial uses (Song, Tang, Wang, & Wang, 2011). In addition, SPI-based packaging films incorporated with nanofillers (e.g., nanocellulose, metal and metal oxide nanoparticles) and active agents (e.g., synthetic and natural antimicrobial agents) have been reported to exhibit enhanced tensile, barrier, thermal, antioxidant and antimicrobial properties (Han, Yu, & Wang, 2018a; Y. Han et al., 2018b; Tao et al., 2022; Xiao et al., 2020; Yu et al., 2018).

In recent decades, many studies have shown that antimicrobial packaging materials, such as sodium alginate (Alves et al., 2019), polycaprolactone (PCL) (Choi et al., 2021), whey protein concentrate (WPC) (Tomat et al., 2019), whey protein isolate (WPI) (Vonasek et al., 2014), gelatin (Weng et al., 2021), acetate cellulose (Gouvêa, Mendonça, Soto, & Cruz, 2015) sodium alginate/gelatin blend, polyvinyl alcohol (PVOH) and sodium caseinate (López de Dicastillo et al., 2021), incorporated with bacteriophages, exhibited antimicrobial properties against various pathogenic bacteria, such as *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Listeria monocytogenes*. Although previous studies have reported the development of antimicrobial packaging materials containing bacteriophages, no studies have described the use of SPI-based films incorporated with bacteriophages for antimicrobial food packaging applications. Hence, the objective of this work was to develop novel SPI-based food

packaging films incorporated with a novel phage (*Escherichia* phage CAM-21) that targets STEC. Color, morphological, mechanical, water vapor barrier, and optical properties of the edible films, phage distribution in the film matrix, and their antimicrobial activity against *E. coli* O157:H7 in broth and raw beef samples were evaluated.

6.2 Materials and methods

6.2.1 Materials and bacterial strains

Pro-Fam® 873 isolated soy protein was obtained from the Archer-Daniels-Midland (ADM) Company (Decatur, IL). A cellulose nanofiber (CNF) suspension was made from the powder form, purchased from the University of Maine, that was produced using bleached wood pulp. Glycerol was purchased from Fisher Scientific (Waltham, MA). Fresh raw bottom round beef and raw beef trimmings were obtained from Meat Market of the University of Missouri (Columbia, MO, USA). *E. coli* O157:H7 strains 3178-85, EDL-933, C7927, 505B and MF-1847 were obtained from the University of Missouri, Food Microbiology Lab culture collection. All bacterial strains were utilized to evaluate the films' antimicrobial activity in broth and beef samples, and *E. coli* O157:H7 strain C7927 was utilized as the host bacteria to produce a phage stock.

6.2.2 Production of bacteriophage stock

One hundred microliters of purified phage solution were mixed with 1 mL of saturated bacterial culture (~10^9 CFU/mL) in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA), and the mixture allowed to sit for 15 min at 37 °C. The phage/bacterial suspension was added into 10 mL of TSB and cultured in a shaker-incubator with shaking

at a speed of 200 rpm for 24 h at 37 °C. After centrifuging at 11,739 × g for 15 min, the suspension was filtered using a PES filter membrane (pore size = 0.22 µm; Merck Millipore, Cork, Ireland), and left overnight at 4 °C. The phage pellet was precipitated by centrifuging at 44,367 × g for 15 min at 4 °C using an ultracentrifuge (Sorvall^{TM} WX+ 80, Thermo Fisher Scientific, Waltham, MA). The phage count was determined using the double-layer agar method.

6.2.3 Film preparation

Film-forming solution (FFS) was prepared by mixing 6% (w/w) SPI and 3% (w/w) glycerol in sterile distilled water at 800 rpm. The pH of the solution was adjusted to 10.0 using 5 M sodium hydroxide solution, followed by heating at 85 °C for 30 min while stirring at 800 rpm. After the solution was cooled down to room temperature, 15% (w/w) CNF, based on the dry weight of SPI, was added, and stirred for 15 min. 1%, 2% and 4% (v/w) CAM-21, based on the weight of FFS, was incorporated into the solution and gently stirred for 15 min. The films were synthesized by solution-casting the FFS into sterile square-shaped 12 cm^2 petri dish and drying at 30 °C for 48 h. A film sample without the incorporation of CAM-21 was used as the control. Four different film samples were prepared, including a SPI film without CAM-21 (S), a SPI film with 1% of CAM-21 (SC1), a SPI film with 2% of CAM-21 (SC2) and a SPI film with 4% of CAM-21 (SC4).

6.2.4 Bacteriophage titer after incorporation in the films

The bacteriophage titer after being incorporated in the films was evaluated according to an earlier technique with some modifications (Alves et al., 2019). Film

samples (1.5 × 1.5 cm^2) were placed in a 12-well plate. 1 mL of saline magnesium (SM) buffer was added into each well and stirred in a shaker incubator at 150 rpm for 3 h at room temperature. The phage count was determined using the double-layer agar method.

6.2.5 Bacteriophage distribution within the films

The bacteriophage distribution within the films was studied based on an earlier technique with some modifications (Alves et al., 2019). A spectral confocal microscope (Leica TCS SP8, Leica Microsystems Inc., Buffalo Grove, IL) was used to observe the fluorescently labelled samples. One microliter of 100 × SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) was added to 0.5 mL of CAM-21 suspension (~10^9 PFU/mL), and the mixture was incubated for 1 h in the dark. The labelled phage suspension was spun down using Illustra MicroSpin G-25 columns (GE Healthcare, Chicago, IL) equilibrated with SM buffer to eliminate excess fluorophores. The labelled phages were incorporated on the film forming solution, as mentioned in Section 6.2.3, and casted in sterile confocal dishes at 30 °C for 48 h. The labelled phages suspension (20 µL) was placed on a glass side and covered with a cover slip. The fluorescent images of labelled phages in the suspension and film samples were obtained using confocal microscopy.

6.2.6 Scanning electron microscopy (SEM)

The microstructure properties of the films were studied using environmental SEM (Quanta 600 FEG; Thermo Fisher Scientific, Waltham MA, USA). The films were placed

on aluminum stubs. Then, the mounted samples were coated with gold before observing using SEM.

6.2.7 Color and opacity

The film color was measured using a colorimeter (CR-410, Konica Minolta Sensing, Inc., Japan). The total color difference (ΔE) of the films was determined based on the L*, a*, and b* values measured. Light transmittance of the films was measured using an UV-Vis spectrophotometer (UV-1900i, Shimadzu Corp., Canby, OR). The transmittance of 0.9 cm × 4.0 cm films at 600 nm was recorded. A test cell without the films was used as the blank. The film opacity was determined based on an earlier method (Yu et al., 2019) using the following equation:

$$Opacity = \frac{-\log(Trans_{600})}{K}$$

where $Trans_{600}$ is the transmittance of the films at 600 nm and K is the film thickness (mm) determined at five different locations using a digital electronic caliper (Model 35-025; iGAGING, San Clemente, CA, USA).

6.2.8 Tensile properties

Tensile studies were conducted using a texture analyzer (TA-HDplusC, Texture Technologies Corp., South Hamilton, MA). Film samples were cut into 1 cm × 5 cm rectangular strips before subjecting them to the tensile measurement. The crosshead speed and initial grip separation were set at 0.5 mm/s and 25 mm, respectively.

6.2.9 Water vapor permeability (WVP)

WVP of the film samples was studied based on an earlier method (Wu et al., 2019). First, 15 g of anhydrous $CaCl_2$ were added into a beaker, which was covered by the film samples and sealed using parafilm. The wrapped beaker was placed in a (75% RH at 25°C) desiccator that contained saturated NaCl solution. For up to 12 h, the weight of the beaker was monitored at various intervals. WVP was measured based on the equation:

$$WVP = (\Delta M \times K)/(\Delta P \times t \times S)$$

where ΔM is the weight variation (g), K is the film thickness (m), ΔP is the vapor pressure difference across the film (Pa), t is the time, and S is the vapor exposed area under test (m^2).

6.2.10 *In vitro* antimicrobial activity

The antimicrobial activity of the films on *E. coli* O157:H7 (strains 3178-85, EDL-933, C7927, 505B and MF-1847) cocktail was evaluated in TSB. *E. coli* O157:H7 strains were cultured in TSB at 37 °C for 24 h. All strains were added in a 1:1:1:1:1 CFU/mL ratio to obtain the bacterial cocktail. Then, the bacterial cocktail was diluted to obtain concentrations of about 10^4 CFU/mL and transferred into sterile test tubes with 5 mL in each tube. Two pieces of each film sample in a dimension of 1 cm × 5 cm were placed in the test tube. The bacterial culture with no film was used as a control. Samples were incubated in a shaker incubator with shaking at 150 rpm and 37 °C. The antimicrobial activity of the edible film was evaluated by conducting a plate count of each sample using MacConkey Sorbitol agar (Difco Laboratories, Detroit, MI, USA) at 0, 3, 6, 9, 12 and 24 h. Total viable counts of the bacteria were determined and expressed in log CFU/g values.

6.2.11 Food application

The antimicrobial activity of the films on *E. coli* O157:H7 (strains 3178-85, EDL-933, C7927, 505B and MF-1847) cocktail was evaluated using fresh raw bottom round beef and raw beef trimmings as the food models. *E. coli* O157:H7 strains were cultured in TSB at 37 °C for 24 h. All strains were added in a 1:1:1:1:1 CFU/mL ratio to obtain the bacterial cocktail. The culture cocktail was then centrifuged at 12,745 ×*g* (10,000 rpm) for 5 min and washed with peptone water. The procedure was repeated for two additional times. Beef samples were cut into 1.5 cm^3 pieces. Fifty microliters of 10^6 CFU/mL bacterial cultures were added and inoculated on the surface of each food sample with a pipet tip. The spiked foods were left under a laminar hood for 15 min to allow for bacterial attachment. Then, 1.5 cm^2 of films were placed onto the inoculated surface of the samples. The sample without film treatment was used as a control. The food samples were then placed in a refrigerator at 4 °C and 25% RH and collected after 0, 1, 3, 5 and 7 days of storage. On each sampling day, the beef samples were placed in sterile stomacher bags, followed by the addition of 90 mL of peptone water (0.1% (w/v)). The samples were homogenized using a Stomacher™ Circulator Model 400 (Seward, Ltd., UK). The serially-diluted homogenates were plated on MacConkey sorbitol agar plates and incubated for 24 h at 37 °C. Total viable counts of the bacteria were determined and expressed in log CFU/g values.

6.2.12 Statistical analysis

All experiments were conducted at least thrice with duplicate (beef studies) or triplicate (all other studies) samples. The collected data were expressed as means

± standard deviations. Data analyses were performed through one-way ANOVA and Tukey's test was conducted by Minitab software (Version 19). A significant difference between mean values was represented by $P \leq 0.05$.

6.3 Results and Discussion

6.3.1 Incorporation and distribution of bacteriophages in the films

The survival capability of bacteriophages in the polymer matrix is one of the important factors in producing phage-based antimicrobial packaging materials for food applications. CAM-21 containing SPI films were developed by incorporating phage suspension in a SPI-based FFS containing CNF and glycerol, as additives. The phage titers found in SC1, SC2 and SC4 were $1.02 \pm 0.04 \times 10^7$ PFU/cm^2, $1.41 \pm 0.16 \times 10^7$ PFU/cm^2 and $2.41 \pm 0.43 \times 10^7$ PFU/cm^2, respectively. The results show that the phages remained stable in the polymer matrix because SPI films offer an ideal environment for phages. The stability of phages can be improved by avoiding and reducing the oxidative damage to viral nucleic acid components (e.g., RNA and DNA) and capsid and stabilizing the structure of viral capsid proteins (Vonasek et al., 2014). For instance, because of their ordered and compact network structure, protein-based films have good oxygen barrier properties that may reduce oxidative damage (Y. Han et al., 2018a). The presence of a plasticizer (e.g., glycerol) and high protein contents, which may stabilize the viral capsid after film drying, may also contribute to phage survival (Vonasek et al., 2014).

A confocal microscopy was utilized to evaluate the spatial distribution of incorporated phages (green) in the films (Fig. 6.1). The phage particles were labelled with SYBR^{TM} Gold stain before incorporating in the SPI films (Fig. 6.1A). The uniform

distribution of phages across the entire surface and depth of the polymer matrix is shown in Fig. 6.1B. Also, phage agglomeration results in the formation of small aggregates, which could be associated with the film drying method or the presence of net opposite charges in the phages' tail and head regions. Similar observations were also reported by Alves et al. (2019) and Vonasek et al. (2014) when using sodium alginate and WPI films, respectively.

6.3.2 Film microstructure

A SEM was utilized to evaluate the surface morphology of different film samples (Fig. 6.2). The control films were homogeneous and compact without phase separation, and the film surface was smooth with the presence of few cracks (Fig. 6.2A). The incorporation of phage particles into the films did not induce apparent phage separation, suggesting their good compatibility in the polymer matrix. However, some pores (shown by the red arrows) were observed (Fig. 6.2B, C). Based on the SEM micrographs, the addition of phage particles had little effect in the film microstructure. Similar results were also observed by Weng et al. (2021) and López de Dicastillo et al. (2021) in various polymer films, such as PVOH, gelatin, sodium caseinate and sodium alginate/gelatin, incorporated with bacteriophages.

6.3.3 Color and opacity

Film color and opacity are important indicators of food packaging materials. As shown in Table 6.1, the film samples showed a yellowish color (large positive b* value), with a great film lightness (high L* value). The incorporation of colorless CAM-21 had no significant effect on the lightness, redness and yellowness of the S film. The ΔE of phage-

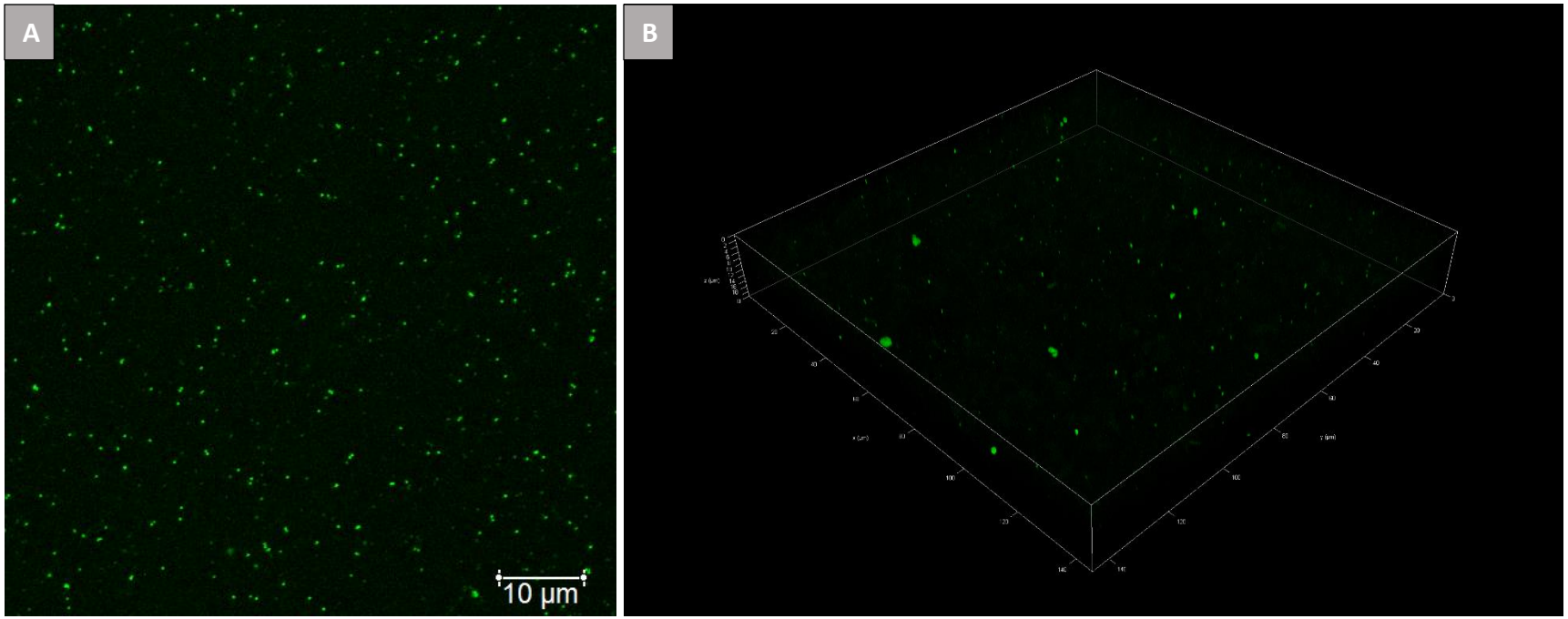


Figure 6.1 Confocal microscopic images of SYBR™ Gold-labelled CAM-21 in the SM buffer (A) and SPI films (B).

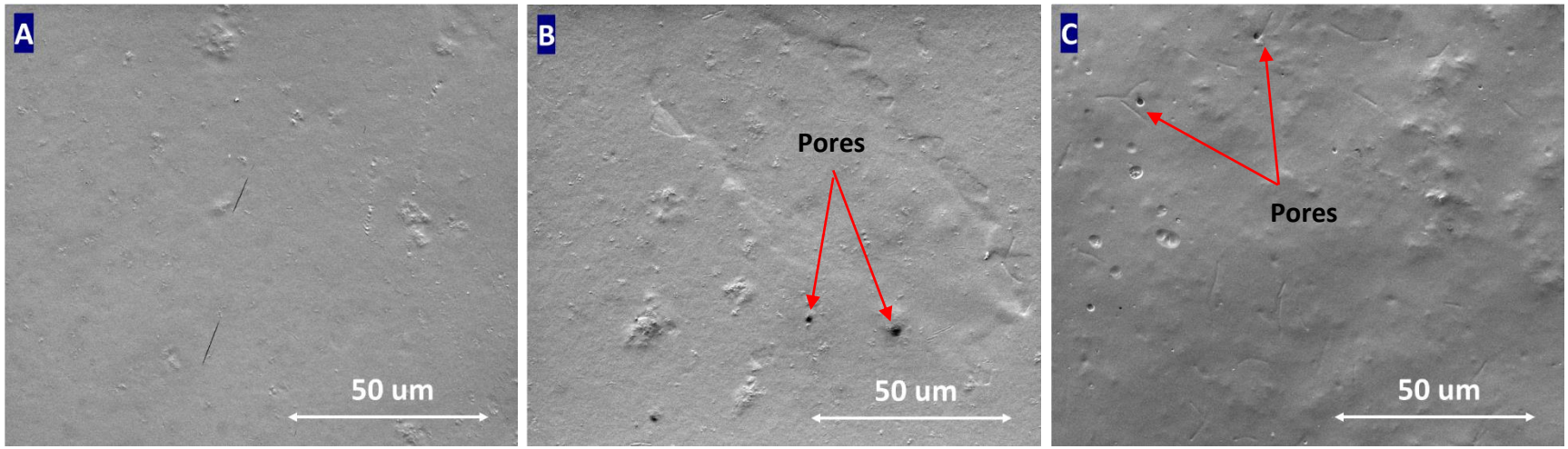


Figure 6.2 SEM observations of the surfaces (A: 0% phage, B: 1% phage and C: 4% phage) of the films. Scale bar: 50 µm. Magnification: 1,000×.

containing SPI films had no significant different with that of the control films. In addition, all the film samples were visually clear and transparent. The incorporation of bacteriophages in the packaging materials slightly increased the film opacity. However, no significant difference in the opacity of SPI films containing different concentrations of bacteriophages was observed. These results confirm that the incorporation of bacteriophages did not significantly affect the optical properties of the SPI films. Similar results were also reported by Weng et al. (2021).

Table 6.1 The influence of incorporated CAM-21 on film color and opacity.

Sample	L*	a*	b*	ΔE	Opacity
S	87.68 ± 1.04^{a}	-1.99 ± 0.06^{a}	20.20 ± 1.68^{a}	16.93 ± 1.90^{a}	1.59 ± 0.10^{a}
SC1	87.97 ± 0.42^{a}	-1.96 ± 0.02^{a}	20.37 ± 0.92^{a}	17.00 ± 0.79^{a}	1.61 ± 0.17^{a}
SC2	86.77 ± 0.80^{a}	-2.02 ± 0.05^{a}	22.11 ± 1.25^{a}	19.01 ± 1.43^{a}	1.68 ± 0.04^{a}
SC4	86.15 ± 0.79^{a}	-2.05 ± 0.07^{a}	22.43 ± 0.89^{a}	19.54 ± 1.11^{a}	1.69 ± 0.09^{a}

Values with the same superscripts in each column were not significantly different ($P > 0.05$).

*The values are given as means ± standard deviation (n = 3).

xΔE: Color change.

6.3.4 Tensile properties

The tensile properties of SPI-based films are shown in Table 6.2. After the incorporation of CAM-21 (1-4%), there was no significant variation in the TS and EAB of the film samples. The results suggest that the bacteriophage concentration utilized in these films were still insufficient to have any negative impact on the film strength and flexibility.

A study has reported that the incorporation of bacteriophages did not significantly affect the tensile properties (e.g., puncture strength and puncture deformation) of gelatin films (Weng et al., 2021). Bacteriophages are very small particles with dimensions of 92.83 nm in diameter and 129.75 nm in length, as provided in Section 5.3.3 above. Hence, the incorporation of low concentrations of bacteriophages is not expected to disrupt the structure of protein-based films that are strengthened by various types of molecular interactions, such as hydrogen and disulfide bonding, electrostatic forces, and hydrophobic interactions. A high concentration of bacteriophages may still cause structural changes in the polymer matrix due to disruption of film homogeneity. In fact, the tensile properties of phage-containing SPI films are much lower than those of conventional packaging materials and need to be improved in the future. The minor variation in TS and EAB of the films could be associated the microstructure of SPI films incorporated with bacteriophages, as discussed in Section 6.3.2 above.

6.3.5 Water vapor permeability (WVP)

One of the most critical aspects influencing food shelf life is the degree of water migration across a packaging material. The WVP of packaging materials depends on the types and concentrations of polymer materials and plasticizer, as well as the nature and amount of antimicrobials added in the polymer matrix (Weng et al., 2021). As compared to the S films, no significant variation in WVP was observed for the films incorporated with CAM-21 (Table 6.2), suggesting that the incorporation of phages does not influence the water affinity of SPI films. The results also indicate that the film structure was not influenced when a low concentration of phages was incorporated. Similar findings were

also obtained by Weng et al. (2021) and Alves et al. (2020), who reported that the WVP of gelatin and sodium alginate films does not significantly affected by the incorporation of bacteriophages.

Table 6.2 The influence of incorporated CAM-21 on TS, EAB and WVP

Sample	TS (MPa)	EAB (%)	WVP ($\times 10^{-10}$ gm^{-1} s^{-1} Pa^{-1})
S	6.35 ± 0.21^{a}	31.95 ± 0.74^{a}	2.56 ± 0.05^{a}
SC1	6.47 ± 0.12^{a}	33.75 ± 1.64^{a}	2.66 ± 0.06^{a}
SC2	6.54 ± 0.17^{a}	32.56 ± 0.63^{a}	2.66 ± 0.06^{a}
SC4	6.53 ± 0.20^{a}	32.79 ± 1.27^{a}	2.60 ± 0.12^{a}

Values with the same superscripts in each column were not significantly different ($P > 0.05$).

*The values are given as means ± standard deviation (n = 3).

6.3.6 *In vitro* antimicrobial activity

The effects of CAM-21 incorporated into the SPI films on *E. coli* O157:H7 growth are shown in Fig. 6.3. For the control groups without film treatment and with S film treatment, their bacterial count increased from 4.3 log CFU/mL to about 9 log CFU/mL in 6 h, suggesting that SPI films do not have any inhibitory effect on *E. coli* O157:H7. After 3, 6 and 9 h of incubation, the bacterial counts in the TSB treated with the phage-containing SPI films (e.g., SC1, SC2 and SC4) were significantly reduced ($P \leq 0.05$) by at least 1.6 log CFU/mL. This could be associated with the short infection time of phages on their host bacteria, which results in bacterial cell lysis after 25-30 min of infection (Choi et al., 2021). The best inhibitory effect was obtained with the SC4 films, which were incorporated with

the highest concentration of CAM-21, and hence, exhibited a maximum reduction of 4.6 log CFU/mL after 6 h of incubation. These results suggest that the antimicrobial activity of the films depends on the concentration of bacteriophages. After 12 h of incubation, the antimicrobial activity of SC2 and SC4 against *E. coli* O157:H7 was still effective. These findings show that the antimicrobial activity of CAM-21 is preserved during the drying process of the FFS, indicating that the addition of bacteriophages protects their infective capability. A similar scenario was also observed when bacteriophages were incorporated into sodium alginate (Alves et al., 2019), acetate cellulose (Gouvêa et al., 2015) and gelatin films (Weng et al., 2021).

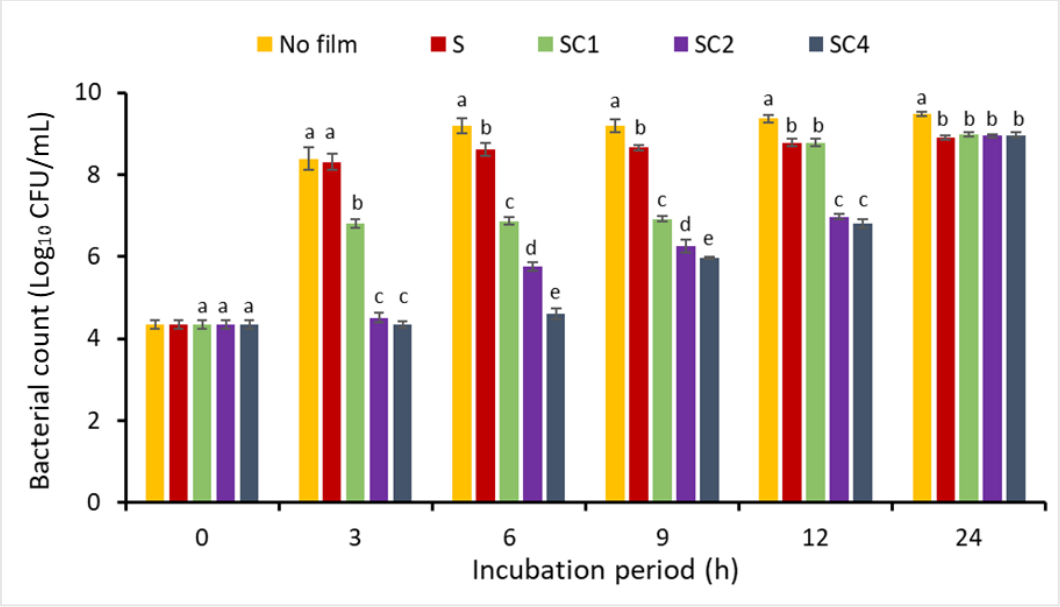


Figure 6.3 Inhibition of *E. coli* O157:H7 by SPI films containing different concentration of CAM-21. Values are presented as means ± standard deviation. The same lowercase letters within the same group indicate that no significant differences ($P > 0.05$).

6.3.7 Food application

The biocontrol effect of CAM-21-containing SPI films against *E. coli* O157:H7 was studied on contaminated raw bottom round beef (Fig. 6.4A). The beef samples that are untreated and treated with the S films exhibited a gradual increase in bacterial count by about 1.5 log CFU/g to about 6.7 log CFU/g after 3 days of refrigerated storage. A microbial load of around 6 to 7 log CFU/g commonly indicates meat spoilage. Hence, these beef samples spoiled on day 3. On the other hand, the beef samples treated with SPI films incorporated with *Escherichia* phage CAM-21 showed antimicrobial activity against *E. coli* O157:H7 by delaying the bacterial growth. The phage-containing films (e.g., SC1, SC2 and SC4) reduced the bacterial count by a maximum of 1.5 and 2.0 log CFU/g after 3 and 5 days of storage, respectively. The microbial loads of all beef samples were increased to at least 6.9 log CFU/g by day 5, indicating that the meat had spoiled. The biocontrol effect of phage-containing SPI films against *E. coli* O157:H7 was also evaluated on raw beef trimmings (Fig. 6.4B). Similarly, both beef samples that are untreated and treated with the S films showed an increase in bacterial count by about 1.8 log CFU/g after 1 day of storage. At day 1, the antimicrobial activity of the SC2 and SC4 films against *E. coli* O157:H7 was greater than that of the SC1 films by delaying the growth of bacteria. The microbial loads of all beef trimmings increased to more than 7 log CFU/g after 3 days of storage, indicating meat spoilage. The difference in the antimicrobial effect may result from the complexity of the real food system containing carbohydrates, proteins and fat, and the level of water content (Choi et al., 2021). For instance, the interaction between phage and *E. coli* O157:H7 may be affected by the amounts of protein and fat molecules in the beef products. The reduced water content may also disrupt the transport of phages to the active site of

target bacteria. The results showed that SPI films containing bacteriophages had a great antimicrobial activity against *E. coli* O157:H7 in beef products, suggesting that they could be used as antimicrobial food packaging for beef preservation.

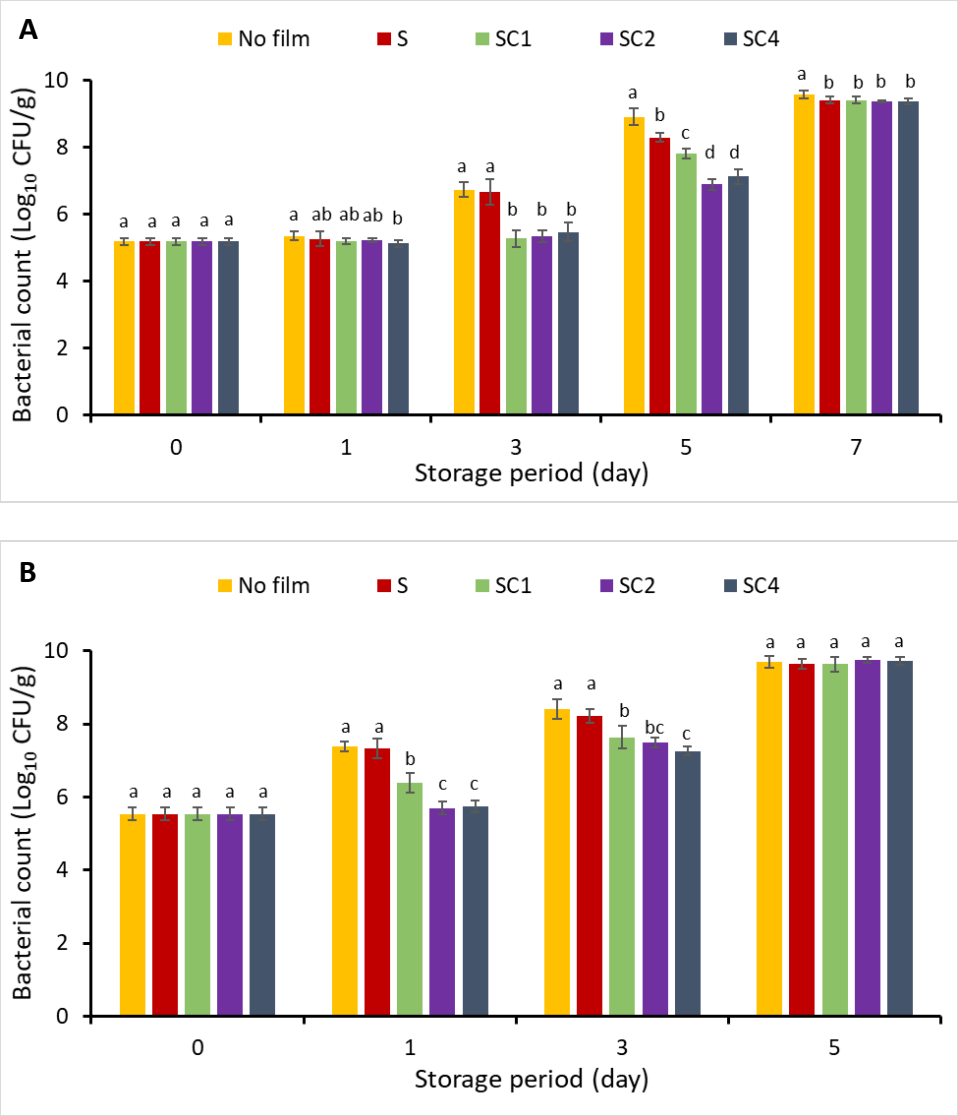


Figure 6.4 Antimicrobial activity of the films incorporated with CAM-21 on *E. coli* O157:H7 on raw bottom round beef (A) and raw beef trimmings (B). Values are presented as means ± standard deviation. The same lowercase letters within the same group indicate that no significant differences ($P > 0.05$).

6.4 Conclusions

In this study, antimicrobial SPI-based films were developed by incorporating *Escherichia* phage CAM-21 in the packaging materials using a solution-casting method. Confocal microscopic imaging revealed the good phage distribution in the film matrix. SEM confirmed that the film microstructure was not affected by the incorporation of CAM-21. The incorporation of CAM-21 did not influence the color, WVP, optical and tensile properties of the SPI films. The phage-containing SPI films exhibited stronger antimicrobial activity against Gram-negative bacterial pathogen (*E. coli* O157:H7) in TSB and raw beef products, which was due to the increased concentrations of antimicrobial agents. These results indicate that the bacteriophage-incorporated SPI films have a great potential to be utilized as antimicrobial food packaging materials for beef preservation.

Chapter 7 Conclusions and Future Direction

7.1 Conclusions

In the first project, antimicrobial composite materials were developed by incorporating cinnamon and clove essential oils (EO) into chitosan (CS)/acetylated starch (ACS) films using a solution casting technique. The addition of EO significantly improved the light, water vapor and oxygen barrier properties of the packaging films. The EO-containing films exhibited antimicrobial properties against spoilage bacteria and a Gram-negative bacterial pathogen (e.g., *E. coli* O157:H7) in a beef model. These results suggest that the composite films have a high potential to be utilized as antimicrobial packaging materials in the food sector.

In the second project, aminosilane-modified bacterial nanocellulose (mBNC) was produced by grafting aminosilane groups onto the surface of bacterial nanocellulose (BNC) using a polycondensation reaction. Polyvinyl alcohol (PVOH)/chitosan (CS)-based composite films were developed with the addition of mBNC and 4-hexylresorcinol (4-HR) using a solution casting method. The addition of mBNC and 4-HR enhanced the optical, mechanical and antioxidant properties of the packaging films. The active composite films inhibited the growth of bacteria on refrigerated raw beef. Hence, food spoilage was retarded, and the shelf life of beef samples was extended. The results showed that the PVOH/CS/mBNCs/4-HR composite film has a great potential to be utilized as an active food packaging material.

In the third project, a novel broad-spectrum *Escherichia* lytic phage, CAM-21, was isolated from the natural environment. The lytic phage, belonging to the *Tequatrovirus* genus of the *Myoviridae* family, was demonstrated to possess a strong lytic activity against

Shiga toxin-producing *Escherichia coli* (STEC), particularly *E. coli* O157:H7. Genes associated with toxin production, virulence, antibiotic resistance, lysogeny and allergens were not found in the genome of CAM-21. CAM-21 could be utilized as a biocontrol agent in foods due to its relatively shorter latent time and antimicrobial activity against *E. coli* O157:H7 in the tested refrigerated foods, milk, ground beef and baby spinach. Hence, CAM-21 is a potential candidate that could be used as a biocontrol agent in foods, especially in fresh produce, meat, and dairy products.

In the final project, antimicrobial soy protein isolate (SPI)-based films were developed by incorporating *Escherichia* phage CAM-21 in the packaging materials using a solution-casting method. The incorporation of CAM-21 did not significantly affect the film microstructure, color, water vapor permeability (WVP), optical, and tensile properties of the films. The active SPI films exhibited antimicrobial activity against *E. coli* O157:H7 in tryptic soy broth (TSB) and raw beef products. These results suggest that bacteriophage-incorporated SPI films have a great potential to be utilized as antimicrobial food packaging materials for preservation of refrigerated foods, particularly meat products.

7.2 Future direction

In this project, various active composite materials were developed for antimicrobial food packaging applications. Although these composite materials have significantly improved in terms of physiochemical, antioxidant, and antibacterial properties, their use in conventional food packaging materials remains limited. This is because a high complexity in real food systems exists, which may affect the functional properties of packaging materials. In addition, some antimicrobial agents, such as EO and plant extracts,

incorporated in the films may influence the sensory properties, such as odor, flavor and aroma, of food products. As a result, antimicrobial agents in packaging materials should be kept to a minimum level while not affecting the antimicrobial activity of the packaging films. Although bacteriophages are stable over a wide pH and temperature ranges, their stability may be affected when they come into contact with a complex food system. Even though the phage stability improves after incorporation into food packaging materials, various encapsulation techniques can be used to further protect the phage, enhancing the antimicrobial activity against target bacteria in food products. Also, phage cocktails can be incorporated to enhance the antimicrobial activity against a wider spectrum of foodborne bacteria.

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