DEVELOPMENT AND CHARACTERIZATION OF A BIO-BASED ANTIMICROBIAL MULTI-LAYERED PACKAGING MATERIAL CONTAINING POLY LACTIC ACID, CHITOSAN, AND GRAPEFRUIT SEED EXTRACT

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ABSTRACT

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Blend solutions containing poly(lactic acid) (PLA) and chitosan (CS) were prepared with the addition of methyldiphenyl diisocyanate (MDI) and coated on PLA film. Increasing the concentration of MDI modified the surface tension and enhanced wettability. The increase in MDI also resulted in increased H-bonding from urethane groups formed within PLA/CS to improve adhesion. These findings were used to improve wettability and adhesion between the PLA/CS coating and PLA film to develop a completely bio-based multi-layer film with antimicrobial capacity. The novel antimicrobial film was prepared by coating CS containing grapefruit seed extract (GSE) on the aforementioned PLA/CS coated PLA film. The effectiveness of the antimicrobial film against *Salmonella* Typhimurium in tomato juice was investigated. A combined effect between the GSE concentration and temperature on the effectiveness of the antimicrobial films against S. Typhimurium in tomato juice was identified. Incorporating GSE into chitosan coatings did not affect the properties of the films initially but did affect these after exposing the films to tomato juice. The tensile strength and elongation at break of films without GSE significantly decreased ($p < 0.05$) while these properties did not change for films containing GSE. Adhesion between the CS coating and PLA film was significantly reduced ($p < 0.05$) after exposing the films to tomato juice. Water vapor and oxygen permeability of the films were not affected by either GSE or tomato juice. CS/GSE coated PLA films have the potential to be an effective bio-based antimicrobial material for food packaging applications.

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1. INTRODUCTION

Packaging materials formed by a single polymer or a polymer blend are not able to meet specific requirements for extending the shelf-life of many food products (Butler and Morris, 2013; Dixon, 2011). Thus, multilayer packaging materials are being developed and utilized for successful commercialization of these products. Multilayer packaging materials can be flexible or rigid with the flexible ones having many applications in the food industry due to the major changes in the way food is produced and retailed today (Dixon, 2011). Currently, petrochemical-based multilayer flexible packaging materials dominate the food packaging market. Environmental concerns caused by the use of these non-renewable and non-biodegradable packaging materials have created a growing interest in the area of biodegradable alternatives originating from renewable sources. However, no single bio-based material can satisfy all needs of a particular food packaging application. Consequently, there is a growing interest in combining bio-based materials for the development of bio-based multilayer flexible packages (Weber et al., 2001).

Over the past several decades, several biopolymers have gained increased attention for their possible benefits in food packaging applications. Chitosan (CS) is one of those biopolymers due to its excellent film forming properties, biodegradability, nontoxicity, and antioxidant and antimicrobial capabilities (Elsabee and Abdou, 2013; Suyatma et al., 2010; Siripatrawan, and Harte, 2010). In addition, CS film can be used as a carrier for compounds due its capability to allow their gradual release and thus, their maintenance at relatively high levels in food products for a long period of time (Peng et al., 2010). However, CS film has a high permeability to water vapor and is brittle and highly soluble under dry and wet conditions, respectively, which limits its use as a packaging material for food (Suyatma et al., 2004; Ye et al., 2008). One strategy used

to overcome these drawbacks, while maintaining the overall biodegradability of the packaging material, is to combine CS with another biopolymer. Among different biopolymers, polylactic acid (PLA) is a promising candidate to be combined with CS. Many attempts have been made to develop blends of CS and PLA to reduce the water vapor permeability, solubility and brittleness of CS (Chen et al., 2005; Grande and Carvalho, 2011; Li et al., 2004; Sebastien et al., 2006; Correlo et al., 2005; Bonilla et al., 2013). However, in all of the aforementioned cases, the addition of CS to PLA decreased the water barrier properties and ductility of the PLA, which limits the use of a blend as the only material for food packaging applications.

The development of a multilayer film made of a PLA/CS blend coated on PLA film will result in a bio-based and biodegradable flexible packaging material with adequate mechanical and barrier properties as well as antimicrobial and antioxidant activity. All of these properties make this new multilayer film a very promising material for food packaging. However, PLA and CS do not readily react with each other due to their differences in polarity (Suyatma et al., 2004; Sebastien et al., 2006), which leads to de-cohesion between layers. A suitable multilayer film used for food packaging applications must require good adhesion between layers to avoid delaminating during use.

Two-component systems based on reactive polyurethane chemistry have been widely used to achieve adhesion in petroleum-based multilayer films used for food packaging (Dixon, 2011). Methylenediphenyl diisocyanate (MDI) is widely used in two-component systems based on reactive polyurethane chemistry because it contains two isocyanate groups. Urethane linkages result from reacting MDI with hydroxyl groups of a polyester or polyether polyol forming a pre-polymer with free isocyanate groups (first component), and the subsequent reaction of the free isocyanate groups of the pre-polymer with the hydroxyl groups of a diol or polyol (second

component). Formation of urethane groups caused by the MDI reaction increases hydrogen bonding (Badri et al., 2010), which enhances adhesion between layers in a multilayer structure. MDI has also been used to improve the compatibility between biopolymers, including PLA/CS blends. The isocyanate groups of MDI readily react with the hydroxyl and carboxylic acid groups on PLA and the hydroxyl and amine groups on CS, forming urethane groups (Suyatma, et al., 2010). Therefore, two-component systems based on reactive polyurethane chemistry could be used to increase the adhesive properties of PLA/CS coating on PLA film to form a suitable multilayer structure for food packaging applications.

A significant increased number of food-borne microbial outbreaks have been reported in the last years. It has been estimated that each year 9.4 million illnesses will be food borne with nontyphoidal *Salmonella* spp. being the most common bacterial pathogen (Scallan, 2011). One possible solution to provide increased safety for food is the development of antimicrobial packages, which fall under the category of active packaging. In antimicrobial packaging, compounds with antimicrobial activity are incorporated into a package to retard or inhibit the growth of microorganisms through direct contact with the food product or by release into the environment surrounding the food product (Almenar and Gartner, 2014). Active packages that are made from bio-based materials in combination with naturally occurring antimicrobial compounds are now receiving increased interest from many organizations, companies, and governments (Del Nobile and Conte, 2013; Almenar and Gartner, 2014).

Chitosan has great potential as an active packaging material because of the characteristics mentioned previously. However, major drawbacks limit its use as an active packaging material. To improve its suitability as an active package material, chitosan has been coated onto polymers having improved moisture barrier and mechanical properties. Torlak and Nizamlioglu (2011)

coated chitosan on polypropylene films and the resulting multilayered film was used to package cheese slices. These multi-layer films reduced the populations of *Listeria monocytogenes, Staphylococus aureus, and Escherichia coli* on the cheese slices during 14 days of storage at 4°C. Another multi-layered film obtained by coating an ethylene copolymer with chitosan was used to package sliced turkey breast (Joeger et al., 2009). The film was found to decrease the populations of *L. monocytogenes* on the sliced turkey breast by 1.7 and 1.2 log after 10 and 15 days of storage at 4°C, respectively. Thus, while chitosan coated films appear to be promising antimicrobial packaging materials, these multi-layer films are not completely bio-based and may therefore not fulfill current needs in regard to sustainable packaging. Since PLA has excellent mechanical properties and is less water soluble than chitosan, the multilayer film will yield an improved antimicrobial packaging material.

Although able to inhibit a wide range of microorganisms, chitosan is not effective against all types of foodborne pathogens. For example, chitosan-based films were unable to decrease *Listeria* on either ham steaks (Ye et al., 2008) or smoked salmon (Ye et al., 2008). In addition, nutrient broth containing 0.1% (No et al. 2002) or 0.5% (Wang, 1992) chitosan was not effective against *Salmonella*. Furthermore, *Salmonella* Typhimurium can develop resistance to the antimicrobial activity of chitosan (Muzzarelli and Muzzarelli, 2007). Antimicrobial agents can be added into the chitosan matrix as a solution, to inhibit a wider range of microorganisms (Sung et al., 2013). Grapefruit seed extract (GSE; sold commercially as Citricidal®) is biodegradable and generally recognized as safe (GRAS) under the Code of Federal Regulations (21 C.F.R. §182, 2013). GSE is inhibitory to both Gram-positive and Gram-negative bacteria (Reagor, 2002) and causes bacterial cell death within fifteen minutes of exposure from disruption of the cell membrane followed by inhibition of enzymatic activities (Heggers et al., 2002). GSE has

been used to enhance the antimicrobial capacity of different types of bio-based films. Red algae films containing GSE have been used as wrappings to reduce the numbers of for *E. coli*- and *L. monocytogenes* on inoculated cheese and bacon (Shin et al. 2012). When GSE was incorporated into rapeseed protein-gelatin films (Jang et al. 2011), the populations of total aerobic bacteria and yeasts and molds on packaged strawberries were lower compared to control strawberries packed in polyethylene terephthalate boxes during storage for 14 days at 4°C. These examples demonstrate the potential of bio-based packaging containing GSE to enhance food safety. However, the mechanical and water solubility drawbacks of these films limit their application as functional packaging materials.

In order to overcome such limitations, GSE has been used in petroleum-based films. Ha et al. (2001) co-extruded low density polyethylene (LDPE) films with a layer of GSE/LDPE and also coated LDPE film with polyamide containing GSE. The polyamide coating with GSE was more effective at inhibiting *E. coli*, *S. aureus*, *Bacillus cereus*, *Bacillus subtilis*, and *Leuconostoc mesenteroides* when compared to co-extruded films due to the effect of heat during co-extrusion on the antimicrobial effectiveness of GSE. Consequently incorporation of GSE as a part of a coating used to form a multi-layered packaging film is a suitable approach to develop antimicrobial packaging materials. However, this approach has not been used to develop functional antimicrobial packages that are completely bio-based and biodegradable to fulfill the growing interest for sustainable packaging.

The objectives of this thesis were: (1) to investigate the effect of MDI concentration on the wettability and adhesion of a PLA/CS blend coated on PLA film in order to develop a bio-based and biodegradable multilayer film suitable for food packaging, (2) to develop and evaluate the antimicrobial activity of a bio-based multilayered packaging film based on chitosan/GSE coating

on PLA against a common food-borne pathogen (*Salmonella* Typhimurium) in tomato juice at different temperatures, and (3) to characterize the mechanical, adhesive, and barrier properties of the antimicrobial film before and after exposure to tomato juice.

2. LITERATURE REVIEW

2.1. Active packaging

Deterioration in food is caused by physicochemical and microbiological changes since fresh foods after harvest are still active biological systems. This results in color, texture and flavor changes, and in some cases possible food safety issues with the food product. The primary role of packaging is to maintain the quality and safety of food under certain conditions for a specific period of time and provide identification and information. Adequate packaging will reduce food losses, improve food safety, and open larger markets and increase options for consumers (Almenar, et al. 2012).

Active packaging allows the food product to interact with the packaging environment, which results in a dynamic way to preserve food (Brody et al. 2008). Active packaging can be defined as the packaging technology where certain additives, known as "active compounds", are incorporated into the packaging material or placed within the packaging container in order to interact directly with the perishable product and/or its environment to extend its quality and/or safety. Current mechanisms that make packages active are: (1) placing the active compound inside the package along with the product (e.g., sachets and labels); and (2) making the active compound part of the material (blended in the bulk polymer matrix or applied to the package as a coating). The main principle regarding active compounds is that they are deliberately added to enhance overall performance of the packaging system (Day, 2008).

While there are many active packaging technologies to preserve the quality of food products (scavenging/emitting oxygen or carbon dioxide, moisture absorbing, flavor management, antioxidants, and antimicrobials), antimicrobial packaging has been proven to be a promising active packaging technology.

2.2. Antimicrobial packaging

Microorganisms present in food products that are associated with quality loss and food safety can be divided into spoilage microorganisms and pathogens. Antimicrobial active packaging technologies can decrease microbial growth. Antimicrobial agents can be incorporated into the polymer, coated, immobilized or surface modified on the polymer, or be an inherent part of the polymer. They can also be placed into permeable sachets that will release them into the package headspace (Suppakul et al. 2003). Antimicrobials used in active packaging function either by direct or indirect contact mechanisms. Direct contact mechanisms preserve food products with either migrating or nonmigrating active components while indirect mechanisms solely rely on migration of active components (Lee and Han, 2011).

Antimicrobial transfer in a migrating antimicrobial package is dominated by diffusion. The antimicrobial initially incorporated into the package material will diffuse until it is desorbed from the surface of the material, then it will be solubilized on the food surface and finally diffused into the food. Therefore, the effectiveness of a migrating antimicrobial depends on its diffusivity in the packaging material, its solubility on the food surface, and its diffusivity in the food. Migrating antimicrobials include organic acids (i.e., benzoic acid), food preservatives (i.e., propionate, and benzoate), bacteriocins (i.e., nisin), chelating agents (i.e., EDTA), extracts from seeds (i.e., grapefruit), aromatic chloro-organic compounds (i.e., triclosan), and others. For example, benzoic anhydride immobilized on low density polyethylene film has been used for cheese and toasted bread (Dobias et al. 2000). Sorbate-releasing plastic films are effective at prolonging cheese shelf life (Han, 1996). Coextruded films containing 1% w/w grapefruit seed extract show antimicrobial effectiveness when wrapping ground beef (Ha et al. 2001).

The correct selection of the antimicrobial agent is essential to the effectiveness of the antimicrobial package because some are strong antifungal agents but not antibacterial. In addition, their effectiveness is dependent on the composition of the food matrix (pH, water activity and type of nutrients). Food additive approval of an antimicrobial agent is a requirement for developing antimicrobial packaging.

2.3. Bio-based antimicrobial packaging

Plastic containers and packaging have exhibited rapid growth in Municipal Solid Waste (MSW) in the United States. The generation of MSW has increased from 3.66 to 4.43 pounds per person per day between 1980 and 2010 (Environmental Protection Agency, 2011). Containers and packaging have contributed the largest portion of MSW with 30 percent of the total 250 million tons and plastic materials comprised of 12 percent of the total reported in 2010 (Environmental Protection Agency, 2011). Emerging initiatives to reduce packaging waste include the use of novel packaging materials such as bio-based polymers or biopolymers, biodegradable plastics, and compostable plastics. The latter is based on the facts that biodegradable and compostable polymers can make a significant contribution to reducing the abundance of plastic packages in landfills and that bio-based polymers can be a possible alternative to reducing dependence on declining petroleum-based resources (Almenar et al. 2012).

Incorporating active compounds into bio-based packaging has the potential to become the most promising form of active packaging. Bio-based active packaging can meet consumer and industry demands by delivering safe and high quality food products with a longer shelf life while creating less of an impact on the environment (Kuorwel et al. 2011). Polymers directly extracted from biomass including polysaccharides (e.g., alginates, cellulose and cellulose derivatives,

starch, chitosan) and proteins (e.g., corn zein, whey protein isolate, soy protein isolate), and polymers produced by classical chemical synthesis from biomass monomers (e.g., poly(lactic acid)) have been used as a basis for developing antimicrobial films and coatings. These biopolymers have been combined with either chemically synthetized (e.g., antibiotics, fungicides) or naturally occurring antimicrobials (plant extracts (e.g., essential oils), enzymes (e.g., lysozyme), bacteriocins (e.g., nisin)) and are effective at reducing both pathogen and spoilage microorganisms. Past and recent developments in this area are compiled in reviews focused on antimicrobial polymers (Cagri et al. 2004; Cha and Chinnan, 2004; Kuorwel, et al. 2011). The most recent developments in the field include the production of the bio-based antimicrobial film using conventional plastic conversion processes like extrusion. Nam et al. (2007) developed antimicrobial pea starch-based materials by extrusion. Joo, et al. (2012) developed antimicrobial poly(lactic acid) (PLA)-based films containing the naturally occurring volatile trans-2-hexenal.

Chitosan, a deacetylated product of chitin, has intrinsic antimicrobial properties and is effective not only on Gram-positive and Gram-negative bacteria but also on yeasts and molds and has the potential to be used for active packaging. Chitosan can form antimicrobial films and coatings, however, the antimicrobial properties will be affected by its molecular weight and concentration. Some examples of chitosan-based active packaging films include chitosan-polysaccharide edible films, chitosan-protein based films, and chitosan complexed with metals (i.e., zinc and copper) (Aider, 2010).

2.4. Polylactic acid and chitosan blends

Chitosan is a promising bio-based and biodegradable packaging material for antimicrobial packaging because it has good film forming properties, is non-toxic, and has

natural antioxidant and antimicrobial activities (Elsabee et al. 2008; Suyatma et al. 2010; Siripatrawan and Harte, 2010). However, chitosan is very sensitive to water which can limit its use as the sole packaging material for food products (Suyatma et al. 2010; Ye et al. 2008). One way to overcome the drawbacks of chitosan is to combine it with other biopolymers. Polylactic acid (PLA) is a biodegradable aliphatic (linear) polyester that has been developed for commercial applications (Nampoothiri, 2010). The mechanical and barrier properties of PLA are desireable for a number of applications that allow it to compete against petroleum-based thermoplastics (Lim et al., 2008).

Most work done with blends involves the mixing of chitosan and PLA solutions (Chen et al. 2005; Grande and Carvahllo, 2011; Li et al. 2004). Hexanoyl chitosan and PLA blends were prepared by a solution casting technique and the thermal and mechanical properties of the resulting films were characterized (Peesan, et al. 2005). The tensile strength and Young's modulus decreased when compared to pure PLA and only partial miscibility between the two polymers was achieved. Other methods of producing PLA and chitosan blends were carried out by extruding and injection molding (Correlo et al. 2005). Increasing the amount of chitosan in the blends decreased the tensile strength and elongation but increased the tensile modulus. The changes in mechanical properties were attributed to incompatibility between the two phases and a lack of interfacial adhesion between chitosan and PLA. The miscibility of PLA and chitosan blends was improved by developing a tertiary blend with PLA, chitosan, and polyvinyl alcohol (PVA) (Grande and Carvahlo, 2011). However, it was determined that chitosan is the major component in the blend dominating the mechanical behavior. Other work with chitosan and PLA blends involves a chemical compatibilization method (Suyatma et al. 2010). This method involved cross linking of PLA and chitosan polymers with methyl diphenyl diisocyanate (MDI).

The functional groups on both PLA and chitosan will readily react with the isocyanate groups from MDI to form urethane linkages. Increasing the amount of MDI would create a homogenous blend and improve the mechanical properties. However, in all the aforementioned cases, the addition of CS to PLA decreased the water barrier properties and ductility of PLA, which limits the use of a blend as the only material for food packaging applications.

2.5. Multilayer packaging

Packaging materials formed by a single polymer or a polymer blend are not able to meet specific requirements for extending the shelf-life of many food products (Butler and Morris, 2013; Dixon, 2011). Thus, multilayer packaging materials are being developed and utilized for successful commercialization of these products. The first high barrier multilayer used in the food industry was introduced in 1983, and since then there has been an increase in momentum to develop new multilayer materials (Schaper, 1991). A multilayer packaging material can be defined as two or more materials with specific properties combined in a single layered structure. Multilayer packaging materials can be flexible or rigid with the flexible ones having many applications in the food industry due to the major changes in the way food is produced and retailed today.

Multilayer packaging films consisiting of chitosan coatings have been tested for their antimicrobial effectiveness (Elsabee et al. 2008; Duan et al. 2007). Elsabee et al. (2008) studied the antifungal and antibacterial effects of chitosan and chitosan/pectin coatings when applied to surface modified polypropylene films. Chitosan was found to be an effective antimicrobial and was able to preserve the quality of whole tomatoes that were stored for 13 days at 4°C. Control tomato samples stored in plain polypropylene films showed evidence of decay during the same storage period. Duan et al. (2007) incorporated lysozyme into a chitosan coating that was applied

to the surface of a commercial barrier film (SARANEX™) that had been corona treated. These coated films were found to significantly reduce the growth of pathogenic and spoilage microorganisms. A greater reduction in the microbial populations was observed with chitosan coatings containing lysozyme. Both these examples demonstrate chitosan as an effective antimicrobial coating in a multilayer structure but they are not completely bio-based. Therefore, there is a need for innovation in antimicrobial packaging that is completely bio-based.

Chitosan has been used as a coating to develop multilayer structures with improved barrier properties (Kurek et al., 2012; Seok-Hoon et al. 2012; Soares et al. 2013). Kurek et al. (2012) used chitosan solutions to coat the surface of polyethylene films. The oxygen permeability was found to be significantly decreased with the chitosan coatings. The oxygen permeability was increased at higher relative humidities, but was still significantly lower than plain polyethylene films. This demonstrates how chitosan can improve barrier properties in multilayer structures. However, this example is not of a completely bio-based multilayer structure. Soares et al. (2012) developed a completely bio-based multilayer structure by immersion and spray coating chitosan on thermoplastic starch/PLA sheets. Improved water solubility and moisture vapor permeability were achieved when chitosan was cross-linked with gluteraldehyde. However, the chitosan coating produced an irregular surface over the films and it was suggested that this was caused by reticulation of the chitosan. Seok-Hoon et al. (2012) also developed a bio-based multilayer structure by coating chitosan or chitosan/clay nanocomposites on PLA. The oxygen barrier properties of the PLA were significantly improved by coating with chitosan and a significant improvement of the water vapor permeability was achieved by adding clay to the chitosan coating. From visual inspection of the SEM cross section of the multilayer

material, there appears to be a gap between the PLA and chitosan signifying an incompatibility between the layers.

The lack of compatibility between layers and adhesion properties in a multilayer film structure can be improved through modifications of the materials. The two aforementioned examples of chitosan/pectin and chitosan lysozyme coatings (Elsabee et al. 2008; Duan et al. 2007) both involved coatings applied to materials that had been corona treated. Peroxide or carboxylic groups will develop over the surface of polypropylene films after a corona discharge and the presence of the functional groups help the chitosan coating adhere to the surface (Elsabee et al. 2008).

Two-component systems based on reactive polyurethane chemistry have been widely used to achieve adhesion in petroleum-based multilayer films used for food packaging (Dixon, 2011). Methylenediphenyl diisocyanate (MDI) is widely used in two-component systems based on reactive polyurethane chemistry because it contains two isocyanate groups. Urethane linkages result from reacting MDI with hydroxyl groups of a polyester or polyether polyol forming a pre-polymer with free isocyanate groups (first component), and the subsequent reaction of the free isocyanate groups of the pre-polymer with the hydroxyl groups of a diol or polyol (second component). The formation of urethane groups caused by the MDI reaction cause an increase in hydrogen bonding (Badri et al. 2010). This hydrogen bonding allows the adhesion between layers in a multilayer structure.

2.6. Grapefruit seed extract antimicrobial films

Grapefruit seed extract (GSE; sold commercially as Citricidal®) is biodegradable and generally recognized as safe (GRAS) under the Code of Federal Regulations (21 C.F.R. §182,

2013). GSE causes bacterial cell death within fifteen minutes of exposure due to disruption of the cell membrane and inhibition of enzymatic activity (Heggers et al., 2002) and posses an antibacterial efficacy comparable to topical antimicrobials with inhibitory effects on both Gram-positive and Gram-negative bacteria (Reagor, 2002). GSE has been used to enhance the antimicrobial capacity of different types of bio-based films. When used as wrappings for *E. coli*- and *L. monocytogenes* on inoculated cheese and bacon, red algae films containing GSE reduced the populations of both microorganisms in both food products (Shin et al. 2012). In another study, GSE was incorporated into rapeseed protein-gelatin films (Jang et al. 2011) and the resulting films were used to form bags and package fresh strawberries. The populations of total aerobic bacteria and yeasts and molds of the bagged strawberries were lower when compared to control strawberries packed in polyethylene terephthalate boxes during storage for 14 days at 4°C. These examples demonstrate the potential of bio-based packaging containing GSE to increase food safety, but the mechanical and water sensitivity drawbacks of these films limit their applications as functional packaging materials. In order to overcome such limitations, GSE has been used in petroleum-based film structures. Ha et al. (2001) co-extruded low density polyethylene (LDPE) films with a layer of GSE/LDPE and also coated LDPE film with polyamide containing GSE. The polyamide coating with GSE was more effective at inhibiting *E. coli*, *S. aureus*, *B. cereus*, *B. subtilis*, and *L mesenteroides* when compared to the co-extruded films due to the effect of the heat in the co-extrusion process on the antimicrobial effectiveness of GSE. This demonstrates that the incorporation of GSE as a part of a coating used to form a multi-layered packaging film is a suitable approach to develop functional antimicrobial packaging.

3. IMPROVING WETTABILITY AND ADHESION OF POLY(LACTIC ACID)/CHITOSAN COATINGS FOR THE DEVELOPMENT OF A BIO-BASED MULTI-LAYER FILM

3.1. Materials and Methods

3.1.1. Materials

Chitosan (CS) with an average molecular weight of 100-300, KDa (Arcos Organics, NJ, USA), polylactic acid (PLA) resin 4060D grade (Nature Works LLC, NE, USA), glacial acetic acid (Sigma Aldrich, MO, USA), methylene diphenyl diisocyanate (MDI) (Sigma Aldrich,,MO, USA), and acetone (Sigma Aldrich, MO, USA) were used to prepare the coating solutions. Commercially available PLA film (Evlon®; BiAx Inc., Ontario, Canada) was used as the base layer for the coating. The heat sealable side of the film which consisted of a thin layer of high molecular weight PLA was the side of the film coated.

3.1.2. Preparation of PLA/CS blend solutions

Plain CS solution (1% w/v) was prepared by dispersing 0.3 g of chitosan in 30 mL of acetic acid solution (1% v/v) using a magnetic stir plate (Barnstead, IA, USA) for 2 hours at room temperature. Subsequently, the CS solution was sonicated for 30 minutes using a homogenizer (Polytron Kinamatica Inc, OH, USA) to remove bubbles. Plain PLA solution (1% w/v) was prepared by placing 0.3 g of PLA resin and 30 mL of acetone inside a leak-proof jar and then stirring both using a magnetic stir plate for 2 hours at room temperature. MDI (0, 0.2, 1, 2, and 3% w/w of the final PLA/CS solution) was added to the plain PLA solution and the resulting solutions were heated in a water bath at 60°C for 1 hour, cooled to room temperature, and then added drop wise to the plain CS solution (20 drops per 30 seconds) while stirring. The PLA/CS blend solutions were heated in a water bath at 80°C for 30 minutes, stirred for 2 hours at room temperature and then held overnight. A control was prepared by adding the PLA solution

drop wise to the CS solution at room temperature (20 drops per 30 seconds) while stirring. After all of the PLA solution was dropped into the CS solution, the mixture was stirred for an additional 2 hours at room temperature and then held overnight.

3.1.3. Preparation of coated PLA films

Each of the PLA/CS blend solutions (treatments and controls) prepared in 3.1.2 was poured onto the surface of a piece of PLA film (30 cm x 20 cm) and then distributed using a K coating bar No. 5 (RK Printcoat Instruments, Litlington, UK). The coating bar was passed over the film surface only once. The coated films were transferred to a fume hood and dried at room temperature overnight. The resulting coated films were used for scanning electron microscopy (SEM) and mechanical pull-off testing characterizations.

3.1.4. Characterization of the chemical structure of the coatings: Fourier Transform Infrared (FTIR) Spectroscopy

Ten mL of each of the PLA/CS blend solutions prepared in 3.1.2 was poured into a petri dish (100 mm x 20 mm) and dried in a fume hood at room temperature overnight. The resulting casted films were removed from the petri dishes and stored at room temperature for 24 hours. Each of the casted films was placed into direct contact with an attenuated total reflectance (ATR) crystal and its FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu FTIR spectrophotometer (Model IRPrestige-21, Shimadzu Scientific Instruments Inc., Japan). All spectra were obtained with a resolution of $4cm^{-1}$ at room temperature. Forty scans were carried out for each sample and averaged.

3.1.5. Characterization of the adhesion between PLA/CS coatings and PLA film: Contact angle

Contact angle was used to determine the wetting properties of the PLA/CS blend solutions prepared in 3.1.2 on the surface of the PLA film via a Krüss contact angle measuring system (DSA10, Hamburg, Germany). PLA film with the heat sealable side facing up was mounted on a glass slide and placed on the measuring stage of the equipment. PLA/CS blend solutions were loaded into a 2.0 mL micrometer syringe (Gilmont Instruments, CA, USA) and mounted on the Krüss contact angle measuring system. Three μL of blend solution was dropped onto the surface of the PLA film by moving the stage towards the drop until proper contact between the drop and the surface of the film was made. Drop shape analysis was used to determine the contact angle by the sessile drop fitting method. Pictures were taken using the camera that was built into the instrument and contact angle values were obtained when the contact time of solution and film were no greater than 30 s. Five measurements on different areas of each film, each equivalent to a drop, were averaged.

3.1.6. Characterization of the adhesion between PLA/CS coatings and PLA film: Mechanical adhesion pull-off testing

Differences in adhesion of the different PLA/CS blend solutions with the PLA film in the coated films prepared in 3.1.3 were determined using a pull-off test for adhesion as described in the European standard EN ISO 4624 (EN ISO, 2003). An Instron tensile tester (Instron 5565, MA, USA) was used to support the test fixture and to apply tensile stress in a direction perpendicular to the plane of the coated film. The support was provided by the gripping mechanism on the tensile tester, which prevented the test fixture from moving out of place. Ball joints were attached to the top and bottom of the test fixture, which ensured that the direction of the tensile stress was always perpendicular to the plane of the test film. Dollies made from

aluminum and with an area of $3.14x10^{-4}$ m^2 were attached to the ball joints. The test film (coating side up) was attached between the two dollies with a bonding agent. Preliminary screening was carried out to determine an appropriate bonding agent that would produce adhesive failure (failure between PLA/CS coating and the PLA film) as opposed to cohesive failure (failure between the bulk of the bonding agent) and would not interact with the coating. The bonding agent that was selected for pull-off testing was Contact Stik™ (DAP®, MD, USA). The test speed for pull-off testing was $8.47x10^{-4}$ m/s and failure occurred within 90 s. The breaking strength ( ) of separating the PLA/CS coating from the PLA substrate was calculated using the following formula:

where is the breaking strength required to separate the coating from the film in Kilopascals (kPa), is the breaking force in Newtons (N) and is the area of the dolly on the testing fixture in square meters (m^2) . Five measurements on different areas of each film specimen were averaged.

3.1.7. Scanning Electron Microscopy

The PLA films coated with PLA/CS blend solutions containing 0, 1 and 3% MDI from section 3.1.3 were cut using a double-edged razor blade. The resulting film cross-sections were sputtered with osmium using a NEOC-AT osmium coater (Meiwafosis Co., Ltd., Osaka, Japan) to create conductive surfaces. The contact area between the PLA/CS blend solution and the PLA film of each film cross-section was visualized using a Jeol high resolution scanning electron microscope (JEOL Ltd., Model 7500F, Tokyo, Japan) with an accelerating voltage of 6 kV and a

working distance of 16 mm at 10000x magnification. For each piece, a surface of 10000 μm x 1000 μm was visualized.

3.1.8. Statistical Analysis

Statistical analyses of the results from contact angle and pull-off test were performed through one-way analysis of variance (ANOVA) and the Tukey-Kramer test to determine any significant differences at a confidence level of 95% ($p \leq 0.05$). All statistical analyses were performed with SAS version 9.3 software (SAS Institute, NC, USA)

3.2. Results and Discussion

3.2.1. Chemical structure of the coatings

The chemical structures of PLA, CS, and PLA/CS blends differing in MDI concentration obtained using FTIR spectroscopy (Fig. 1) were compared to identify the effect of MDI content on the chemical interactions between the functional groups of PLA and CS in the PLA/CS blends. The peak observed at 1745cm^{-1} in pure PLA (Fig. 1a), which has been attributed to C=O stretching of ester groups (Grande and Carvalho, 2011; Peesan et al. 2005) was observed at 1755cm^{-1} in all of the PLA/CS blends (Fig. 1c-f). This same upward shift has already been reported for blends of CS with PLA (Grande and Carvalho, 2011). The intensity of this peak was higher in the absence of MDI and is most likely due to the heterogeneous nature of the PLA/CS blend without MDI, leading to a higher composition of PLA in the analyzed wavelength. The peak at 1755cm^{-1} has also been associated with C=O vibrations in urethanes and reported to increase in intensity with the concentration of MDI (Suyatma et al. 2010). In agreement, the intensity of this peak increased with the addition of MDI from 1% to 3% (Fig. 1d-f). This indicates that the change of MDI from 1% to 2% or from 2% to 3% creates more

urethane groups between PLA and CS, and consequently, a higher degree of cross-linking between both polymers.

The peaks observed around 1550cm^{-1} and 1400cm^{-1} in pure CS (Fig. 1b) have been attributed to NH_3^+ and NH_2 groups from CS, respectively, and have been identified previously in PLA/CS blends (Sebastien et al. 2006). The peak at 1550cm^{-1} in pure CS (Fig. 1b) was shifted to 1535cm^{-1} in all of the PLA/CS blends (Fig. 1c-f) possibly due to interactions between the OH groups on PLA and the amino groups on CS. This same upward shift has been observed in starch-chitosan composite films and reported as specific interactions between the OH groups on starch and the amino groups on chitosan (Xu et al. 2005). The intensity of the peak at 1535cm^{-1} increased along with increasing the amount of MDI in the blend. The higher absorption band at 1535cm^{-1} caused by the addition of MDI has been reported to be due to the NH bonds in urea and urethane formed by the compatibilization of CS and PLA (Suyatma et al. 2010). This higher absorption band is another indication that the amount of urethane groups and degree of cross-linking was increased along with the increase of MDI from 1% to 3% in the PLA/CS blends.

A small peak was observed at 1700cm^{-1} (Fig. 1 e-f). This peak represents C=O stretching associated with H-bonding between urethane groups that have been formed from the reaction between the N=C=O on MDI and the OH groups on PLA and CS (Badri et al. 2010; Yilgor et al. 1999). The intensity of this peak increased along with increasing the amount of MDI in the blend except for 1%MDI, which suggests that the amount of H-bonding produced by 1%MDI may be too low to be detectable by FTIR. This is an indication that there is an increase in H-bonding associated with the amount of MDI added to the PLA/CS blends. A characteristic peak for N=C=O group on MDI should appear around 2266cm^{-1} if it remains un-reacted in a blend

(Suyatma et al. 2010; Badri et al. 2010). This peak was not identified in any of the PLA/CS blends with MDI, which confirms that all of the MDI reacted with the PLA and CS.

In summary, increasing the amount of MDI increased the number of urethane groups leading to a higher degree of cross-linking between PLA and CS polymer chains as well as an increase in H-bonding. This affected the adhesion properties of the PLA/CS blends as shown in sections 3.2.2-3.2.3. Additionally, all of the MDI reacted with the PLA and CS and therefore there were no diisocyanate monomers remaining that could lead to food safety concerns related to this multilayer.

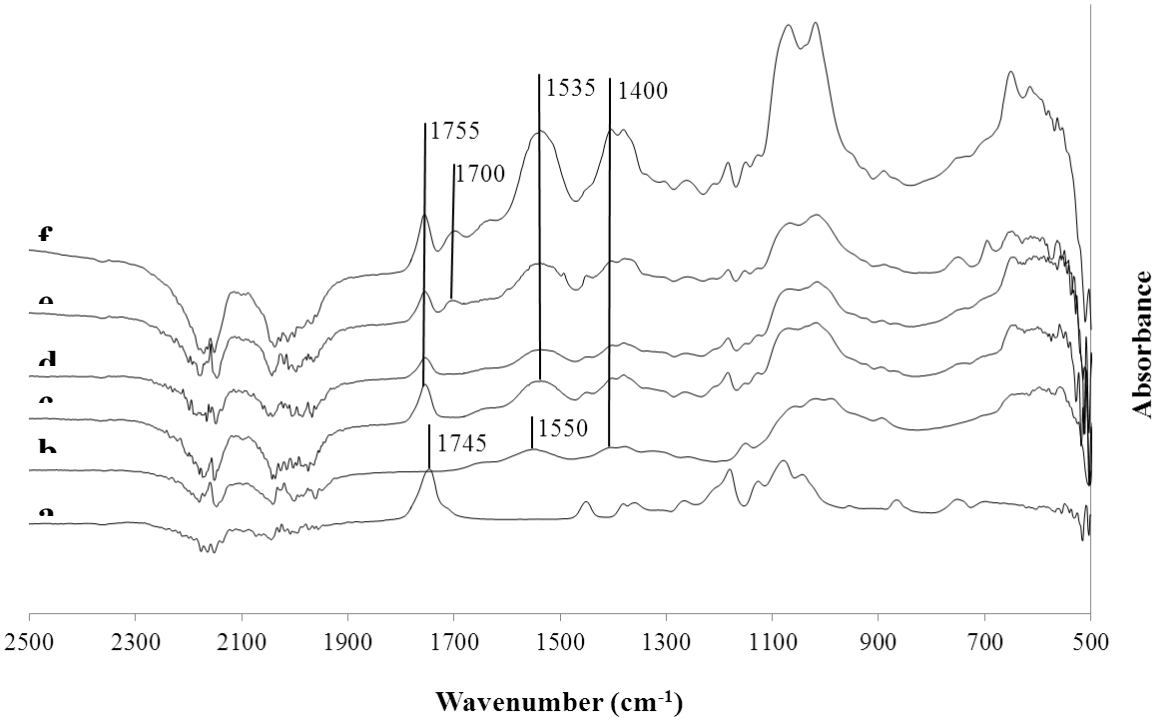

Figure 1. ATR-FTIR spectrum for PLA (a); CS (b); and PLA/CS with MDI concentrations of 0% (c); 1% (d); 2% (e); and 3% (f)

3.2.2. Wettability of PLA/CS blend solutions on PLA film

Fig. 2 shows the effect of MDI on the contact angle (wettability) of PLA/CS blend solutions dropped on PLA film. The contact angle of the PLA/CS blend shifted to a lower value with increasing MDI concentration. This indicates that the addition of MDI to the PLA/CS blend solution improves the wettability of the PLA/CS coating with the PLA film. This improved wettability can be attributed to a modification in surface tension of the PLA/CS blend solution caused by MDI. As PLA is compatibilized with CS, there is a decrease in the cohesive forces of the pure polymers, subsequently affecting the surface tension. A similar reduction of the cohesion energy of starch was observed when natural rubber was compatibilized with starch using glycidyl methacrylate (Pichayakorn et al. 2014). Additionally, polar interactions of H-bonding caused by the formation of urethane groups during the reaction of PLA and CS with MDI may have also enhanced wettability.

Interestingly, the heat treatment was effective at enhancing the wettability between the PLA/CS blend solution and PLA film (from 46.0° to 41.1°; ($P \leq 0.05$)). Both PLA and CS contain highly reactive groups and therefore, they could have reacted if free radicals are formed in the system by heating, thus modifying their surface tension and consequently, increasing wettability. Most polymers decompose as they are heated, subsequently producing carbon-centered free radicals that can promote polymerization (Singh and Bahari, 2002).

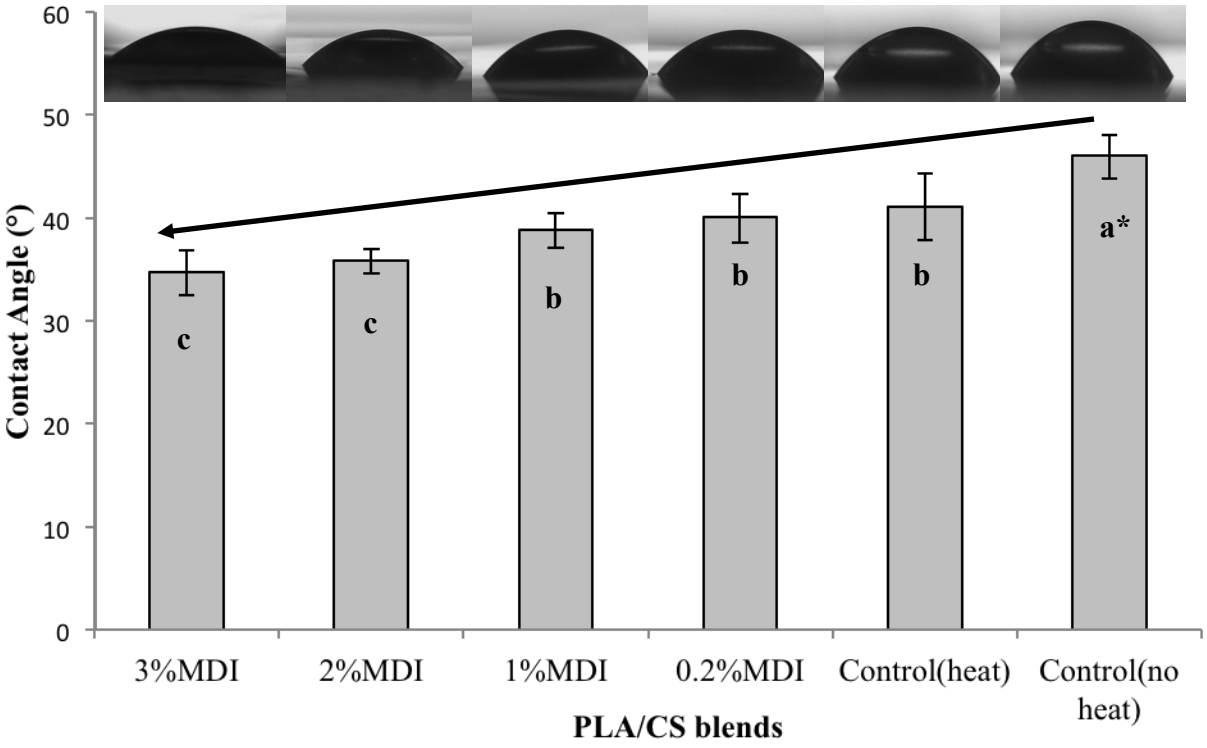

Figure 2. Contact angle of PLA/CS blend solutions with different concentration of MDI on the surface of PLA film. *Different letters indicate significant differences ($P \leq 0.05$) between PLA/CS blend solutions

3.2.3. Adhesive breaking strength of PLA/CS coatings on PLA film

The adhesive failure at the interface between PLA/CS coatings and PLA film was evaluated by measuring breaking strength using an adhesion pull-off test (Table 1). An amount of 1%MDI or higher and heating was necessary to obtain a PLA/CS coating with a significantly ($P \leq 0.05$) increased breaking strength compared to that of the PLA/CS coatings without MDI and without heating. Among the PLA/CS coatings prepared by heating, an amount of 3%MDI was necessary to significantly ($P \leq 0.05$) increase the breaking strength of the PLA/CS coating. The increase of MDI yielded a blend solution that better wetted the PLA surface as well as an increased H-bonding due to the more urethane groups formed between PLA and CS. The more

H-bonding led to more intermolecular forces between the PLA/CS coating and the PLA film and subsequently, to a higher breaking strength. Urethanes are known to make very good adhesives because they have the ability to effectively wet most surfaces and readily form hydrogen bonds to a substrate (Szycher, 2013).

Table 1. Breaking strength (kPa) of PLA/CS coatings on PLA film

	Concentration of MDI (%) in PLA/CS blend solutions					
	0		0.2	1	2	3
	no heat	heat				
Breaking Strength (kPa)	$212 \pm 15^{a*}$	228 ± 30^{abc}	234 ± 37^{abc}	273 ± 32^{bcd}	288 ± 47^{dc}	303 ± 19^{d}

*Different letters indicate significant differences ($P \leq 0.05$) between PLA/CS blend solutions

3.2.4. Cross-section morphological characterization of the interface between PLA/CS coating and PLA film

Figure 3 shows the cross-section morphology of PLA film coated with PLA/CS blends differing in MDI concentration (0, 1 and 3%). As observed, the concentration of MDI has a remarkable influence on the physical interaction between the PLA/CS blend coating and the PLA film. PLA/CS blend coatings containing 0% MDI had no contact (physical entanglements) with the PLA film (Fig. 3(a)). This can be attributed to the weak wettability of the PLA/CS blend solution and poor adhesion between the PLA/CS blend coating and the PLA film as shown in Fig. 2 and Table 1. Physical entanglements between the two layers were formed when MDI was added to the blend and increased along with increasing the MDI concentration, as shown in Fig. 3(b) and Fig. 3(c). These results correlate with the contact angle and pull-off test results which show the improved wettability and adhesion between the PLA/CS blend and the PLA film with

increasing the MDI concentration. The cross-section micrographs also show that the fracture surface of the PLA/CS coating without MDI is not flat (Fig. 3(a)). The observed small bulges indicate incompatibility between PLA and CS. These bulges were reduced with the addition of 1%MDI to the PLA/CS blend (Fig. 3(b)) and disappeared (smooth fracture surface) when the concentration of MDI was increased to 3% (Fig. 3(c)), which shows better compatibility between PLA and CS with increasing the MDI concentration.

The variability in the thickness of the coatings (0%MDI versus 1% and 3%MDI) can be explained by differences in homogeneity between the blends. According to Sebastien et al. (2006) variations in thickness between films obtained from PLA/CS blends can be attributed to the heterogeneity of the blend (Sebastien et al. 2006). From visual inspections, coatings containing 0%MDI were not able to effectively coat the PLA film which yielded to an un-uniform coating. When MDI was added to the coating solutions (even at 0.2% level), the coatings were all able to uniformly coat the PLA film.

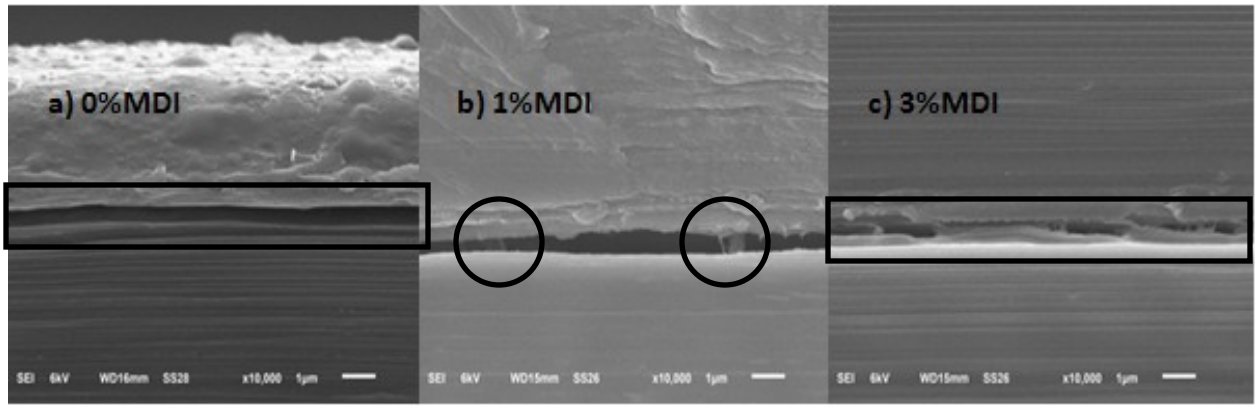

Figure 3. Cross-section morphology of PLA film coated with PLA/CS blends with MDI concentrations of 0% (a), 1% (b) and 3% (c)

4. ANTIMICROBIAL, MECHANICAL, AND BARRIER PROPERTIES OF A COMPLETLEY BIO-BASED MULTI-LAYER FILM CONTAINING POLY(LACTIC ACID), CHITOSAN, AND GRAPEFRUIT SEED EXTRACT IN FRESH TOMATO JUICE

4.1. Materials and Methods

4.1.1. Materials

Chitosan with an average molecular weight of 100-300 kDa was obtained from Arcos Organics (NJ, US). Glacial acetic acid was purchased from Sigma Aldrich (MO, US). Grapefruit seed extract (GSE) (Citricidal®, 60% grapefruit extract and 40% vegetable glycerine) was obtained from Biochem Research (CA, US). Polylactic acid film (Evlon®) was purchased from BiAx Inc. (Ontario, Canada). Tryptic soy broth (TSB), trypticase soy agar (TSA), and yeast extract (YE) were purchased from Becton, Dickinson and Company (NJ, US). TSA and TSB were supplemented with 0.6% (w/v) YE to produce TSAYE and TSBYE, respectively. Ferric ammonium citrate (0.5%) and sodium thiosulfate (0.3%) were purchased from Sigma Aldrich (MO, US) and used to supplement TSAYE to produce TSAYE-FS. An avirulent strain of *Salmonella* Typhimurium (LT2) was obtained from Dr. Michelle Danyluk (University of Florida, FL, US).

4.1.2. Bacterial culture preparation

S. Typhimurium was stored at -80°C in TSB and glycerol. Before use, the organism was streaked onto TSAYE plates. A single colony of the organism was transferred to TSBYE and incubated for 24 hours at 37°C. The organism was transferred to another tube of TSBYE and incubated an additional 24 hours at 37°C. The final concentration of the organism was approximately 10^9 CFU/mL determined by serial dilutions and direct plate counting.

4.1.3. *Salmonella*-inoculated tomato juice

Greenhouse grown tomatoes were purchased from a local supermarket and pureed using a blender sterilized with ethanol and strained through two layers of sterilized cheesecloth. The resulting liquid was further filtered using Whatman #42 filter papers (General Electric, CT, US) to obtain tomato juice. The pH of the tomato juice was recorded using a pH meter (Corning, MA, US). The bacterial culture prepared in 4.1.2. was used to inoculate the tomato juice so the resulting juice contained approximately 10^4 CFU/mL of *S.* Typhimurium determined by serial dilutions and direct plate counting.

4.1.4. Antimicrobial activity of GSE

Different amounts of GSE diluted in sterilized distilled water were added to the *Salmonella*-inoculated tomato juice prepared in 4.1.3. to obtain final concentrations of 20, 50, 75, and 100ppm of GSE. Three replications of tomato juice with different concentrations of GSE were incubated at 23, 10, or 4°C for 3 days. The number of viable cells of *S.* Typhimurium was enumerated by direct plating on TSAYE-FS initially, and after 1, 2, and 3 days. Triplicate samples of *Salmonella*-inoculated tomato juice containing sterilized distilled water instead of diluted GSE served as controls. The limit of detection (LOD) for *S.* Typhimurium was 1 log CFU/mL.

4.1.5. Preparation of chitosan/GSE coated PLA films

The chitosan/GSE coated PLA films (47 μm) consisted of three layers: PLA film (38 μm), chitosan/PLA adhesive layer (4μm) and chitosan/GSE layer (5μm). The chitosan/PLA adhesive layer was obtained following the procedure described in 3.1.2. The chitosan/GSE layer was obtained by preparing a chitosan solution (2% w/v) by dispersing 1.0 g of chitosan in 50 mL

of acetic acid solution (1% v/v) with 0.1 mL (10% v/w) or 0.3 mL (30% v/w) of GSE and stirring for 2 hours at room temperature. The amounts of GSE were chosen based on the results obtained in 4.1.4. After stirring, the chitosan/GSE solutions were sonicated for 30 minutes. The chitosan/PLA adhesive solution was coated on the PLA film and the chitosan/GSE solutions were coated onto the PLA/chitosan adhesive layer with a K coating bar #7 on an automatic coating table (RK Printcoat Instruments, Model K303, Litlington, UK). The films coated with the chitosan/PLA adhesive solution were transferred to a fume hood and dried at room temperature overnight. The final coated films were dried in the same way. Control films (0% v/w) were prepared as described above but without GSE. Three different chitosan/PLA adhesive solutions and chitosan/GSE solutions per treatment were used to prepare the films in order to get three replicates per treatment.

4.1.6. Antimicrobial activity of chitosan/GSE coated PLA films

Qualitative evaluation of the antibacterial activity of chitosan/GSE coated PLA films against *S.* Typhimurium was carried out using an agar diffusion method. The bacterial culture prepared in 4.1.2. was diluted to 10^8 CFU/mL and 100 µL of this suspension was spread over the TSAYE-FS plates to have an overlay with approximately 10^7 CFU/mL. Four chitosan/GSE coated PLA films (0 and 10% v/w) were prepared in duplicate (8 samples total), cut to 2.0 x 2.0 cm and overlaid on TSAYE-FS plates with the chitosan/GSE coating side exposed to the bacteria. The TSAYE-FS plates were incubated for 24 hours at 37°C and then the plates were observed for clear zones of inhibition.

Quantitative evaluation of the antibacterial activity of chitosan/GSE coated PLA films against *S.* Typhimurium was carried out using the tomato juice prepared in 4.1.3. Triplicate chitosan/GSE coated PLA films containing 0, 10 and 30% GSE (v/w) were cut to 4.5 x 4.5cm

and placed into screw cap tubes with 10 mL of tomato juice. The tubes were incubated at 23, 10, or 4°C for 3 days. Three days was chosen because it would be enough time to show if the films are effective at inhibiting *S.* Typhimurium in tomato juice. The number of viable cells was enumerated by direct plate counts on TSAYE-FS for *S.* Typhimurium initially, and after 1, 2, and 3 days. The limit of detection (LOD) for *S.* Typhimurium was 1 log CFU/mL.

4.1.7. Characterization of chitosan/GSE coated PLA films

The barrier, mechanical, and adhesive properties of the chitosan/GSE coated PLA films (0 and 30%v/w) were determined before and after exposure to tomato juice for 10 days at 7°C. 7°C was chosen as the storage temperature because it was in-between the temperatures studied. A storage period of 10 days was used to mimic an average storage period of tomato juice.

4.1.7.1. Barrier properties

Water vapor transmission rates (WVTR) of the films were measured using a Permatran W Model 3/33 Water Permeability Analyzer (MOCON, MN, US) according to the American Society for Testing Materials (ASTM) method F1249 (ASTM, 2013). Three replications were tested at 23 ºC and 100% RH.

Oxygen transmission rates (OTR) of the films were measured using an 8001 Oxygen Permeation Analyzer (Mocon, MN, US) according to the ASTM method D3985 (ASTM, 2010). Three replications were tested at 23 ºC and 0% RH.

In all cases, the permeability coefficients (kg m m^{-2} s^{-1} Pa^{-1}) were obtained as follows:

$P = (TR \times l)/\Delta p$

where TR is the transmission rate value (kg m^{-2} s^{-1}), l (m) is the film thickness, and Δp is

the partial pressure differential across the film (Pa).

4.1.7.2. Mechanical properties

An Instron universal testing instrument Model 5565 (Instron Engineering, MA, US) was used to measure the tensile properties (tensile strength, elongation at break, and modulus of elasticity) of the films according to ASTM method D882 (ASTM, 2012). The thickness of the films was measured in five areas with a digital micrometer (Testing Machines Inc., DE, US), averaged to the nearest 0.001mm and were cut to have a width of 2.54 cm. The initial grip separation and cross head speed were 50.8 mm and 50.8 mm/min, respectively. Six strips from each treatment (two per replicate) were tested in both the machine and transverse directions of the films at 23°C and 55%RH.

4.1.7.3. Adhesive properties

The adhesion between the coating layer and the PLA film was determined using a pull-off test for adhesion as described in the European standard EN ISO 4624 (EN ISO, 2003). The Instron tensile tester mentioned above was used to support the test fixture and apply tensile stress in a direction perpendicular to the plane of the coated film. Ball joints were attached to the top and bottom of the test fixture, which ensured that the direction of the tensile stress was always perpendicular to the plane of the test film. Dollies with an area of 3.14×10^{-4} m^2 were made from aluminum and attached to the ball joints. The test film (coating side up) was attached between the two dollies with a bonding agent. The bonding agent that was selected for pull-off testing was Contact Stik™ (DAP®, MD, US). The test speed for pull-off testing was 8.47×10^{-4} m/s and failure occurred within 90 s. The breaking strength ( ) of separating the coating layer from the PLA film was calculated using the following formula:

where is the breaking strength in Kilopascals (kPa), is the breaking force in Newtons (N) and is the area of the dolly on the testing fixture in square meters (m^2). Six films (two per replicate) from each treatment were tested at 23°C and 55%RH.

4.1.8. Statistical Analysis

Statistical analyses of the results were performed through one-way analysis of variance (ANOVA) and the Tukey-Kramer test to determine any significant differences at a confidence level of 95% ($p \leq 0.05$). All statistical analyses were performed with SAS version 9.3 software (SAS Institute, NC, USA).

4.2. Results and Discussion

4.2.1. Antimicrobial activity of GSE against S. Typhimurium in tomato juice at different temperatures

The antimicrobial effects of GSE on S. Typhimurium in tomato juice as a function of GSE concentration (0, 20, 50, 75, and 100 ppm) and temperature (4, 10, and 23°C) were studied for 3 days. The results are presented in Fig. 4 (logs of growth) and Table 2 (log reductions as a difference from the control (0 ppm of GSE) at each storage day). The latter was used to facilitate comparison of the different growth of *S.* Typhimurium at all three temperatures. The acidic pH of the tomato juice (pH = 4.3) did not inhibit the growth of *S.* Typhimurium as shown by its increase from 4.5 log to 5.8 log at 10°C and to 8.0 log at 23°C after 3 days in the absence of GSE. Similar effect of pH and temperature on the growth of other *Salmonella* species in tomato juice has been reported (Li et al., 2010; Weissinger et al., 2000).

Both GSE concentration and temperature had an effect on the antimicrobial capacity of GSE against *S.* Typhimurium. GSE concentrations of 50 ppm or higher were found to significantly ($p \leq 0.05$) decrease the population of *S.* Typhimurium in tomato juice at all three temperatures and by increasing the concentration of GSE, a greater log reduction was observed. Also, an increase in the log reduction was found to be caused by the increase of temperature. Therefore, a combined effect between the GSE concentration and temperature was identified. The greatest log reduction was observed in tomato juice containing 100 ppm GSE and stored at 23°C. However, tomato juice that was stored at 23°C with 100 ppm GSE was not able to reduce the *S.* Typhimurium to a population below the LOD. The only treatment that was able to reduce the *S.* Typhimurium population below the LOD was 100 ppm GSE and stored at 10°C. This occurred after 1 day and additional growth was prevented for the remaining 2 days. In other work, oregano essential oil and nisin were found to be more effective as antimicrobials against *Salmonella enteritidis* at 10°C versus 4°C (Govaris et al., 2010). It has been reported that increasing the temperature increases the activity of antimicrobial compounds (Mackowiak, et al. 1982). Since GSE concentrations of 50 ppm and higher were shown to be effective at inhibiting the growth of *S.* Typhimurium in tomato juice at all three temperatures. GSE concentrations of 60 and 180 ppm, which were equivalent to 10 and 30% (v/w) when incorporated into the film, were chosen and the antimicrobial effectiveness of the films was determined and compared.

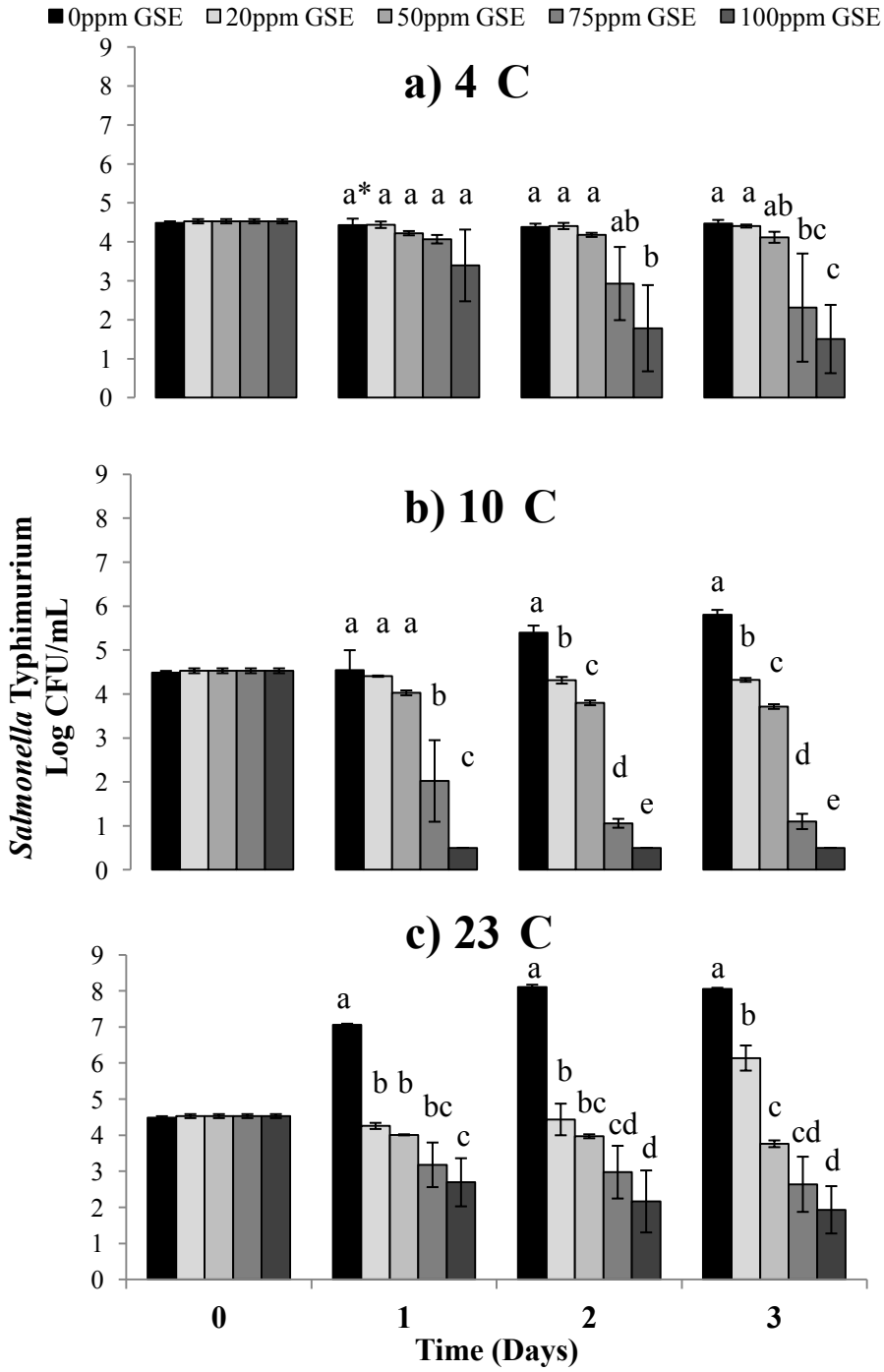

Figure 4. Antimicrobial activity of different GSE concentrations (0, 20, 50, 75, and 100 ppm) against *S.* Typhimurium in tomato juice at (a) 4°C, (b) 10°C, and (c) 23°C.
*Lowercase letters indicate significant differences between GSE concentrations (0, 20, 50, 75, 100 ppm) at days 1, 2, and 3 ($p \leq 0.05$).

Table 2. Reductions of *Salmonella* Typhimurium (log CFU/mL) in tomato juice containing GSE (20, 50, 75, and 100 ppm) at 4, 10, and 23°C.

Time (Days)	Temperature (°C)	Log reduction CFU *Salmonella* Typhimurium /mL tomato juice			
		20*	50	75	100
1	4	-0.01 a**A***	0.21 aA	0.36 aA	1.03 aA
	10	0.14 aA	0.52 abA	2.52 bcA	4.04 cB
	23	2.80 aB	3.04 aB	3.88 abB	4.36 bB
2	4	-0.03 aA	0.20 aA	1.45 abA	2.60 bA
	10	1.08 aB	1.59 bB	4.33 cB	4.89 dB
	23	3.67 aC	4.13 aC	5.13 abB	5.94 bB
3	4	0.07 aA	0.36 aA	2.16 abA	2.97 bA
	10	1.48 aB	2.09 bB	4.70 cB	5.30 dB
	23	1.91 aB	4.29 bC	5.41 bcB	6.12 cB

*ppm of GSE
**Lowercase letters indicate significant differences between GSE concentrations (rows) ($p \leq 0.05$).
***Uppercase letters indicate significant differences between temperatures for each GSE concentration (columns) ($p \leq 0.05$).

4.2.2. Antimicrobial activity of chitosan/GSE coated PLA films

4.2.2.1. Qualitative study

Figure 5 displays the results from the agar diffusion test with chitosan coated PLA films with and without GSE. Chitosan coated PLA films without GSE were not able to inhibit the growth of *S.* Typhimurium as shown by the absence of a zone of inhibition (Fig. 5a). A possible reason for the lack of antimicrobial activity of these films is that chitosan was not able to migrate from the polymer matrix and diffuse through the agar medium and consequently, this could not interact with the microorganism. Additionally, *S.* Typhimurium has been reported to be able to build up resistance to cationic polymers through mutations (Muzzarelli and Muzzarelli, 2007; Pranting and Andersson, 2010), and if that were the case, the inhibitory action of the chitosan coating would not be shown. Similarly to this study, Pranoto et al. (2005) reported no inhibitory

effect on *S.* Typhimurium for plain chitosan films used as controls, which were used to compare against chitosan films incorporated with garlic oil, potassium sorbate, and nisin that did demonstrate antimicrobial activity. Plain chitosan films also showed no inhibition with the agar diffusion method when tested against *L. monocytogenes* (Coma et al., 2002, Zivanovic et al., 2005). By adding GSE to the chitosan coating, the multilayer film was able to inhibit the growth of *S.* Typhimurium. This inhibitory effect is shown by the clear zone under the contact area between the coated film and inoculated media, as well as a clear zone of about 2 mm surrounding the film (Fig. 5b). Based on these results, GSE was desorbed from the film and able to diffuse into the agar medium. Other studies involved with incorporating GSE into red algae, gelatin, alginate and carrageenan, and polyamide coated polyethylene films have shown the inhibitory effect of GSE against various pathogenic organisms using agar diffusion methods (Shin et al., 2012; Jang et al., 2011; Cha et al., 2002; Ha et al., 2001).

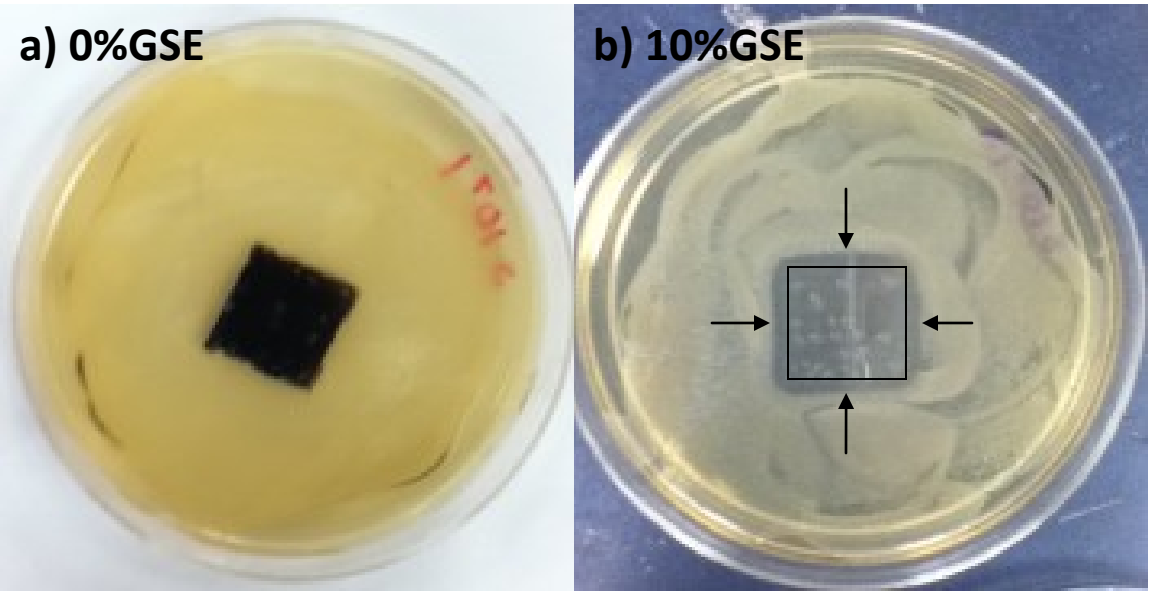

Figure 5. Qualitative antimicrobial activity of chitosan coated PLA film containing (a) 0% GSE and (b) 10% GSE against *Salmonella* Typhimurium.

4.2.2.2. Quantitative study

Based on the qualitative results obtained by agar diffusion, chitosan coated PLA films containing 10% GSE were shown to posses antimicrobial activity against *S.* Typhimurium. However, the agar diffusion method more closely simulates the interaction between the antimicrobial films with a solid food product rather than a liquid and it is of note that agar cannot really simulate a food matrix. In addition, this method cannot compare the effects of variables like GSE concentration in the film and temperature on *S.* Typhimurium growth. Therefore, chitosan coated PLA films differing in GSE concentration (0, 10, and 30%) were submerged in tomato juice and stored at three different temperatures (4, 10, and 23°C) for 3 days to determine their antimicrobial effects on *S.* Typhimurium as a function of GSE concentration, temperature, and time.

The results are displayed in Fig. 6 (logs of growth) and Table 3 (log reductions as a difference from the control (0%GSE) at each storage day). The latter was used to facilitate comparison due to the different growth of *Samonella* at all three temperatures. The results obtained at each day and at all three temperatures prove that the populations of *S.* Tyhphimurium are significantly ($p \leq 0.05$) reduced by increasing the concentration of GSE in the chitosan coatings. Furthermore, there is also an effect of temperature ($p \leq 0.05$) on the antimicrobial effects of the multilayer films, where at higher temperatures a greater reduction in *S.* Typhimurium populations was observed. Chitosan coated PLA films containing 10% GSE were not able to reduce *S.* Typhimurium populations at 4°C. However, at 10 and 23°C, these films significantly ($p \leq 0.05$) decreased *S.* Typhimurium populations. The films containing 30% GSE showed reduction in *S.* Typhimurium populations at all three temperatures but were more effective at the higher temperatures (10 and 23°C). Temperature is known to be one of the main

factors affecting migration rates of components of packaging materials into food (Bhunia et al. 2013). The increase of temperature could have increased the migration of GSE from the chitosan matrix to the tomato juice and increase the interaction between GSE and the microorganism. Additionally, it has been reported that increasing the temperature increases the activity of antimicrobial compounds and Gram-negative bacteria are more susceptible to higher temperatures when compared to Gram-positive bacteria (Mackowiak, et al. 1982). This same effect of temperature on antimicrobial effectiveness was also observed when GSE was added directly to the tomato juice.

The greatest reduction in *S.* Typhimurium populations was observed from chitosan/GSE coated PLA films containing 30% GSE stored in tomato juice at 23°C. However, these films were not able to reduce *S.* Typhimurium populations below the LOD. Coatings with 30% GSE stored at 4 and 10°C were able to reduce *S.* Typhimurium populations to below the LOD after 3 and 2 days of storage, respectively. These films stored at 10°C were also able to maintain the *S.* Typhimurium populations below the LOD during day 3 as well. At all temperatures, 10% GSE coatings were not effective at reducing the *S.* Typhimurium populations to below the LOD.

Similarly to the agar diffusion results, chitosan coated PLA films containing 0% GSE did not show any inhibitory effects against *S.* Typhimurium when they were submerged in tomato juice at all three temperatures. A possible explanation is that chitosan is not able to diffuse from the film into the liquid tomato juice. Therefore, direct contact between the microorganism and the chitosan polymer would never be achieved. Additional explanations have been reported. The number of bacteria may exceed the inhibitory effects of chitosan or that the chitosan must be dissolved in order to have inhibitory action since the molecules are too tightly bound when it is a film (Zivanovic et al., 2005).

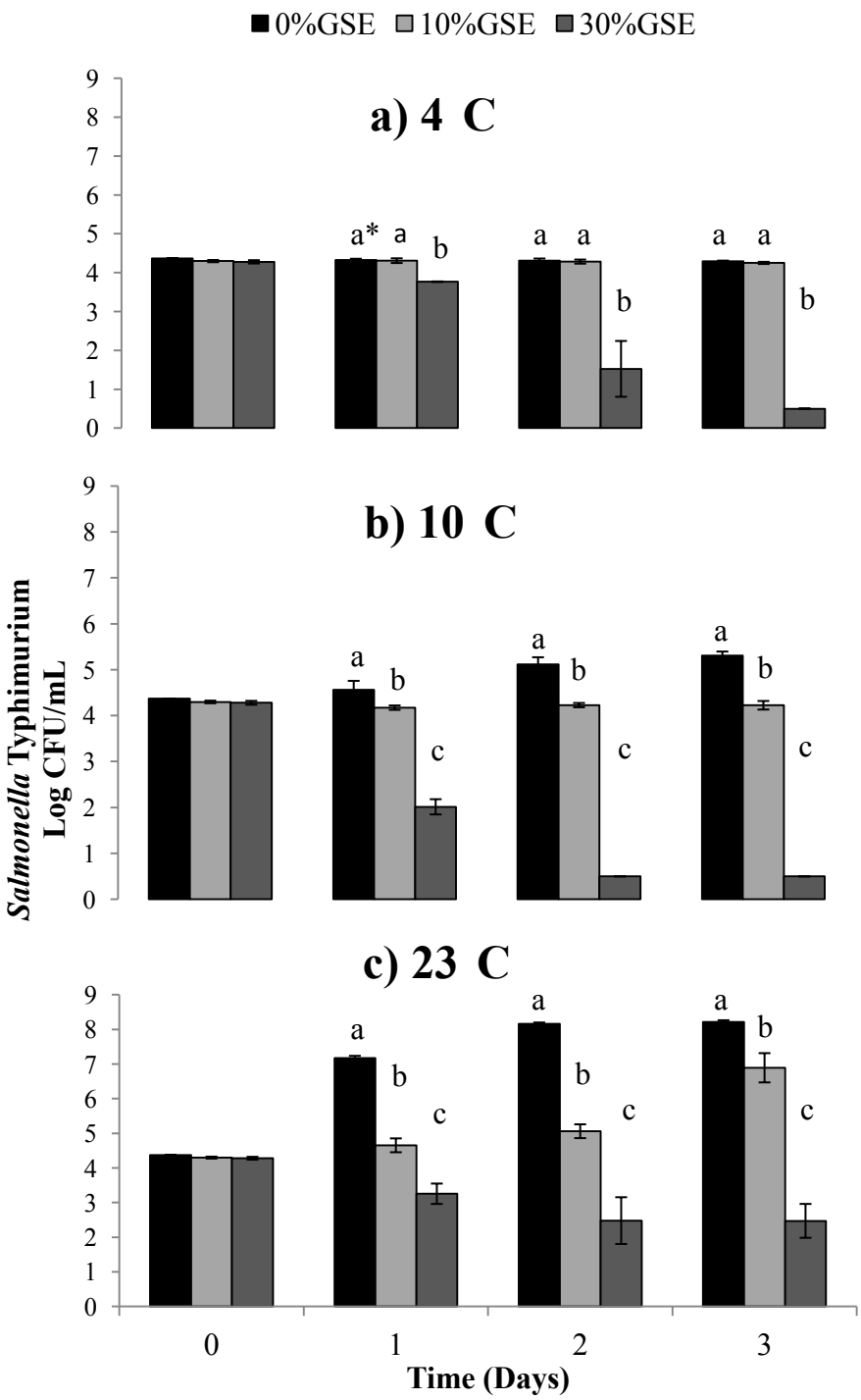

Figure 6. Antimicrobial activity of chitosan coated PLA films containing 0, 10 and 30% GSE against *S.* Typhimurium in tomato juice at (a) 4°C, (b) 10°C, and (c) 23°C. *Lowercase letters indicate significant differences between GSE concentrations in films (0, 10, and 30%) at days 1, 2, and 3 ($p \leq 0.05$).

Table 3. Reductions of *Salmonella* Typhimurium (log CFU/mL) in tomato juice containing chitosan coated PLA films (10 and 30% GSE) at 4, 10, and 23°C.

Time (Days)	Temperature (°C)	Log reduction CFU *Salmonella* Typhimurium /mL tomato juice	
		10%GSE	30%GSE
1	4	0.01 a*A**	0.55 bA
	10	0.39 aA	2.55 bB
	23	2.52 aB	3.92 bC
2	4	0.02 aA	2.78 bA
	10	0.88 aB	4.61 bB
	23	3.10 aC	5.68 bB
3	4	0.04 aA	3.79 bA
	10	1.08 aB	4.80 bB
	23	1.32 aB	5.74 bC

*Lowercase letters indicate significant differences between GSE concentrations (rows) ($p \leq 0.05$).
**Uppercase letters indicate significant differences between temperatures for each GSE concentration (columns) ($p \leq 0.05$).

4.2.3. Characterization of chitosan/GSE coated PLA films

4.2.3.1. Mechanical properties

Table 4 displays the tensile strength, elongation at break, and modulus of elasticity in machine and transverse directions of the chitosan coated PLA films with and without GSE before and after being exposed to tomato juice at 7°C for 10 days. At day 0, there were no significant differences between the tensile strength, elongation at break, and modulus of elasticity of the chitosan coated PLA films with and without GSE, indicating that the antimicrobial does not affect the mechanical properties of the films. However, GSE did influence the effects of the tomato juice on the mechanical properties of the films. The tensile strength and elongation at break of the chitosan coated PLA films containing 30% GSE were not affected during exposure to tomato juice, while the films without GSE had significantly ($p \leq 0.05$) lower tensile strength

and elongation at break after exposure. This difference could be attributed to the 40% of glycerol contained in GSE, which by its hygroscopic nature could be binding water and therefore protecting the PLA film from degradation. Degradation of PLA film caused by hydrolysis has been reported. This is due to an increase in water content in the film and can be catalyzed by the presence of carboxylic acids (Siparsky et al. 1998). Therefore, the high amount of water and the acids present in the tomato juice induced degradation in PLA causing it to change its mechanical properties. The loss of elongation at break in PLA films has also been correlated to water in other studies (Ho et al. 1999; Holm et al. 2006; Kranz et al. 2000).

The modulus of elasticity significantly ($p \leq 0.05$) increased in both orientations for both types of coated films after exposure to tomato juice for 10 days. This indicates that the films became more rigid after being exposed to tomato juice. An explanation for this is that plasticizers used in the production of PLA film may be leaching and causing an increase in the modulus of elasticity. The leaching of acetyl triethyl citrate used as a plasticizer for PLA/starch/maleic anhydide blends was reported to increase the modulus of elasticity of the blend (Zhang and Sun, 2004). After exposure to tomato juice, no significant differences between the different concentrations of GSE were observed in the modulus of elasticity in the transverse orientation of the film. However, coatings with 0% GSE in the machine orientation had a significantly ($p \leq 0.05$) higher modulus of elasticity when compared to coatings with 30% GSE. Overall, the mechanical properties of the chitosan coated PLA films were best maintained by the addition of GSE in the chitosan coatings.

4.2.3.2. Adhesive properties

The effect of both GSE and exposure to tomato juice on adhesion between the coating layer and PLA film was determined and the results are displayed in Table 4. At day 0, there were no significant differences ($p > 0.05$) between the breaking strengths of the coatings with and without GSE. This indicates that the antimicrobial does not affect breaking strength between coating and PLA film. However, after exposure to tomato juice, the breaking strengths of the coatings with GSE were significantly ($p \leq 0.05$) lower than those of the coatings without GSE. The differences in breaking strengths can be attributed to the 40% glycerol contained in the GSE. Glycerol bound water due to its hygroscopic nature and swelled the chitosan matrix. This most likely facilitated the diffusion of glycerol towards the chitosan/PLA layer that interfered with the bonding between the chitosan/PLA layer and PLA film, therefore reducing adhesion. Glycerol coatings on the surface of ultra high molecular weight polyethylene were found to interfere with atmospheric plasma surface treatments and interfacial bonding between polyethylene and epoxy (Ji et al., 2012).

The results also show that the breaking strengths of both coatings were significantly ($p \leq 0.05$) reduced compared to the initial values when the films were exposed to tomato juice. Absorption of the tomato juice components by the coatings interfered with the bonding layer causing reduced adhesion. It has been reported that absorption of components from foods by multilayered packaging materials may alter their properties and cause delamination (Stollman et al. 2000). However, delamination of the coatings with and without GSE from the PLA film after exposure to tomato juice was not observed.

4.2.3.3. Barrier properties

The water vapor permeability (WVP) and oxygen permeability (OP) of chitosan coated PLA films with and without GSE before and after exposure to tomato juice are presented in Table 3. There were no significant differences ($p > 0.05$) in WVP between chitosan coated PLA films with and without GSE initially and after 10 days of exposure to tomato juice. This indicates that the antimicrobial did not affect WVP of the multilayer film. WVP of both films was not affected by exposure to tomato juice ($p > 0.05$). The WVP of both multilayer films was very similar to that reported for PLA film (2.2×10^{-14} kg m m^{-2} s^{-1} Pa^{-1}) by Gonzalez-Buesa et al. (2014). This indicates that chitosan coatings are a poor barrier to water. There were no significant differences ($p > 0.05$) in OP between chitosan coated PLA films with and without GSE initially and after exposure to tomato juice. The OP of both multilayer films is lower than values reported for PLA film (5.67×10^{-18} kg m m^{-2} s^{-1} Pa^{-1}). This indicates that the chitosan coating is improving the oxygen barrier of the PLA film. In other work involving chitosan coatings on PLA, it was reported that chitosan coating caused a significant decrease in the oxygen transmission rate through the films (Park et al. 2011).

Table 4. Mechanical, adhesive, and barrier properties of chitosan coated PLA films containing 0% and 30% GSE before and after 10 days of exposure to tomato juice at 7°C.

Amount of GSE in the film	Time (Days)	Tensile Strength (MPa)		Elongation at Break (%)		Modulus of Elasticity (MPa)		Breaking Strength (kPa)	Water Vapor Permeability ($\times 10^{-14}$ kg m m^{-2} s^{-1} Pa^{-1})	Oxygen Permeability ($\times 10^{-19}$ kg m m^{-2} s^{-1} Pa^{-1})
		Machine Direction	Transverse Direction	Machine Direction	Transverse Direction	Machine Direction	Transverse Direction			
0%	0	77 a*A**	111 aA	142 aA	78 aA	2683 aA	3830 aA	276 aA	1.97 aA	1.48 aA
30%		72 aA	106 aA	140 aA	77 aA	2663 aA	3781 aA	260 aA	1.98 aA	2.06 aA
0%	10	64 aB	68 aB	8 aB	18 aB	2983 aB	3996 aB	195 aB	1.86 aA	1.84 aA
30%		72 bA	101 bA	157 bA	78 bA	2860 bB	3942 aB	154 bB	2.24 aA	1.62 aA

*Lowercase letters indicate significant differences between concentrations of GSE (0% and 30%) in the chitosan coated PLA films at each day ($p \leq 0.05$).

**Uppercase letters indicate significant differences between exposure times (0 and 10 days) to tomato juice for each type of film ($p \leq 0.05$).

5. CONCLUSIONS

Both wettability and adhesion between PLA/CS coating and PLA film can be improved by increasing the concentration of MDI in the blend. The increase of MDI results in an increase in urethane groups that: (1) modify the surface tension of the PLA/CS blend solution leading to increased wettability and consequently, makes it a more effective coating on PLA film, and (2) increases H-bonding forces required for improved adhesion between the PLA/CS coating on PLA film. Improved wettability and adhesion between PLA/CS coating and PLA film leads to the development of a completely bio-based multi-layer flexible packaging film for food packaging applications.

Chitosan/GSE coated PLA film has the potential to be an effective bio-based antimicrobial material for food packaging applications. The antimicrobial activity of chitosan/GSE coated PLA films against *Salmonella* Typhimurium in tomato juice was found to be greatly affected by the concentration of GSE as well as the temperature. The films were most effective at reducing the populations of *S.* Typhimurium with higher concentrations of GSE and stored at higher temperatures. However, at these conditions the films were not effective at reducing *S.* Typhimurium populations below the LOD in tomato juice while they were at 4 and 10°C after 3 and 2 days of storage, respectively. Incorporating GSE into chitosan coatings did not affect the properties of the films initially but they were affected after exposing the films to tomato juice. The mechanical properties after exposing the films to tomato juice were best maintained when GSE was present in the chitosan coatings. However, the presence of GSE reduced the adhesion between the coatings and PLA film after exposure to tomato juice. The water and oxygen barrier properties of the multilayer films were not affected by GSE in the coating or exposure to tomato juice.

6. FUTURE WORK

The results of this study indicate that the incorporation of GSE into chitosan coatings is effective at inhibiting the growth of *S.* Typhimurium in an acidic liquid food product (tomato juice). The coated films were also formed into pouches and used to package fresh-diced tomatoes (Appendix 1). These pouches were not found to be an effective package for reducing the populations of *S.* Typhimurium in the diced tomatoes. Therefore, future work would include:

1) Develop a different packaging design with the multilayer films that may be more effective at inhibiting *S.* Typhimurium.

2) Increasing the amounts of GSE in the coatings to see if they would be more effective at inhibiting *S.* Typhimurium in packaged fresh-diced tomatoes.

3) The films and pouches could be examined for their effectiveness against other types of pathogenic microorganisms (e.g. *L. monocytogenes*, *E. coli*, etc.).

4) The films and pouches could be evaluated on different types of fresh liquid or solid food products other than fresh-diced tomatoes.

5) Different types of natural antimicrobial compounds other than GSE can be incorporated into the chitosan coating and evaluated.

APPENDIX

APPENDIX. PHYSIOCHEMICAL AND SENSORY QUALITY OF FRESH-DICED TOMATOES PACKAGED IN CHITOSAN/GSE COATED PLA POUCHES

Materials and methods

Preparation of chitosan/GSE coated PLA pouches

Chitosan/GSE coating solutions (0 and 30%GSE) were prepared as described in 4.1.5. For preparation of the pouches, plain PLA films were marked with a rectangle measuring 24cm x 12cm. After coating, the solution around the marked area was removed with the flat edge of a ruler to allow sealability and then the coating was dried. The coated films were heat sealed (105°C) using an impulse sealer to form 12cm x 12 cm pouches with the coating on the inside. Three different coating solutions per treatment (0% and 30% GSE) were used to prepare the pouches in order to get triplicates per each treatment.

Fresh-diced tomato processing and packaging for physico-chemical and sensory evaluation

Commercial processing was simulated. Briefly, the stem scars on the tomatoes were removed and then the tomatoes were diced using a commercial dicer (IN, US), washed with cold water, and placed on a shaker table to remove excess liquid. The tomato dices were transferred to a 4°C walk-in cooler to be packaged. 150 g of diced tomatoes were weighed out into the chitosan/GSE coated PLA pouches or control pouches prepared as described above. After filling the pouches, they were heat sealed and stored at 7°C for 10 days. The pouches were prepared in triplicate for each treatment and the diced tomatoes were evaluated for weight loss, texture, color and pH at days 0, 3, 7, and 10.

The commercial processing of diced tomatoes used for physico-chemical evaluation could not be used for sensory evaluation because of safety considerations. Fresh roma tomatoes were hand diced using knives, washed with cold water, and placed in a colander to remove excess liquid. The tomato dices were transferred to a 4°C walk-in cooler to be packaged. 150 g

of tomatoes were weighed out into the chitosan/GSE coated PLA pouches or control pouches prepared as described above. After filling the pouches, they were heat sealed and stored at 4°C for 14 days. The pouches were prepared in duplicate for each treatment and the diced tomatoes evaluated for aroma, color, hardness, sweetness, sourness, and overall appearance at days 0, 3, 7, 10, and 14.

Physiochemical evaluation methods

The quality of fresh diced tomatoes packaged in chitosan/GSE coated pouches was evaluated based on weight loss, texture, color, pH. The weight loss was measured by weighing all of the filled pouches initially (day 0) and at each sampling date (days 3, 7, and 10). From the recorded pouch weights the percentage of weight loss could be calculated with the following equation:

$$\frac{Initial\ pouch\ weight\ (g) - Stored\ pouch\ weight\ (g)}{Initial\ pouch\ weight\ (g)} * 100\%$$

Texture was measured using a texture analyzer (TA-XT2i, Stable MicroSystem, MA, USA) equipped with a 5 blade Kramer compression cell using a 50kg load cell. 100 g of diced tomatoes from each pouch was measured and loaded into the cell. The test speed was performed at 3.0 mm/s until the blades completely passed through the sample. The firmness was recorded as the maximum force (N) required for the blades to pass through the tomatoes.

Color of the diced tomatoes was measured on both the skin and flesh surfaces using a Hunter Lab colorimeter (LSXE, HunterLab, VA, US). From each treatment the L*, a*, and b*

values were recorded on the skin and flesh of 30 tomato dices. From the a* and b* values, the chroma (C*) and hue angle (H°) color parameters were calculated from the following equations:

$$C* = \sqrt{a*^2 + b*^2}$$

$$H° = \tan^{-1}\left(\frac{b*}{a*}\right)$$

The left over diced tomatoes from each pouch were collected from the texture measurements and pureed with a blender for pH measurements using a pH meter (Corning, MA, USA). The pH probe was inserted into the pureed tomatoes and the pH was recorded twice for each pouch.

Quantitative descriptive sensory analysis (QDA) methods

Panelists trained for QDA were selected based on their ability to discriminate. A preliminary screening assessment using triangle tests was administered to potential panelists. From the screening results, ten panelists were selected to be trained to evaluate fresh-diced tomatoes. Quality parameters evaluated by the panelists included aroma, color, hardness, sweetness, sourness, and overall appearance. During training, intensity scaling was explained and standard references were created to cover the types of changes expected during storage. All sensory characteristics were evaluated based on unstructured, anchored 15-cm horizontal line scales. The same ten panelists were used to evaluate the tomatoes throughout the entire storage. Sample cups of diced tomatoes were prepared in duplicate for each panelist and given to them in cups labeled with 3-digit random numbers.

Statistical Analysis

Statistical analyses of the results were performed through one-way analysis of variance (ANOVA) and the Tukey-Kramer test to determine any significant differences at a confidence level of 95% ($p \leq 0.05$). All statistical analyses were performed with SAS version 9.3 software (SAS Institute, NC, USA).

Results and Discussion

Physiochemical Evaluations

The weight loss of the diced tomatoes was not significantly affected by the addition of GSE into the chitosan coating and both treatments had a total weight loss of 4% by the end of storage after 10 days. The firmness of the diced tomatoes was significantly reduced after the first three days of storage for both treatments, from 168N initially to 132N and 131N for 0% and 30% GSE, respectively. The firmness of other cut tomato products has also been reported to decrease during storage and is affected by the temperature, with a greater loss of firmness at 10°C when compared to 2°C (Artes et al. 1999). By the end of storage (day 10), tomatoes packaged in pouches with coatings containing 0% GSE were significantly less firm than pouches with coatings containing 30% GSE, 78N and 107N, respectively. This indicates that GSE may have an effect on preventing the softening of diced tomatoes during storage.

The pH of the diced tomatoes rose slightly during storage and became significantly higher for both treatments, from an initial pH of 4.5 to a pH of 4.6 by day 7. This increase in pH during storage is suggested to be caused by a decrease in organic acids, which could be affected by microbial deterioration of the tomatoes (Ordizola-Serrano et al. 2008). It is also suggested that an increase in physiological activity will degrade the organic acids in fresh-cut products (Buta et al. 1999). By the end of storage the pH had decreased in both treatments, where the 30% GSE

treatment was lowered to 4.5, which was not significantly different from the initial pH. The pH of the 0% GSE treatment was lowered to 4.3 and was significantly lower than the initial pH of 4.5. This sudden decrease in pH can possibly be attributed to a reduction in the O_2 levels inside the package causing anaerobic metabolism in the diced tomatoes, resulting in fermentation (Solomos, 1997). These results suggest that incorporating GSE into the chitosan coating may help to prevent the extent of fermentation within the diced tomatoes.

There were no significant changes in any of the color parameters over the storage period. Similar results were shown in stored sliced tomatoes, where no significant changes in lightness, chroma, or hue angle was observed during a storage time of 4°C for 21 days (Ordizola-Serrano, et al. 2008). These results indicate that incorporating GSE into the chitosan coating is not affecting the skin and surface color of the diced tomatoes during storage.

Table 5. Weight loss, texture, and pH quality evaluations of fresh diced tomatoes packaged in chitosan/GSE pouches (0% and 30% GSE) stored at 7°C for 10 days

		Time (Days)			
Amount of GSE in film	Quality Parameter	0	3	7	10
0% GSE	Weight Loss (%)	0.0 a*	0.89 b	1.7 c	4.0 dA**
30% GSE		0.0 a	0.97 b	1.6 c	3.9 dA
0% GSE	Firmness (N)	168 a	132 b	137 b	78 cA
30% GSE		168 a	131 b	122 b	107 bB
0% GSE	pH	4.49 a	4.55 a	4.61 b	4.28 cA
30% GSE		4.49 a	4.59 b	4.59 b	4.52 aB

*Lowercase letters indicate significant differences between storage days (0, 3, 7, and 10 days) for each type of film (rows) ($p < 0.05$)
**Uppercase letters indicate significant differences between type of film (0 and 30% GSE) for each quality parameter (columns) ($p < 0.05$)

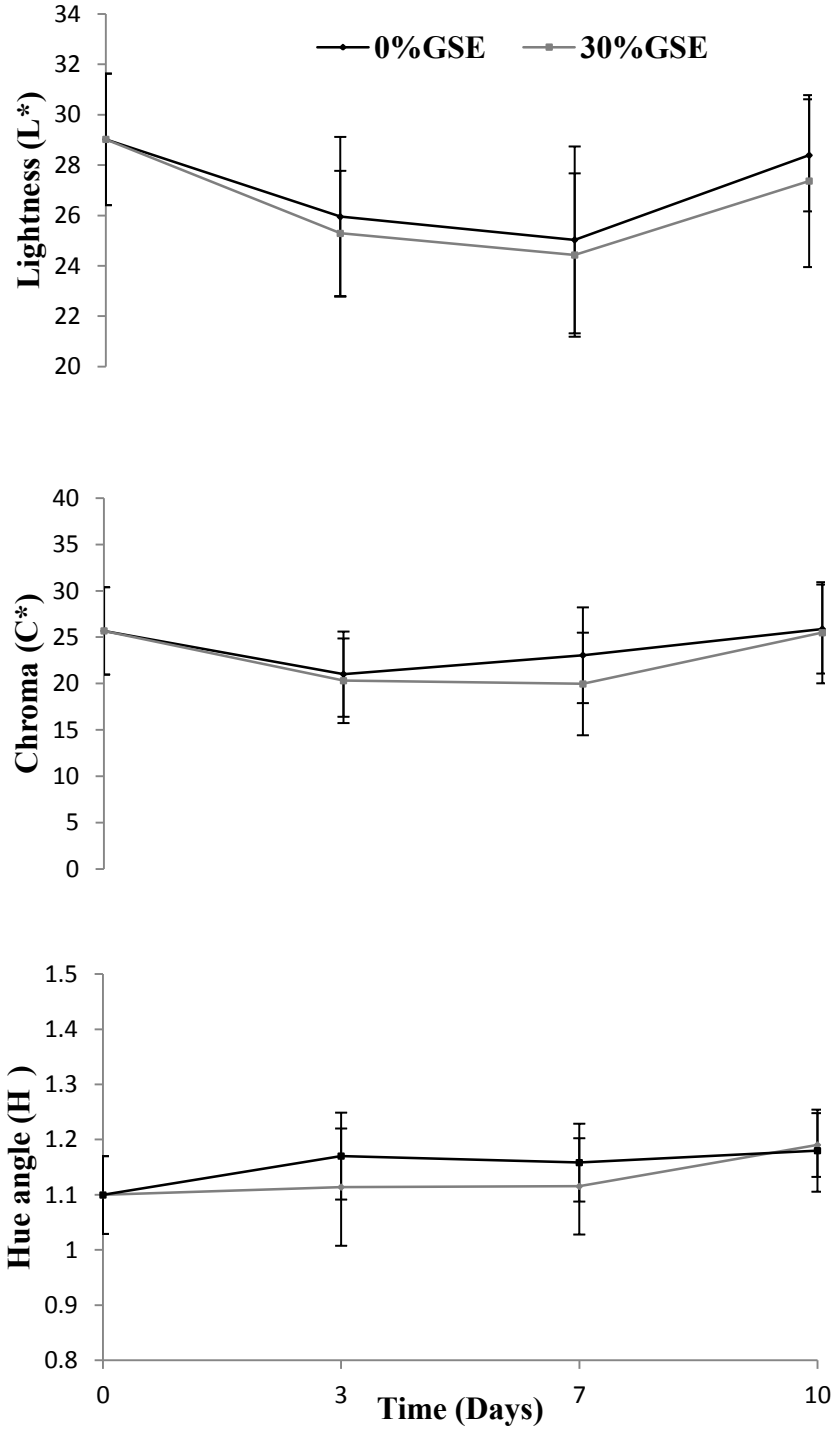

Figure 7. Color parameters a) Lightness, b) chroma, and c) hue angle of the skin of the diced tomatoes packaged in chitosan/GSE pouches (0% and 30% GSE) and stored at 7°C for 10 days

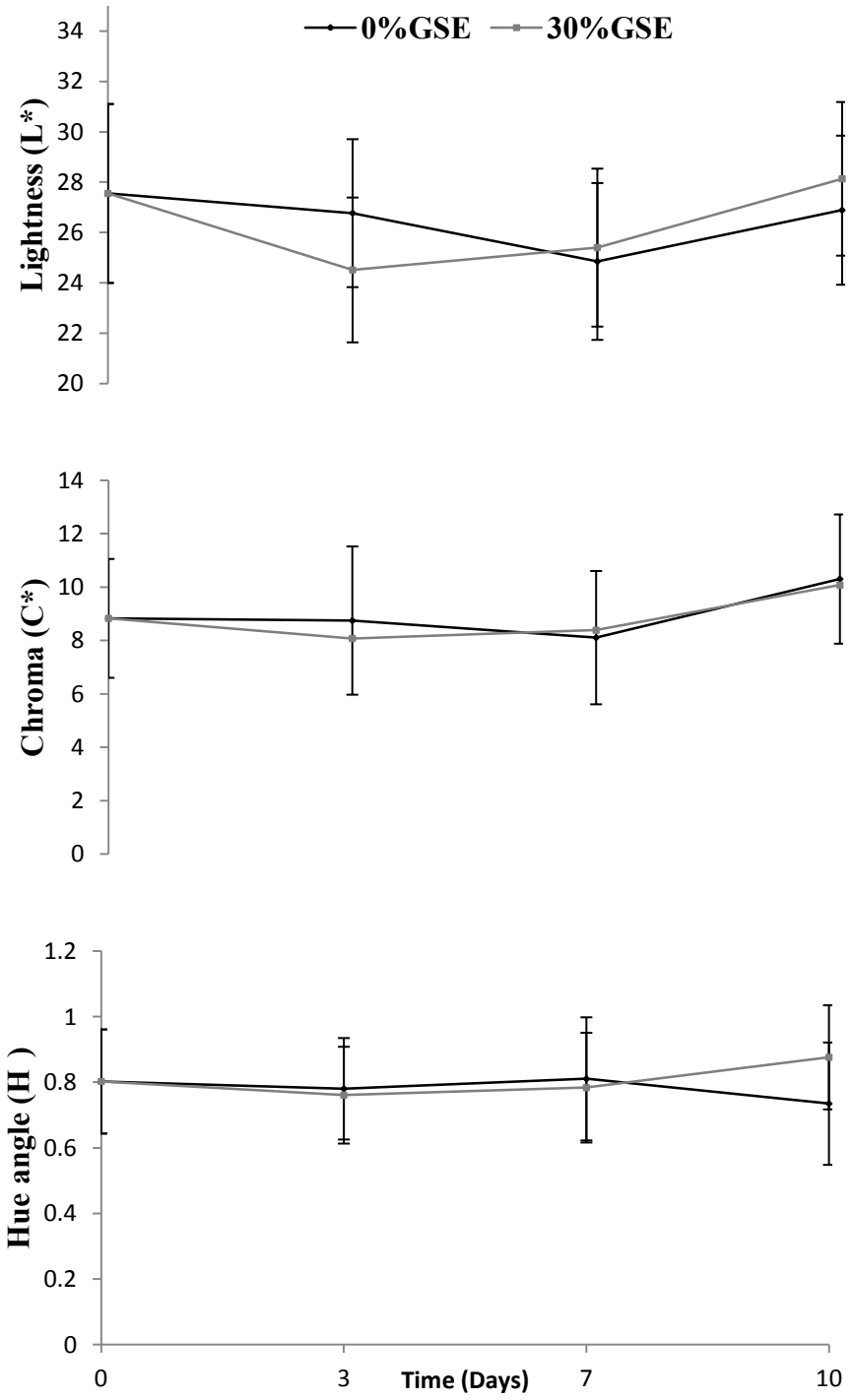

Figure 8. Color parameters a) Lightness, b) chroma, and c) hue angle of the flesh of the diced tomatoes packaged in chitosan/GSE pouches (0% and 30% GSE) and stored at 7°C for 10 days

QDA sensory evaluations

No significant differences were observed between the two treatments for any of the sensory attributes that were evaluated. This indicates that incorporating GSE into the chitosan coating does not negatively affect the sensory qualities of the diced tomatoes. Additionally, panelists were not able to perceive any off attributes associated with the chitosan coating without GSE. Significant differences in the intensity of the sensory attributes were perceived to be solely dependent on the storage time. The shelf life of the diced tomatoes for both treatments in chitosan coated PLA pouches was determined by trained panelists to be 10 days when stored at 4°C.

The intensity of the off aroma was found to significantly increase after 3 days of storage but still retained good quality throughout 10 days of storage. Good aroma quality descriptors identified by the panelists included fresh-cut grass, sweet, and raw potato. Over time the intensity of these good quality aromas had disappeared and off aroma descriptors of fruity and moldy became more intense. These negative aroma qualities became very prominent by day 14 of storage and signified a complete loss in product quality. A significant difference in the color of the diced tomatoes was identified only after 14 days of storage and the red color of the diced tomatoes was perceived by the panelists as being more intense. By day 14 of storage, diced tomato samples had visible mold growing on them and were not used for hardness, sweetness, or sourness evaluations because these tests required panelists to put the samples in their mouths. The mean values for hardness decreased over the storage time, but only became significantly different after day 3. The sweetness and sourness of the diced tomatoes did not significantly change throughout the storage. Finally, the overall appearance of the diced tomatoes became significantly different after 3 days of storage and did not change throughout day 10. By day 14,

the overall appearance became significantly more intense in the amount of defects, which were characterized by the mold growth, wet appearance and increased translucency of the flesh.

Currently, there are no studies involved with sensory evaluation of diced tomatoes packaged in PLA pouches with chitosan coatings so it is difficult to compare sensory results. However, whole tomatoes coated with different chitosan complexes showed no significant differences in color, texture, taste, flavor, and overall acceptability when compared to uncoated tomatoes (El-Beltagy et al. 2013). The sensory quality of different types of packaged cut tomato products other than dices has been evaluated in other work without chitosan (Antunes et al. 2013; Gil et al. 2002; Aguayo et al. 2004). Sensory evaluation of quartered tomatoes placed in plastic trays wrapped with polyvinyl chloride determined that the quality was preserved for 10 days when stored at 4°C (Antunes et al. 2013). In addition, the sensory quality of cut tomato slices was determined to be greatly affected by temperature showing better quality at 0°C vs. 5°C (Gil et al. 2002; Aguayo et al. 2004) and at 2°C vs. 10°C (Artes et al. 1999). Therefore it would be expected that diced tomatoes packaged in PLA pouches with chitosan coatings would exhibit different sensory qualities during storage when stored at different temperatures and better quality could be retained at a lower storage temperature.

Table 6. QDA sensory evaluation of packaged fresh-diced tomatoes stored at 4°C for days 14 days. Scores are based on 15-point scales (1- very slight to 15- very intense)

Sensory Attribute	Amount of GSE in film	Storage Time (Days)				
		0	3	7	10	14
Off Aroma	0% GSE	1.6 a*A**	3.2 bA	4.4 bcA	4.8 cA	11.6 dA
	30% GSE	1.6 aA	3.4 bA	3.9 bcA	5.6 cA	11.2 dA
Color	0% GSE	5.5 aA	5.8 aA	5.1 aA	4.9 aA	7.9 bA
	30% GSE	5.5 aA	4.1 aA	5.3 aA	5.1 aA	7.8 bA
Hardness	0% GSE	3.9 aA	2.8 bA	2.7 bA	2.3 bA	***
	30% GSE	3.9 aA	2.8 bA	3.0 bA	2.5 bA	***
Sweetness	0% GSE	2.3 aA	1.7 aA	1.9 aA	1.7 aA	***
	30% GSE	2.3 aA	2.1 aA	1.6 aA	1.9 aA	***
Sourness	0% GSE	1.4 aA	1.4 aA	1.5 aA	1.4 aA	***
	30% GSE	1.4 aA	1.4 aA	1.6 aA	1.5 aA	***
Overall Appearance	0% GSE	1.6 aA	3.7 bA	5.0 bA	5.2 bA	10.6 cA
	30% GSE	1.6 aA	4.1 bA	4.7 bA	5.9 bA	9.8 cA

*Lowercase letters indicate significant differences between storage days (0, 3, 7, and 10 days) for each type of film (rows) ($p < 0.05$)

**Uppercase letters indicate significant differences between type of film (0 and 30% GSE) for each sensory attribute (columns) ($p < 0.05$)

***These evaluations required panelists to place the samples in their mouths. Panelists did not evaluate the samples because of visible mold.

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