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# Natural and value-added approaches for pathogen control

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**Natural and value-added approaches for pathogen control**

by

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## ABSTRACT

Infectious diseases caused by established foodborne pathogens, multidrug-resistant bacteria and opportunistic fungal pathogens pose serious threats to both public health and the economy. The aim of this work was to provide new approaches based on natural or value-added agricultural materials to address these important threats. Chapter 1 of this thesis provides a general introduction and description of how the work is organized. Chapter 2 reviews the literature related to our topics of research and Chapter 8 provides general conclusions. The remaining chapters report on the various projects that were conducted on the topics of natural and value-added approaches for pathogen control. Specifically, the cationic vegetable oil-based polyurethane dispersions and coatings described in Chapters 3, 4 and 5 of this work displayed promising antimicrobial properties against multiple human pathogens of both clinical and foodborne concern. The antimicrobial properties of these coatings were determined using a variety of cultural and instrumental approaches. Cultural methods included disk diffusion tests, time course plating and broth microdilution assay using a Bioscreen C Microbiological Reader. Instrumental approaches included flow cytometry for determination of cell membrane permeability, fluorescence and light microscopies for determining changes in cell and colony morphology and spectrophotometry for measuring the release of intracellular compounds. The use of multiple antimicrobial testing strategies allowed us to more fully characterize the cellular effects of polymer treatment and to gain clearer insight into polymer mode(s) of action (MOA). An understanding of MOA may help identify potential benefits and limitations of the practical use of these polymers in medical or food-related environments.

Over the last three decades, *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* have emerged as the three most prevalent bacterial pathogens causing outbreaks in fruits and vegetables. Natural antimicrobial systems capable of inactivating these pathogens could provide attractive clean-label solutions for enhancing produce safety. In Chapters 6 and 7, antimicrobial hurdle systems comprised of GRAS natural antimicrobials having complementary or interactive modes of action were developed and examined. Systems containing Grape Seed Extract, long-chain sodium polyphosphate (polyP) and various organic acids were found to be effective against *E. coli* O157:H7 and *S.* Typhimurium in both broth culture and in lettuce extract, a simulant for the plant-based organic materials expected in produce processing waters and which may challenge the efficacy of applied antimicrobials. Data gathered using various methods demonstrated that use of polyP resulted in enhancement of GSE against *L. innocua* or *L. monocytogenes*. Lastly, pronounced differences in the antimicrobial efficacy of two commercial GSE's were observed, suggesting that intrinsic biological or preparative variations resulting in different levels of antimicrobially-active polyphenolic components might be important for practical use of this ingredient. A greater understanding of the biochemical basis of these variations, coupled with quality control and standardization is needed before GSE can be widely adopted by the fresh produce industry.

Together, the studies presented in this thesis provide natural and value-added approaches for pathogen control that may be useful in both medical and food production applications.

## CHAPTER 1. GENERAL INTRODUCTION

### Introduction

The emergence of multidrug-resistant bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) or *Acinetobacter baumannii* and opportunistic yeasts such as *Candida albicans* represent serious threats to both the public health and the world economy. As a community-acquired foodborne outbreak of MRSA demonstrates, line between organisms of clinical concern and those of importance to food safety is no longer as clearly defined as once thought *(5)*. New approaches for controlling the proliferation of these robust and difficult to control organisms in clinical or food production environments, such as the biorenewable vegetable oil-based coatings examined here, may be key to limiting the potential for their nosocomial or foodborne transmission.

Over the past decade, produce has emerged as the leading category for foodborne disease, being associated with 696 outbreaks (17% of total foodborne outbreaks) and 25,222 illnesses (24% of total foodborne illnesses) *(1)*. The most commonly identified pathogens in produce products were *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes*. Although chemical sanitizers such as chlorine and hydrogen peroxide are predominantly used, there is increasing interest in the fresh produce industry to use "greener" or more "natural" interventions considering the potential risk caused by chemical sanitizers *(4)*. Natural multicomponent antimicrobial systems capable of synergistically inactivating bacterial pathogens would provide attractive clean-label solutions for enhancing produce safety. The metal-chelating long-chain

polyphosphate BEKAPLUS FS has been found to enhance the antimicrobial effects of polyphenol-rich peanut skin extract and plant essential oils *(7)*. Other groups have also explored the combination of Grape Seed Extract (GSE) and organic acids for use in controlling bacterial pathogens in produce *(2)*. Although a promising approach, high levels of organic acids may have deleterious effects on produce quality *(6)*. We hypothesized that polyP could be used to enhance the antimicrobial effects of organic acids and/or Grape Seed Extract, which, like peanut skin extract, is also high in antimicrobial polyphenolic compounds. With this strategy, we hypothesized that we could be able to use lower concentrations of each individual compound while reducing negative organoleptic effects *(6)*.

In the work that comprises this thesis, the antimicrobial properties of both polyurethane coatings and the natural antimicrobial hurdle system were determined using a variety of cultural and instrumental methods, including disk diffusion, time course plating, Bioscreen-based broth microdilution, flow cytometry, light and fluorescence microscopy, and a colorimetric method for measuring the chelating activity of the antimicrobial polymer. These methods provided insight on the mode of action and cellular impact of the individual antimicrobials used, or on their combinations.

The overall goal of the work included in this work was to develop both natural and value-added means for control of pathogens in medical or food production environments or in fresh produce. The objectives developed to achieve this overall goal included: 1. To determine the antimicrobial effects of three kinds of vegetable oil-based cationic polyurethane coatings against foodborne or nosocomial pathogens using multiple antimicrobial testing methods; 2. To demonstrate the contribution of individual hurdle compounds towards inactivation of pathogens

and to demonstrate the ability of the system to inactivate pathogens in a fresh produce system and 3. To explore the mode of action of the various antimicrobials examined in objectives 1 and 2 using a diverse set of cultural and instrumental approaches.

## Dissertation organization

This dissertation is a collection of work that includes a literature review (Chapter 2) followed by five Journal articles (Chapter 3-7) and general conclusions (Chapter 8). Chapter 3 was published in ChemSusChem *(8)*. Chapter 4 was published in Macromolecular Materials and Engineering *(3)*. It is the author's intent to submit Chapter 5 to Polymer Chemistry. For Chapter 3-5, I have performed and reported the antimicrobial evaluation work for polymer coatings, while the rest of work was performed and reported by Ying Xia or Thomas Garrison. It is the author's intent to submit Chapter 6 to Journal of Food Protection. A poster based on this paper 'Natural antimicrobial system for inhibition of pathogens in fresh produce' was presented at United Fresh in Chicago, IL 2014 and an oral presentation was presented at Graduate and Professional Research Conference of Iowa State University. It is the author's intent to submit Chapter 7 to Journal of Food Protection. With exception of Chapter 1 and Chapter 8, references can be found at the end of each chapter and follow the format of each specified journal.

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## CHAPTER 2. LITERATURE REVIEW

### 1. Antimicrobials applied to fresh produce

Based on CDC foodborne outbreak data from 2002 to 2011, the Center for Science in the Public Interest reported that the top category for foodborne outbreaks was produce, with 667 outbreaks and 23,748 illnesses, accounting for 17% of total foodborne outbreaks and 24% of total foodborne illnesses over the period in question *(45)*.

### 1.1 Bacterial pathogens in fresh produce

### 1.1.1 *Escherichia coli*

*Escherichia coli* (*E. coli*) is a Gram-negative, nonsporulating, facultatively anaerobic, rod-shaped bacterium, and a normal inhabitant of the intestinal tract of humans and warm-blooded animals and birds *(154)*. Some *E. coli* are well known to provide many health benefits to the host, however, there are some groups of *E. coli* that can cause severe diarrheal diseases in humans *(54)*. Based on their ability to produce toxins, to adhere and to invade epithelial cells, pathogenic *E. coli* are subdivided into six groups, Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) *(54)*. The symptoms and mechanisms of infection caused by different groups of pathogenic *E. coli* are somewhat distinct and may also show some overlapping characteristics. Of these, contaminated food or water are well known sources for the first four pathogenic groups, among which EHEC is often implicated in major foodborne outbreaks.

**EHEC and *E. coli* O157:H7**

The EHEC group is a subset of Shiga toxin-producing *E. coli* (STEC) that is of special concern to food microbiology and medicine for two reasons. One is that foodborne transmission of EHEC is more common than with other diarrheagenic *E. coli*; the other is that EHEC cause hemorrhagic colitis which may lead to the life-threatening conditions Hemolytic Uremic syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) *(54)*. *E. coli* O157:H7 is currently the predominant EHEC serotype reported and accounts for about 75% of the EHEC infections worldwide *(54)*. The letter "O" in the serotype designation refers to the cell wall (somatic) antigen, while the letter "H" refers to the flagellar antigen. Other non-O157 EHEC serotypes are emerging as cause□of foodborne illnesses. The most common non-O157: H7 serotypes□isolated from clinical infections in the United States are O26, O45, O103, O111, O121 and O145, collectively known as the "Big Six" *(54)*.

EHEC are characterized by the production of Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) and the presence of the virulence factor Locus of Enterocyte Effacement (LEE) *(54)*. Stx1 shows 98% homology to the Shiga toxin (Stx) of *Shigella dysenteriae* Type I *(174)*. Stx1 and Stx are differentiated by three nucleotides and one amino acid and is neutralized by antibodies to Stx *(91)*.

*E. coli* O157:H7 possesses these three virulent factors Stx1, Stx2 and LEE pathogenicity island *(117)*. The infective dose of *E. coli* O157:H7 is estimated to be as low as 10 to 100 cells *(79)*. The food vehicles most often implicated in O157:H7 outbreaks are raw or undercooked ground beef and beef products. Produce such as lettuce, spinach, and alfalfa sprouts, is

increasingly being implicated in O157:H7 infections *(189)*. *E. coli* O145 is an emerging non-O157 STECs that can produce the same illness as *E. coli* O157.

**Major EHEC outbreaks associated with produce: O157:H7 and O145**

Fresh spinach, 2006

CDC *(31)* reported that a large multi-state *E. coli* O157:H7 outbreak associated with fresh spinach caused 199 people to be ill, 102 of whom were hospitalized, 31 whom developed HUS and three of whom died as a result of the infection. A cattle ranch nearby the spinach farm and contaminated irrigation water were considered to be two sources of the strain responsible for the outbreak *(42)*.

*E. coli* O145 in shredded romaine lettuce, 2010

In May of 2010, CDC *(32)* reported an outbreak of *E. coli* O145 in 5 states. Of the 30 people known to be affected, 12 were hospitalized and 3 developed HUS. Many clinical laboratories did not have abilities to test for *E. coli* O145 and limited public health surveillance data were available for it. Therefore, it was more difficult to identify than *E. coli* O157. Evidence implicated romaine lettuce from one processing facility as the source of this outbreak.

Romaine lettuce, 2011

In March of 2012, 58 persons infected with *E. coli* O157:H7 were reported from 9 states. Thirty-three people were hospitalized, and 3 developed HUS. It was determined that the same batch of lettuce harvested from Farm A was used to supply both the grocery store Chain A locations and the university campus in Minnesota *(33)*.

Organic spinach and spring mix blend, 2012

A multistate Outbreak of *E. coli* O157:H7 infections occurred from October to November 2012, was linked to pre-packaged organic spinach and spring mix blend produced by a produce company in Massachusetts. A total of 33 persons infected with *E coli* O157:H7 were reported from five states, with 13 hospitalized and two developing HUS *(35)*.

### 1.1.2 *Salmonella, S.* Typhimurium and major outbreaks

*Salmonella* is a non-spore forming, Gram-negative, rod-shaped, facultatively anaerobic bacterium belonging to the family *Enterobacteriaceae (54)*. The genus *Salmonella* includes over 2,500 serotypes, which are commonly referred to by their serotype names *(54)*. For instance, two common foodborne pathogens *S.* Enteritidis and *S.* Typhimurium are two serotypes of *Salmonella enterica* subsp. *enterica*. The current convention for *Salmonella* nomenclature is that reported by Brenner et al. *(19)*. The optimum pH for growth of *Salmonella* is around neutrality *(91)*. The optimal growth temperature is 35-37°C at neutral pH. *S.* Typhimurium and *S.* Enteritidis were shown to be able to grow at low temperatures in foods stored at 2-4°C *(3)*. It is generally recognized that *Salmonella* does not grow below a water activity of 0.93 *(59)*.

The virulence factors are *Salmonella* Pathogenicity Islands (SPIs), Type III Secretion Systems (TTSSs), some factors involved in epithelial cell invasion and induction of enteritis and macrophage survival and systemic infection *(143)*. SPIs often contain multiple genes essential for a specific virulence phenotype. For instance, SPI-1 encodes genes necessary for *Salmonella* to invade intestinal epithelial cells and induce enteritis. Whereas SPI-2 encodes genes related with intracellular replication. Like all the other *Salmonella*, the virulence genes for *S.* Typhimurium are those clustered within SPIs and the others chromosomally or on virulence plasmids, including the spv operon, flagella, adhesins, and essential components for biofilm

formation *(52)*. So far five SPIs have been reported to have a major contribution to pathogenesis of *S*. Typhimurium *(52)*.

*Salmonella* can cause two types of illness, non-typhoidal salmonellosis and typhoid fever. The symptoms of non-typhoidal salmonellosis can be nausea, vomiting, abdominal cramps, diarrhea, fever, headache, but this illness is generally self-limiting among healthy people with intact immune systems. It is usually caused by serotypes other than *S*. Typhi and *S*. Paratyphi A. Typhoid fever is more serious and has a higher mortality rate than does non-typhoidal salmonellosis, which is caused by *S*. Typhi and *S*. Paratyphi A *(54)*. *Salmonella* serotypes Enteritidis, Typhimurium and Newport are the top three in the genus causing foodborne illness outbreaks from 2006 to 2010 in the United States*(37)*.

**Major *Salmonella* outbreaks linked to produce**

*S*. Typhimurium in tomatoes, 2006

Based on a CDC report *(30)*, in this outbreak a total of 183 individuals were infected with *S*. Typhimurium from 21 states. All patients resided east of the Mississippi River except for a Washington state resident. Additionally, 2 patients were from Canada, one of whom had traveled to an affected state in the U.S. Of all patients with available clinical data, 22 (12%) were hospitalized. Analysis data indicated that tomatoes consumed at restaurants were the food responsible for this outbreak.

*S*. Braenderup in mangoes, 2012

According to a report from CDC *(36)*, this multistate outbreak 127 persons were infected with *S*. Braenderup and 33 were hospitalized. Investigation showed that mangoes imported from Mexico was the source of this outbreak.

*S*. Typhimurium and *S*. Newport in cantaloupe, 2012

CDC*(34)* reported that a producer southwest of Indiana was inspected due to an outbreak of salmonellosis caused by *S*. Typhimurium and *S*. Newport in summer of 2012. It was linked to fresh whole cantaloupe grown on this farm that resulted in 261 illnesses in 24 states, with 84 hospitalizations and 3 deaths. Based on the FDA report, the initial contamination of the cantaloupes may have occurred in the production fields and was spread to the packing house by operations and practices. The pathogens contaminating cantaloupes also likely proliferated during storage and transport to market.

*S*. Saintpaul in imported cucumbers, 2014

In June of 2014, CDC reported a total of 84 persons were infected with *S*. Saintpaul, with 17 hospitalized. Cucumbers from two suppliers were reported as the food that most of those stricken ate before illness.

**1.1.3 *Listeria*, *Listeria monocytognenes* and a major outbreak**

*Listeria* species are gram positive, facultative anaerobic, non-spore or capsule forming bacilli. Sometimes cells are coccoid and look like streptococci *(54)*. Some *Listeria* species have peritrichous flagella and are motile when cultured at 20-25°C, but show weak or no movement at 37°C *(101)*. Based on the data from phenotyping and genotyping method such as Pulsed-field gel electrophoresis, multi-locus sequence typing or whole-genome sequencing, eight *Listeria* species are currently recognized: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L marthii and L. rocourtiae (176)*. *L. monocytogenes* is the major human pathogen the genus.

Unlike many other pathogens, *L. monocytogenes* can survive and grow in high salt or low temperature (below 1°C). The maximum temperature for the growth of *Listeria* spp. is about 45□C. Optimum pH at which *L. monocytogenes* can grow ranges from pH 6 to 8 *(91)*. The minimum and maximum pH of for growth of this pathogen varies by strain, but some strains can survive in the wide pH range from 4.1 to 9.6 *(91)*.The minimum water activity $a_w$ at which *L. monocytogenes* can survive in foods is as low as 0.90 in certain conditions, which is lower than most foodborne pathogens *(176)*.

Key virulence factors for *L. monocytogenes* include, internalin (InlA, InlB), ActA , LLO and phospholipase C *(107, 176)*. The surface protein internalin (InlA) initiates epithelial cell invasion through binding to its receptor in epithelial cells. With surface protein InlB, *L. monocytogenes* is shown to invade different types of host cells including hepatocytes, fibroblasts, epithelial and endothelial cells. Another listerial surface protein, ActA, not only plays a role in internalin-independent uptake of *L. monocytogenes* by epithelial cell but also is a major virulence factor of actin-based intracellular motility. All isolated colonies *L. monocytogenes* on a blood-agar plate shows hemolytic activity due to listeriolysin O (LLO), which was recognized as another major virulence factor. LLO is a thiol-activated, cholesterol-dependent, pore-forming toxins. With cholesterol is its receptor, LLO assists *L. monocytogenes* in entering the cytosol from phagosomes without damaging the plasma membrane of the infected cell. Internalization of *L. monocytogenes* protects this pathogen from host immune responses *(176)*. There are two types of phospholipase C involved in host cell invasion by *L. monocytogenes* and spread to adjacent host cells: phosphatidyl-choline phospholipase C and phosphatidyl-inositol phospholipase C *(176)*.

Listeriosis is a serious bacterial infection, most commonly caused by *L. monocytogenes*.
It only accounts for a very small fraction of all illness caused by known foodborne pathogens,
but there is and increased clinical concern due to its high case-fatality rate and the expanding
population of immunocompromised individuals. Listeriosis carries a high mortality rate, with
27.6% of all bacterial foodborne disease deaths caused by listeriosis *(145)*. Listeriosis affects
primarily immunocompromised persons such as newborns, pregnant women, the elderly and
adults with compromised immune systems. Listeriosis in pregnant women may result in
stillbirth, fetal loss, neonatal infection or premature delivery. The rate of fetal mortality among
pregnant women diagnosed with listeriosis is between 16 and 45% *(145)*. It is now known that
almost all cases of human listeriosis are foodborne *(145)*. Therefore, in order to control human
listeriosis, it is of significance to understand how to control food contamination with *Listeria*.

According to the CDC, in 2011 a multistate outbreak of listeriosis linked to whole
cantaloupes from a Colorado farm infected 147 people, led to a 99% hospitalization rate (143
hospitalized) and 4 deaths, including 1 miscarriage. In September of 2011, after it was found that
cantaloupe was linked to this outbreak, the FDA issued several press releases on recall of
cantaloupe the producing farm as well as from food processors.

## 1.2 Sources of microbial contamination

The majority of microorganisms present on vegetables and fruits are spoilage bacteria,
yeasts and molds, but bacterial pathogens are not infrequent, causing numerous outbreaks
associated with fresh produce in the past two decades. Contamination of fresh produce by human
pathogens can happen at any point from farm to fork such as during production, preharvest- and
postharvest-handling, storage, transport and preparation *(76)*. On the farm, seeds, organic

fertilizers such as animal manure and slurry, contaminated soil and irrigation water contaminated by fecal material or sewage overflow are sources of direct contamination of vegetables and fruits *(128, 144)*. It was also reported that the type of irrigation system might also affect the microbial safety of fresh produce. Flood and spray irrigation has the greatest risk since water can directly flow onto produce leaves. Ground water is influenced by surface water and may also be vulnerable to contamination *(53)*. Solomon et al. *(167)* found that spray irrigation introduced much more *E. coli* O157 than surface irrigation on lettuce.

In addition, wild and domestic animals are potential reservoirs for human pathogens and may assist the spread of pathogen in the agricultural production setting. All these contaminant factors are dependent on and intertwined with each other and potentially threaten the safety of fresh produce. Field personal and employees handling fresh fruits and vegetables, the equipment, wash and hydrocooling water, the cold facilities and packaging materials may all serve as potential sources of contamination *(76)*.

## 1.3 Interactions between produce and pathogens (attachment, biofilm, infiltration and internalization)

Although some bacterial pathogens and spoilage organisms are removed by simple washing or killed by current sanitizing methods such as chlorine washing solution, a significant portion of these microbes are still able to survive and proliferate on the surface. Understanding the factors influencing the ability of pathogens to attach to, survive or grow on fresh produce is significant to develop appropriate sanitizing methods and antimicrobial agents and therefore improve food safety of raw ready-to-eat vegetables and fruits. These factors include attachment pathogen attachment, their interaction with plant tissues and other microorganisms, produce

characteristics (pH, leaching of nutrients, desiccation) and improper handling during processing, storage, transport etc. *(5, 131)*.

Attachment, as a first step of establishment of bacterial human pathogens on the plant surface, plays a significant role in contamination fresh produce. Some surface appendages such as fimbriae, flagella, pili, polysaccharides (EPS, LPS, CPS), and outer membrane proteins contribute to attachment of bacterial pathogens to plant surfaces *(184)*, which are also categorized as five attachment factors *(128)*. Considering their prominent surface location, the pili, flagella and fimbriae of human pathogens likely initiate attachment. For example, flagella might assist cells in moving along a surface until an optimal attachment site is recognized and a tight plant-microbe binding interactions are formed. It was reported that enterotoxigenic *E. coli* (ETEC) and *S.* Senftenberg use flagella to attach to intact salad leaves *(9, 165)*. *E. coli* O157:H7 and *S. enterica* can move on the plant surface resulting in the successful colonization around damaged regions *(44)*, where nutrient leakage and enzymolysis usually occur to facilitate the growth of pathogens *(5)*. In some bacterial systems, two kinds of interactions were identified for binding of bacteria to plant tissues: binding of bacteria to plant carbohydrates and attachment of complex bacterial polysaccharides to plant lectins. It is possible that human pathogens might combine these two strategies to build tight binding to plant tissue surfaces *(128)*. Additionally, cell surface charge and hydrophobicity of bacterial cells are likely to affect their adherence to the waxy cuticle of leaf surfaces *(24)*.

The interactions between plant microbiota and foodborne pathogen contribute to inhibition or colonization of pathogens in the phyllosphere (leaf zone) and rhizosphere (root zone) of fresh produce. Human pathogens must compete for resources with the resident

microflora present in these niches. Native plant microbiota may have competitive advantages over bacterial human pathogens. They grow fast, have highly efficient mechanisms for nutrient uptake, or produce antimicrobial compounds capable of inhibiting pathogens *(5)*. The epiphytic microbe *Enterobacter asburiae* was found to reduce *E. coli* O157:H7 20-30 fold when co-inoculated, suggesting that *E. aburiae* is more competitive for nutrients required by both itself and the pathogen *(43)*. However, when microorganisms use different carbon and energy sources, they might be able to coexist in the phyllosphere. In the same study, *Wausteria paucula* enhanced survival of *E. coli* O157:H7 by six-fold in the phyllosphere. A lack of overlapping substrate utilization profiles suggested that *W. paucula* did not compete directly with *E. coli* O157:H7 for nutrients *(43)*.

Schuenzel and Harrison *(162)* found 3% of produce epiphyte isolates produced inhibitory compounds that inhibited at least one of the following pathogens:, *E. coli* O157:H7, *Staphylococcus aureus, Listeria monocytogenes* and *Salmonella* Montevideo. Most of the inhibitory isolates were Gram-negative, including *Aeromonas* spp. and *Pseudomonas* spp. Antimicrobial peptides bacteriocins were the inhibitory substances found in Gram-positive produce epiphyte microbes. The mode of action was not clear. In another similar study *(93)*, the microflora isolated from fresh-cut spinach and lettuce had ability to inhibit *E. coli* O157:H7 and 97.7% of inhibitory isolates were Gram-negative bacilli including *Pseudomonas*, *Pantoea*, *Klebsiella*, *Enterobacter*, *Aeromonas*. Some of the inhibitory metabolites were found to be acid and antimicrobial peptides. Bacteria with the greatest antimicrobial activity against *E. coli* O157:H7 were isolated before treating lettuce and spinach using chlorine. This brings us to the concern that this treatment not only decreased the foodborne pathogens but also reduced the

population of beneficial antagonistic microflora. In another study of commercial lactic acid bacteria product LactiGuard$^{TM}$ *(26)*, during refrigerated storage, 8.0 $\log_{10}$ CFU $g^{-1}$ LAB of this product was found to inhibit foodborne pathogens *E. coli* O157:H7 and *S. enterica* on spinach by 1.6 and 1.9 $\log_{10}$ CFU $g^{-1}$ respectively. L-Lactic acid and a bacteriocin-like inhibitory substance were two antimicrobial metabolites produced by LactiGuard$^{TM}$. It is apparent that understanding the interaction between microflora on produce and foodborne pathogen would help us develop intervention methods to control human pathogens in fruits and vegetable.

The interactions of some human pathogens with the plant microflora may also be advantageous to their growth or survival after they migrate onto plants. Some human pathogens actively interacting with microflora and exopolymer matrix of fresh produce could aggregate on the surface and form biofilms under certain conditions. Biofilms may represent an important means by which pathogens may become established and persist on fresh produce surfaces. The biofilm environment may protect organisms from environmental stresses such as desiccation and antimicrobial treatment, or serve as an environment for gene transfer. *Salmonella* Thompson was found to form large heterogeneous aggregates with *Pantoea agglomerans* on the surface of cilantro leaves *(15)*. Further research needs to be done to determine the effects of the ecology of human pathogen heterogeneous biofilms on fruit and vegetable safety.

The exopolysaccharide layer of biofilm was produced by microbes is capable of maintaining water and nutrients for cell growth and also protect members from the natural or stressed environment. The physiological characteristics of bacterial cells within a biofilm are different from those cells isolated on the plant surface. The exopolysaccharide matrix can facilitate attachment to plant surfaces, supply nutrients to cells within a biofilm, maintain a pH

conducive to pathogen survival and absorb volatile compounds produced by the plant *(136)*. The bacteria in biofilms also work in cooperatively by degrading xenobiotic compounds and offering byproducts as a carbon source for other members *(136)*.

Biofilms on plant tissues may allow foodborne pathogens to survive or even grow in stressful environments and act as barriers to effective penetration of sanitizers. *Salmonella* Typhimurium biofilms on parsley reduced the effectiveness of chlorine disinfection after storage, not immediately after contamination, which suggested that the biofilm matrix did not contribute to initial adhesion and survival of *Salmonella* on parsley, but that pre-existing biofilms protected the cells inside from disinfection treatment *(111)*. Further study is needed regarding the impact of the biofilm environment on survival of pathogens on fresh produce surfaces.

The biofilm environment may also promote development of antibiotic resistance. *Klebsiella pneumoniae* cells became less sensitive to both ciprofloxacin and ampicillin in a biofilm. The slow growth and stressed status of cells within a biofilm may contribute to antibiotic resistance *(4)*. Brooun et al. *(21)* showed that this increased resistance is most likely not caused by multidrug resistant pumps, but by a small portion of super-resistant cells within the biofilm. These cells were resistant to increasing levels of ciprofloxacin. Although the mechanism was not clear, a subpopulation of super-resistant *Pseudomonas aeruginosa* cells contributed to increased resistance of biofilms to quinolones and tobramycin *(21)*.

## 1.4 Infiltration and internalization

Internalized microorganisms are those embedded within plant tissues. Once internalized, microbes are protected from environmental stresses and surface sanitizers and are not removed by simple washing. Natural openings such as stomata and lenticels, the blossom end and stem

scar can provide entry points into deeper tissues. Damage by other microbes or insects and birds may facilitate infiltration of bacteria into the inner tissue of fresh produce *(131)*. Golberg et al. found that *Salmonella* Typhimurium was able to penetrate iceberg lettuce leaves through open stomata, displaying a high degree of leaf internalization *(73, 106)*. Hou et al. studied the ability of epiphytes to become internalized in lettuce leaves. The results showed that plant epiphytes were frequently detected in surface-sterilized iceberg leaves *(87)*.

Four pathways for internalization have been described for fresh produce, including by aerosol, water channels, through wounds and by aqueous cell suspensions *(7)*. Aerosols of bacteria and viruses may be able to move long distances and enter through stomata on leaves. Some fresh produce surface apertures such as stomata serve as a water channel, which provide an entrance for microorganisms. Wounds release fluid from damaged vacuoles and plasmalemma, and also connect the intercellular air-space of the plant with the surrounding environment. The cell sap released from ruptured cells creates instant fluid channels, which facilitates the internalization of bacterial pathogens. Compared with intact fresh produce, fresh-cut fresh produce has more readily available nutrients and higher water activity, which support the growth of a variety of foodborne pathogens. Many studies indicated that bacteria including pathogens in water or aqueous suspensions of bacteria could infiltrate into fresh produce through the surfaces during harvest and handling leading to internalization of bacteria in deeper tissues. The cooling of fresh produce results in a reduction of gas pressures in the apoplast, which leads to the pressure differential between leaf surface and internal space. This pressure differential might be a reason for infiltration of bacteria. In addition, submerged vegetables or fruits at the bottom of containers would be exposed to a hydrostatic pressure, which not only force water directly into surface apertures of fresh produce products, but also squeeze them and then cause air to bubble

out of natural openings on the surface. Once this hydrostatic pressure is removed, the products would expand back to its original size and water with bacteria would be drawn into inside tissue *(7, 131)*. Various studies found that a transient pressure caused by immersion of warm fruits in cold water can lead to infiltration of bacteria *(8, 23, 24)*. Additionally some other factors might affect bacterial infiltration into fruits and vegetables such as the addition of surfactants as well as the type of cooling system.

From a food safety perspective, after human pathogenic bacteria are internalized in those products, the bacteria need to survive in fresh produce to pose a heath hazard to people. Several factors play important roles on the survival and growth of internalized bacterial pathogens such as physical and chemical characteristics of the fruit and vegetable, type of bacteria, conditions of postharvest processing and handling. In order to prevent and control the bacterial infiltration in vegetables and fruits, optimum conditions of temperature and hydrostatic pressure must be used.

**1.5 Prevention and control of contamination**

In October of 1997, President Bill Clinton announced a produce safety initiative, entitled "Initiative to Ensure the Safety of Imported and Domestic Fruits and Vegetables". In response to this directive, the FDA and USDA issued "Guidance for Industry - Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" *(53)*.  This is a voluntary, science-based program focusing on best practices in production and packing of fresh produce. To reduce, eliminate or control microbial food safety hazards of fresh and fresh-cut produce, effective multidisciplinary intervention strategies are needed, farm-to-fork. Strategies included in this review include HACCP, chemical and physical approaches and use of natural antimicrobials.

### 1.5.2 Chemical Sanitizers

Some chemical sanitizers, such as free chlorine, chlorine dioxide ($ClO_2$), hydrogen peroxide ($H_2O_2$), and peracetic acid (PAA) are oxidative biocides. They pass easily through cell walls and membranes and react with internal cellular components, leading to apoptosis and necrotic cell death within host tissues. Additionally, they can also damage microbial structures and cause the cells to release intracellular components *(60)*.

**Chlorine**

Chlorine is currently the predominant sanitizer used in produce industry, as it is relatively inexpensive and effectively kills a broad range of pathogens. It also leaves very little residue on produce surfaces. The widely used forms are sodium hypochlorite, calcium hypochlorite, and liquid chlorine. Recommended concentrations range from 50 to 200 ppm, with treatment times of 1-2 minutes *(11)*. The reductions of 1 to 3 $\log_{10}$ CFU/g are achievable when using washing solutions containing approximately 20-200 ppm free chlorine*(5)*.

The pH, temperature and the presence of organic matter alter the effectiveness of chlorine. The form of free available chlorine is Hypochlorous acid (HOCl). The equilibrium between hypochlorous acid and the hypochlorite ion ($OCl^-$) is dependent on pH of the solution. Typically, pH ranging from 6.0 to 7.5 is used in sanitizer solutions in order to prevent corrosion of equipment and maintain chlorine efficacy at the same time *(146)*. Solubility is maximal at 4°C. However, in order to prevent the infiltration of pathogens into the produce, the temperature of chlorine washing solution should be kept at least 10°C higher than that of the produce itself *(11)*. Because chlorine can react with organic matter to form potentially carcinogenic trihalomethanes (THMs), alternatives to chlorine have been investigated *(51, 75, 139)*

Alternatively, the chlorine stabilizer T-128 has been studied for its ability to enhance the efficacy of chlorine. Nou and others *(140)* reported that it decreased the depletion rate of free chlorine in soil solution and significantly reduced the population of *S. enterica* and *E. coli* O157:H7 in the presence of high organic loads (0.25% to 2.0% lettuce extract). In the same study, the cross-contamination was found to occur between *E. coli* O157:H7 and *Salmonella* on iceberg lettuce due to depletion of free chlorine in the presence of lettuce extract. At a concentration of 0.05% or 0.1%, T-128 effectively reduced this cross-contamination. T-128 was also reported, when combined with free chlorine, to significantly enhance inactivation of *Salmonella* or *Pseudomonas* in biofilms on stainless steel *(166)*.

**Chlorine dioxide (ClO2)**

Chlorine dioxide has been studied and used as a sanitizer for fruits and vegetables since it was allowed for use in washing fruits and vegetables by the Food Drug and Administration *(53)*. It can denature the proteins primarily because it can covalently modify their tryptophan and tyrosine residues *(142)*. Unlike free chlorine, chlorine dioxide is not affected by pH because it does not hydrolyze in water to form HOCl. The presence of organic matter does not decrease its efficacy as observed in chlorine solutions.

Chlorine dioxide can be applied in aqueous or gaseous form. The effectiveness of gaseous $ClO_2$ was evaluated to extend the shelf life of minimally processed carrot, lettuce, and cabbage*(74)*. The $\log_{10}$ CFU/g reductions of *Salmonella* achieved on blueberries, strawberries, and raspberries were 2.4 to 3.7, 3.8 to 4.4, 1.5, respectively, with the treatment of 8.0 mg/liter of $ClO_2$ gas *(173)*. Trinetta et al. *(181)* used high-concentration-short-time $ClO_2$ gas to treat

pathogens inoculated on tomatoes, cantaloupe and strawberries. With the treatment of 10 mg/l $ClO_2$ for 180 s, *Salmonella* was reduced by 5 $log_{10}$ CFU /$cm^2$ on three produce surfaces, while *E. coli* and *Listeria* were reduced by 3 $log_{10}$ CFU /$cm^2$. Considering convenience and health concerns, chlorine dioxide- releasing films were developed to reduce *Salmonella* spp. and *Escherichia coli* O157:H7 inoculated on grape tomatoes by more than 3 logs *(155)*.

The main disadvantage of $ClO_2$ is that there is a maximum allowable concentration of 3 ppm in whole produce and post-application rinsing with potable water is needed, making the use of $ClO_2$ on fresh produce less desirable *(56)*.

**Hydrogen peroxide ($H_2O_2$)**

Hydrogen peroxide is a strong oxidizer and can damage cellular components, especially the cell membrane, leading to bacterial death *(154)*.The use of $H_2O_2$ on whole and fresh-cut produce has been investigated in recent years. $H_2O_2$ produces no residue since catalase in most fruits and vegetables can breakdown residual levels to water and oxygen.

When lettuce leaves were treated with 2% $H_2O_2$ at 50°C, the population of *E. coli* O157:H7 and *Salmonella enteritidis* were reduced by 4 $log_{10}$ CFU per leaf, 3 $log_{10}$ CFU per leaf respectively *(118)*. The treatment of fresh cut cantaloupes with 2.5% hydrogen peroxide for 2 min reduced the total bacterial numbers by 2.6 $log_{10}$ CFU/$cm^2$ *(183)*. However, in another study, hydrogen peroxide (3%) only reduced *Salmonella enterica* on organic leafy greens by less than 1 log CFU/g *(135)*. When combined with 37.8 mJ/$cm^2$ UV light at 50°C, 1.5% $H_2O_2$ reduced surface-associated *Salmonella* by around 4.00 $log_{10}$ CFU and internal counts by 2.80 $log_{10}$ CFU, which was significantly higher than $H_2O_2$ or UV alone*(83)*. The disadvantage is that $H_2O_2$ can

cause extensive browning of some produce like berries, and mushrooms, lettuce, and impact their quality.

**Peroxyacetic Acid (PAA)**

Peroxyacetic acid is a strong oxidizer made from hydrogen peroxide and acetic acid. Unlike chlorine, it is stable in the presence of organic matter in aqueous solution and not corrosive to equipment. PAA is most effective in acidic environments with pH between 3.5 and 7, but activity decreases rapidly at pH above 7. Besides inappropriate pH, high temperatures and metal ion contamination also decrease its effectiveness. Peroxyacetic acid has been used as a sanitizer for vegetables and fruits for years. Samadi et al. *(159)* found 2.8 logs reduction of the total mesophilic bacteria on mixed raw vegetables were achieved after treatments with peroxyacetic acid of 100 ppm for 15 min. The efficacy of PAA to reduce the natural flora depends on the type of produce. The reduction of 0.4 to 3.5 log10 CFU/g were achieved for carrots, iceberg lettuce, and fresh-cut leek, white cabbage *(188)*. The combination of PAA with other chemicals showed better effectiveness against microorganisms. Grace Ho et al. *(77)* found that in comparison with PAA, lactic acid or chlorinated water alone, a novel sanitizer containing peroxyacetic acid and lactic acid (LA-PAA) achieved a more than 8 log reduction of *L. innocua* and *L. plantarum,* which showed synergism existed between LA and PAA in terms of antimicrobial activities. In addition, there are some commercial sanitizers developed based on PAA and $H_2O_2$, such as Tsunami, SaniDate 5.0, SaniDate 12.0, and StorOx 2.0. Kim and others *(103)* reported that larger than 4.00 log CFU/apple of *E. sakazakii* reduction was obtained after the treatment of 40 µg/ml Tsunami 200 for 1 min.

**Acidified sodium chlorite (ASC)**

Acidified sodium chlorite is a combination of sodium chlorite and citric acid in aqueous solution with broad-spectrum germicidal activity. It has been approved for use on fresh produce and the other food products like meat, poultry and seafood with the concentration ranging from 500 to 1200 ppm *(55)*. Ruiz-Cruz et al. *(158)* studied the efficacy of acidified sodium chlorite in reducing *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* populations on fresh-cut carrots. They found that ASC was more effective than chlorine or peroxyacetic acid treatments in reducing pathogens. One hundred, 250, and 500 ppm of ASC reduced the populations of inoculated pathogens by 1, 1.5, and 2.5 $\log_{10}$ CFU/g, respectively. ASC of 100 to 500 mg/L had the same antimicrobial ability against the native microflora and *Escherichia coli* on tatsoi baby leaves as NaClO, sensory quality attributes remained unchanged at 5°C but not at the abusive temperature of 10°C during 11 days of shelf life *(180)*. Elena et al. *(48)* reported that *in vitro*, *E. coli* was reduced by $4.3 \pm 0.9$ log and $7.8 \pm 1.7$ $\log_{10}$ CFU/mL with a 3 min treatment of 100 mg/L NaClO and 20 mg/L ASC (pH 4.6), respectively. However, there was no significant difference between these sanitizers for *E. coli* when tested in lettuce and spinach, likely due to the presence of organic matter.

**Quaternary ammonium compounds**

Quaternary ammonium compounds ("quats") are cationic antimicrobial surfactants. Compared with other chemical sanitizers, they are noncorrosive, relatively stable at high temperatures and in the presence of organic matter, and are able to penetrate food contact surfaces. Because of their surfactant activity, quats are able to form a residual antimicrobial film

on hard surfaces. Polymers containing quaternary ammonium salts exhibit high antimicrobial activity *(99)*. In addition, antibacterial soybean-oil-based cationic polyurethane (PU) coatings incorporating ammonium cations developed by Xia et al. *(193)* had inhibitory effects against *L. monocytogenes* NADC 2045, *S.* Typhimurium ATCC 13311 and *S.* Minnesota R613. However, the effectiveness of quats varies according to microorganism type. Feliciano et al. *(58)* reported that 200 ppm quaternary ammonium (OASIS 146 Multi-Quat, Ecolab, Saint Paul, MN) was less effective in reduction of murine Norovirus (MNV-1) in milk and virus stock suspension than was 200 ppm chlorine (sodium hypochlorite).

### 1.5.4 Natural antimicrobials

**Organic acids**

Acids are useful in controlling many types of microbes as many cannot grow well at pH values below 4.5. Organic acids may also possess bactericidal abilities. Weak organic acids have better inhibitory activities than strong acids since their hydrophobic properties allow the undissociated form to penetrate the cell membrane and influence intracellular pH *(177)*. Their antimicrobial activity depends on their concentration, the pH of the solution and the ratio of protonated form vs. deprotonated form.

Some organic acids, such as lactic, citric and acetic acids not only act as food preservatives, but also as sanitizing agents for reduction of microorganism numbers on fresh produce. *S.* Poona on the surface of cantaloupe was reduced by 2.5 $\log_{10}$ CFU/cm$^2$ after treatment with 2% lactic acid for 2 min *(187)*. Neal et al. *(139)* found that the reductions of population of *E. coli* O157:H7 and *Salmonella* were 2.7 $\log_{10}$ CFU/g, 2.3 $\log_{10}$ CFU/g, respectively with the treatment of 2% L-lactic acid for 2 min at 55°C on spinach (*Spinacia*

*oleracea*) leaves. Park et al. *(147)* studied the antimicrobial effect of organic acids against a pathogen cocktail of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on whole red organic apples and lettuce. After treating the apples with 1% and 2% organic acids for 10 min, the $\log_{10}$ CFU/apple reductions of the pathogen cocktail were 0.52 to 2.78, 1.69 to larger than 3.42, 1.52 to larger than 3.42 by treatment of acetic acid, lactic acid, and citric acid, respectively. For lettuce, the $\log_{10}$ CFU/g reductions of acetic, lactic, and citric acid were 1.13 to 1.74, 1.87 to 2.54, 1.85 to 2.86, respectively. However, Fouladkhah et al. *(62)* did not find a significant difference in capability between a water control treatment and 5% acetic acid for reduction of original microflora on cantaloupe melons at 25°C.

Recently, the combinations of different organic acids were studied to test their inhibitory activities. A greater than 4.95 log reduction of *Salmonella enterica* on tomato stem scars was achieved with the treatment of 5.1% total combination of acetic, lactic, and levulinic acids, or with the treatment of 6% combination of lactic acid and acetic acid *(81)*. However, 0.25 g/100 g citric acid and 0.50 g/100 g ascorbic acid treatments at 10°C for 2 min were not effective in removing biofilms of *Listeria monocytogenes* and *Escherichia coli* on lettuce, indicating a need to further understand mechanisms of attachment for biofilms on produce surfaces *(197)*.

**Plant Extracts Rich in Polyphenols (PERP)**

Consumers are becoming more concerned about the potential harm of chemical sanitizers. This concern has motivated research on natural antimicrobials for control of microorganisms on fresh produce. A large amount of agricultural by-products are plant tissues rich in phytochemicals, which may have chemical and biological significance. Plant polyphenols have a variety of potential health benefits such as antioxidant, antiviral and antimicrobial

properties *(80)*. The high cost of isolation, purification and examination of these chemicals have led to increased interest in inexpensive plant extracts such as Grape Seed Extract (GSE) from agricultural waste streams.

PERPs such as GSE, apple or olive extracts have also been studied to inhibit pathogens on leafy greens. Moore et al. *(135)* studied the inhibitory effects of apple extract, hibiscus concentrate, olive extract, and hydrogen peroxide formulations against *Salmonella enterica* on organic leafy greens. Olive extract exerted the greatest effectiveness, achieving 2 to 3 $\log_{10}$ CFU/g reductions at 1, 3, and 5% concentrations for four types of organic leafy greens (organic romaine, iceberg lettuce, organic adult and baby spinach). Apple extract reduced 1 to 2 log CFU/g of *Salmonella* on day 3 on various leafy greens. Hibiscus concentrate achieved an overall 1 log CFU/g reduction for all leafy greens. In contrast, the maximum reduction by hydrogen peroxide (3%) was only about 1.0 $\log_{10}$ CFU/g. The bactericidal activities of the health-promoting food-based plant extract powders (nutraceuticals) were also studied against the foodborne pathogens. The bactericidal activities (BA50) was defined as the percentage of the sample in the mixture solution required to achieve 50% decrease in CFUs of bacterial culture. BA50 of apple skin extract, olive pomace and GSE against *S. aureus* were 0.002%, 0.008%, and 0.016%.

**Grape Seed Extract (GSE)**

GSE is by-product of wine and juice processing and rich in polyphenols, the most promising type of natural antimicrobials and antioxidants. 60-70% of the grape's total phenolic compounds is reported in the seeds, which made GSE to have antimicrobial activities*(38)*. Gladine et al. *(72)* found, compared with rosemary extract, citrus extract and marigold extract,

grape extract had the highest polyphenols content and strongest reducing potential. Three percent grape seed extract (GSE) exhibited the maximum antimicrobial ability against *E. coli* O157:H7 (3.8 log CFU/g reduction) on spinach and iceberg lettuce when using electrostatic spraying at day 14 of storage *(67)*. Bisha et al. *(14)* also found MIC of GSE against *L. monocytogenes* Scott A was as low as 50 µg/mL in aqueous solution. In the same study, *L. monocytogenes* inoculated on tomato surfaces was reduced by about 2 log units with the treatment of 0.125% GSE for 2 min.

The polyphenols determined for a variety of grape seeds were mainly of low molecular weight. The most abundant polyphenols in GSE are monomeric flavan-3-ols (catechin, epicatechin, epicatechin 3-O- gallate), followed by dimeric flavan-3-ols (also called condensed tannins or procyanidin B1, B2) and trimeric flavan-3-ols. They collectively accounted for more than 96% of the total polyphenolic compounds in grape seed *(80, 116)*. Knowing the conformational and geometrical feature is of great significance to understand the mode of action of antioxidant and antimicrobial activities. These polyphenolics contain multiple aromatic rings attached with multiple hydroxyl groups and other additional function groups like C=O carbonyl group and carbon carbon double bond. Figure 1 showed the structures of monomeric (Catechin and epicatechin) and dimeric flavan-3-ols (Procyanidin B1 and B2) commonly found in GSE. The planar structure allows conjugation and electronic delocalization. Those attached groups donate H atoms to scavenge free radicals during hydrogen atom transfer and electron transfer process, provide sites for metal ion chelation (hydroxyl and carbonyl groups), which have an influence on antioxidant, antimicrobial abilities of GSE *(114)*.
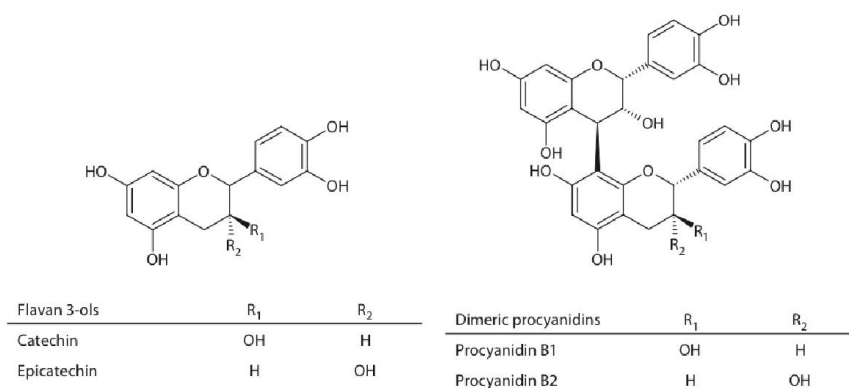
Fig 1. The structures of common polyphenols in GSE *(96)*

**Metal chelation by polyphenols and GSE**

The chemical structure of polyphenols such as number of phenolic hydroxyl groups and their position play an important role on chelating activity *(86, 133)*. The deprotonated phenolic groups allow an oxygen possess a high charge density forming a "Hard ligand" with metal cations. A pyrone oxygen may also bind metal ions because of its partial delocalization of the lone pairs. While some polyphenols with complex structure such as glycosylated hydroxyl group of phenol lose its ability to bind metal ions. Therefore, some polyphenols have multiple binding sites for metal chelation and are capable of polymerization and oligomerization *(86)*.
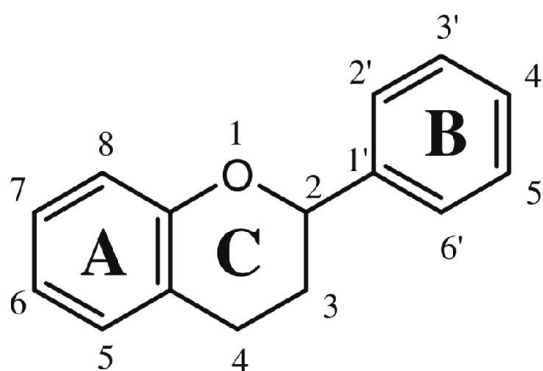


Fig 2. The typical flavan ring structure of polyphenols in GSE *(133, 149)*

Mladenka et al. *(133)* had even more detailed explanation of the relationship between chemical structure and iron chelation binding sites. The most abundant polyphenols in GSE shown in Fig 1 share a typical structural three-membered flavan ring system (Fig 2.), which was also common by most polyphenolic compounds. In their findings they concluded that 6,7-dihydroxy conformation was the most effective iron binding site of flavonoids, which was structurally similar to deferoxamine, a clinically used iron chelator. The 3-hydroxy-4-keto structure and the catecholic B ring were associated with a substantial iron chelation at neutral condition. Kim et al. *(102)* and Ma et al. *(126)* found (-) -epigallocatechin-3-gallate and GSE bioactive dietary polyphenols inhibit heme iron absorption mainly by decreasing iron export, possibly by forming nontransportable complexes with iron through metal chelation. Karamac *(98)* found that $Cu^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ were able to chelate nuts tannin, which offered the possibility that other metal ions might have chelation ability with polyphenols or GSE.

**Polyphosphate (polyP)**

Inorganic polyP is a linear or cyclic polymer of several to even hundreds of orthophosphates linked by high-energy phosphoanhydride bonds. It ubiquitously exists in every cell of bacteria, fungi, plants and animals as an important intracellular compound with numerous and varied biological functions, which serve as a significant regulatory physiological role in the growth and adaption to environmental stress for the cells *(105)*. PolyP kinase is the enzyme to synthesize and metabolize polyphosphate. Kornberg and others *(105)* indicated that this enzyme is essential for virulence of *Salmonella enterica* species and other pathogens.

Exogenous polyP is a widely used food additive with a variety of functions such as thickening agent, buffering agent, emulsifier and cation sequestrant due to polyP's water binding capacity and its metal ion chelation ability*(186)*. Food and Drug Administration has listed it as GRAS (generally recognized as safe) food additive with antimicrobial properties to prevent spoilage of food *(57)*. A large number of polyphosphates with different chain lengths from pyrophosphates to hexametaphosphates have numerous applications in cosmetics and foods. The major function in foods is to increase dispersion of food constituents and food stability. They can also serve as chelating agents in cosmetic formulations. Pyrophosphates are compounds with two P atoms and tripolyphosphates have three P atoms while the long chain polyphosphates such as sodium tetrametaphosphate (STMP) and sodium hexametaphosphate (SHMP) are with four or more P atoms *(115)*. The long chain polyphosphate are not pure compounds, instead they are actually mixtures of different phosphates with different chain-length *(49)*.

Recently polyP has attracted considerable attention as direct antimicrobial agents *(112, 127, 134, 141)* or as an indirect antimicrobial enhancer to increase the sensitivities of bacteria to hydrophobic drugs that do not permeate through the outer membrane of gram-negative bacteria *(186)*. This is hypothesized to occur dud Due to the outer membrane barriers, some hydrophobic antimicrobials are less effective against gram-negative bacteria than against gram-positive bacteria. Because polyP acts to chelate structurally important divalent cations present in the outer membrane, polyP can be expected to more inhibitory against gram-negative bacteria. The mode of action of antibacterial ability of polyP is that it has highly charged anionic nature and therefore enables to chelate structurally essential multivalent metal ions such as $Ca^{2+}$ and $Mg^{2+}$

of the cell wall, resulting in unstable and permeabilized cell walls and damaged cell membrane *(104)*. Consequently intracellular macromolecular compounds protein and DNA would be released causing cell lysis *(112)* . Wazer and Campanella *(190)* reported, based on dissociation constant of the complex between polyphosphate (n=5) and metals, the complex stabilities for the cations in their studies were $Mg^{2+} > Ca^{2+} > Fe^{2+} > Zn^{2+} > Mn^{2+}$. It indicated that the antibacterial ability of polyphosphate could be reversed by the addition of polyvalent metal ions $Mg^{2+}$, $Ca^{2+}$ and $Fe^{3+}$*(50, 113, 195)*, which further confirmed that antibacterial ability is due to chelation with these metal ions structurally and functionally essential in cell walls.

A well-know metal ion chelator EDTA can damage the outer membrane of gram-negative bacteria and increase uptake of other antimicrobial agents such as lysozyme and hydrophobic antibiotics, dyes and detergent *(185)*. Without EDTA those antimicrobials are not effective against bacteria. Similarly polyP, also as a chelator, also has ability to permeabilize the outer membrane by removing $Mg^{2+}$ and $Ca^{2+}$*(186)*.

**Bacterial cell wall structure and metal ion chelation**

Both Gram-positive and Gram-negative bacteria are protected by a complex cell envelope that performs a variety of functions. To develop effective antimicrobial agents, the structure of cell wall is essential and important to know. Gram-positive bacteria posses densely functionalized peptidoglycan (PG), the most abundant PG-linked glycopolymers- wall teichoic acids (WTA) extended through and beyond the cell wall and lipoteichoic acid (LTA) anchored to the plasma membrane*(22, 171)*. WTA has been thought as possible targets for novel antimicrobial agents such as antibacterial drugs and vaccines*(172, 191)*. WTA has high affinity

to bind divalent metal ions such as $Mg^{2+}$, $Ca^{2+}$ *(100, 109, 178)*, which are structurally and functionally essential for growth and survival of bacteria. Similarly, the lipopolysaccharide (LPS) layer of Gram-negative bacteria has a highly anionic density and therefore has been implicated as the major binding sites for metal ions such as $Mg^{2+}$ and $Ca^{2+}$, which are essential components of cell wall structure *(12, 110)*. It is proposed that, the agents that are able to bind and steal metal divalent ions from WTA-metal ions complex in Gram-positive bacteria or from LPS layer attached to outer membrane of Gram-negative bacteria and therefore permeabilize their cell walls, could act as antimicrobial agents. Two examples are sodium polyphosphate and GSE used in this study.
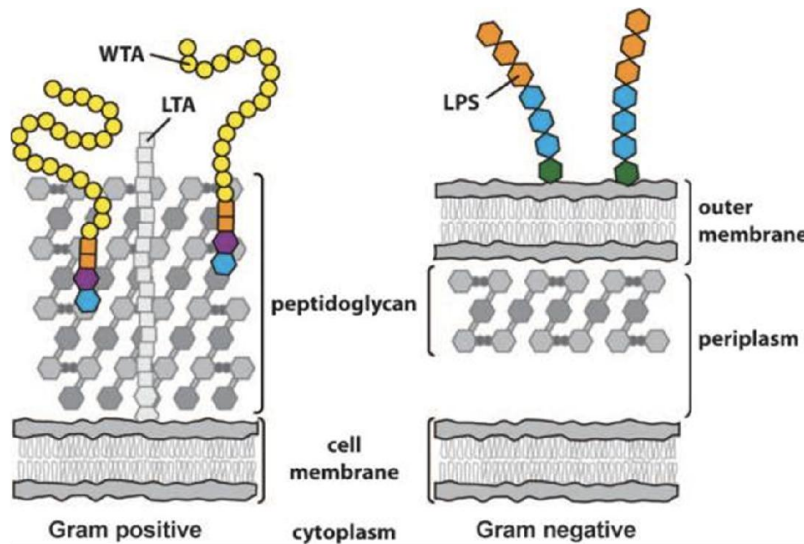


Fig 3. Cell wall structures of Gram-positive and Gram-negative bacteria *(171)*

**Plant Essential Oil**

Plant essential oils (EOs), as another type of alternative naturally derived antimicrobials, have attracted the attention of researchers and have been studied in different vegetables and fruits

in recent years. The antimicrobial effects of mint and basil EOs were studied against

*E. coli* O157:H7 and *S.* Typhimurium inoculated on lettuce and purslane during refrigerated

storage by Karagözlü et al. *(97)*. Both EOs at concentration of 0.08 ml/L were the most effective

antimicrobial treatment against two pathogens in these two vegetables. Most antibacterial studies

of EOs were conducted during the washing step, Ponce et al. *(151)* performed different

application such as spray, immersion and lactose capsule applications for tea tree, clove and

rosemary EOs on processed romaine lettuce. They found the romaine lettuce maintained

accepted quality after spray application during the entire storage period. Azizkhani et al. *(6)*

found among those tested EOs, Zataria multiflora Boiss (zataria) (10%) exerted the strongest

antimicrobial effectiveness against *E. coli* O157:H7 (by 3.5 log cfu/g) and the endogenous

microbiota of baby-leaf salads stored for 5 days at 7°C by being sprayed after chlorine sanitizing

washing step.

The inhibitory ability of 800 ppm Cinnamaldehyde-Tween and 800 ppm Sporan®(a

commercial combination of clove, rosemary, and thyme oil)-acetic acid was similar to 5 ppm

chlorine to reduce the *E. coli* O157:H7 and *Salmonella* on iceberg and romaine lettuce on the

first day of storage *(194)*. In order to be effective, the plant essential oils, as food-grade

antimicrobials, are generally applied at high levels that are often impractical. In addition, their

high levels often result in unacceptable sensory attributes. The sesquiterpenoids, often contained

in plant essential oils, are a group of biological synthesized 15-carbon isoprenoid compounds

which have recently been reported as cytotoxic anticancer agents *(39)*. Farnesol, a natural

sesquiterpenoid, was reported to show antimicrobial activity against *S. epidermidis* and *S.*

*aureus*. It also caused disruption of the cytoplasmic membrane and consequently the release of

cellular compounds, and also decreased the biofilm formation of *C. albicans (153)* and *S. aureus (90)*. Brehm-Stecher and Johnson *(18)* found that the low level of sesquiterpenoids such as nerolidol and farnesol had the ability to increase bacterial permeability and sensitize *Escherichia coli* to the antibiotic polymyxin B. Brilhante et al. *(20)* reported that the combination of this compound with β-lactams decreased MIC (minimum inhibitory concentration) against *Burkholderia pseudomallei*. However, farnersol has not been studied in fruits and vegetables, which might be a potential enhancer to other sanitizers commonly used in produce.

### 1.5.5 Hurdle technology

Although as alternatives of chemical sanitizer, aromatic EOs from plants such as oregano and plant extract have been studied for many years to meet consumer needs for natural food additives, their efficacies of each alone might not be effective to ensure the food safety of fresh produce. Meanwhile, researchers have tried to combine different kinds of antimicrobials together or use other hurdle technology to get synergistic effects against foodborne pathogens or use other hurdle technology. Hurdle antimicrobial technologies have been studied in meat products for years. Hulankova and coworkers *(88)* found that combination of oregano essential oil (OEO) and caprylic acid (CA) had additive antibacterial effects when used in minced beef. This study showed combination of 0.5% CA, 0.2% OEO (v/w) and 0.1% of citric acid was able to reduce lactic acid bacteria and *L. monocytogenes* by 1.5 log CFU/g, more than 2.5 log CFU/g respectively after 10 days at 3 °C.

The antimicrobial activity of oregano and thyme against *L. monocytogenes* was increased by essential oil at acidic pH conditions and higher protein concentrations, which can be used as a function of ingredient manipulation *(82)*. Natural plant extracts such as grape seed

extract, pine bark extract and green tea extract rich in polyphenolic compounds as well as organic acid (lactic acid, citric acid and malic acid) have been studied as natural antimicrobials in meat products, but at relatively high levels, which might affect sensory properties.  One percent grape seed extract and 1% pine bark extract alone can reduce about 1.7 log of *E. coli* O157:H7 and 2.0 log of *S.* Typhimurium, and *L. monocytogenes* after 9 days and both effectively retained the redness in cooked beef during storage *(2)*. Gadang et al. *(66)* reported that whey protein isolate coating incorporated with nisin, malic acid, grape seed extract and EDTA decreased 1 log cycles of *S.* Typhimurium after 28 d at 4 °C and decreased 4.6 log cycles of *E. coli* O157:H7 on a Turkey frankfurter system.

All of these previously discussed decontamination methods that are applicable to fresh produce have advantages and disadvantages. The number of foodborne outbreaks and illnesses has not decreased with the increasing number of novel methods developed. Despite efficacies of these alternative methods, chlorine is still used primarily in fresh produce industries. However, people pay more and more attention to their health and prefer to eat minimally processed vegetables and fruits, so the effort must be made to find effective novel natural sanitizers to take the place of chlorine. The sesquiterpenoid farnesol has been studied for years to enhance the effectiveness of antibiotics, which is potentially used to enhance the sanitizers for fresh produce. The enhancement of farnesol or other sesquiterpenoid may reduce usage levels of sanitizers or obtain stronger antimicrobial activities at their current levels used.  Another promising antimicrobial system might be plant extracts rich in polyphenols combined with organic acid and sensitizer polyP, at low level of each ingredient, can synergistically permeabilize the outer membrane of gram negative foodborne pathogen, damage the cell wall and membrane and then

release cellular components, killing the pathogen cells to a large extent. It can act as a natural complex antimicrobial system in fresh produce without sacrificing sensory characteristics such as color, flavor and texture. Malic acid, citric acid, tartaric acid, Grape Seed Extract and Green Tea Extract and polyP are considered as "natural" additives, and are generally recognized as safe. Their combination tends to be a novel natural antimicrobial system applied to fresh produce.

## 2. Agricultural and value-added antimicrobial polymer coatings

### 2.1 Waterborne PU

Recently considerable research has focused on the development of bio-renewable-based materials using various agricultural commodities to replace petroleum-based polymer materials. Polyurethane (PUs) is one of the most versatile polymers and has a wide range of applications. Environmentally friendly waterborne polyurethane dispersions (PUDs) have found its wide applications as coatings and adhesives due to their advantageous characteristics over conditional solvent based polymers*(150, 192)*. The key advantage of waterborne coatings over organic based coatings is low hazardous emissions of hazardous air pollutants from volatile organic compounds that are often generated by non-aqueous solvents. More recently, the development of waterborne PUDs made with bio- renewable oils especially vegetable oils has gained significant attention of research into environmentally friendly protective and decorative coatings due to ready availability, green origin, inherent biodegradability and relative low cost of those oils *(192)*.

### 2.2 Vegetable oil based PU

Vegetable oils consist mainly triglycerides formed of various fatty acids and glycerol. Most fatty acids are long straight chain unsaturated fatty acids with an even number of carbons and the double bonds in between such as palmitic acid, linoleic acid, linolenic acid, oleic acid,

stearic acid. The physical and chemical properties of the vegetable oils depend heavily on the degree of their unsaturation. It is noted that different vegetable oils consist of different compositions of fatty acids depending on the plant where oils are extracted from and the growing conditions *(192)*. Lu and Larock synthesized several vegetable oil based anionic PUDs from vegetable oils such as castor oil, soybean oil and rapeseed oil, which exhibited comparable thermophysical and mechanical properties to those PUs synthesized from petroleum-based polyols *(122, 123)*. Among all the vegetable oils used to develop PUs, castor oil is the only one that naturally contains hydroxyl groups (about 2.7 per triglyceride) that is significant during the production of PUs, so it has gained considerable attention before it was determined that polyols were produced from other vegetable oils *(137)*. A novel biodegradable plastic was developed from new waterborne PUD synthesized with natural occurring castor oil and thermoplastic starch, which exhibited improved physical properties *(125)*.

For the other vegetable oils that do not have hydroxyl groups, it is required to contain a considerable number of carbon-carbon double bonds, which can be converted into polyols that can react with diisocyanates to produce PU. Soybean oil has a 4.6 double bonds per triglyceride and is commonly used to produce polyols for making PU*(150)*. Lu and Larock synthesized soybean oil based waterborne PUs with high performance, showing that it is a very promising approach to develop environmentally friendly dispersions from renewable agricultural valued added materials*(122)*. However, cationic PUDs prepared by soybean oil polyols are of much greater interest because of their distinctive antimicrobial abilities *(170)*.

## 2.3 Cationic polymer

Cationic polymers are able to bind to bacteria and other microorganisms with negatively charged cell membrane, and disrupt cell structure, resulting in permeabilization of the cell wall and cell death *(99)*. Macromolecules might react effectively with gram positive bacteria cells because their peptidoglycan layer of cell wall is loosely packed and these active molecules could penetrate into the cells through cell wall and cytoplasmic membrane. In comparison, gram negative bacteria have an outer membrane of lipopolysaccharides, which effectively protects the cytoplasmic membrane from antimicrobial agents *(138)*. Some polymer coatings containing an amine group or cationic peptides such as Chitosan have positive charges and therefore are well known to have antimicrobial properties *(28)*. Polymers prepared by modified poly derivatives with quaternary ammonium and phosphonium salts exhibit effective antimicrobial activity *(99)*. The backbone of the antimicrobial PU polymer is able to bind metals ions ($Ag^+$, $Cu^{2+}$, $Fe^{3+}$). These ions are well known to have broad-spectrum antimicrobial activity against microorganisms without cross-resistance with antibiotics. Polymers containing $Ag^+$, $Cu^{2+}$, $Zn^{2+}$ were able to significantly reduce *S. epidermidis* cells adherent to polymer surface. $Ag^+$ polymer exhibited the best inhibitory effects and showed synergetic fashion in the presence of ciprofloxacin*(63)*. In another study by the same group, the presence of silver and ciprofloxacin in polymer also acted synergistically with ciprofloxacin to inhibit both *S. epidermidis* and *P. aeruginosa* for at least one month. This novel approach may be used to develop antimicrobial biomaterials for medical equipment or materials to prevent or reduce microbial colonization on their surfaces without increasing antibiotic resistance *(64)*.

## 2.4 Antibacterial vegetable oil cationic PUDs and their application

Lu and Larock have previously developed novel cationic polyurethane dispersions PUDs from vegetable oil-based polyols *(123)* and examined the hydroxyl functionality of polyols on thermal and mechanical properties of PUDs *(124)*. The effects of different cationic soybean oil based polyols on antibacterial properties of soybean oil based PUDs and PU coatings were firstly reported by our group *(193)*. In this study all PUs had better antibacterial ability against gram positive *L. monocytogenes* than gram negative *S.* Typhimurium. The "deep rough" mutant of S. Minnesota with intact outer membrane (OM) is largely susceptible to all PU films. All this together indicated that OM of gram negative bacteria play a significant role protecting them from antibacterial polymer. Whereas the peptidoglycan layer of gram positive bacteria is thick but relatively porous, so that PUs can easily diffuse through the cell wall leading to destructive action. We also developed excellent cationic PUDs with soybean oil based polyols and reported the effects of amino ratio and degree of crosslink on thermo-mechanic and antibacterial properties *(68)*. Using multiple antimicrobial testing methods, the antibacterial properties of these materials have been evaluated against two most common bacterial pathogens associated with foodborne illnesses *S.* Typhimurium and *L. monocytogenes* as well as methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to commonly used antibiotics.

Infectious diseases caused by pathogenic microorganisms have been a critically important global healthcare issue, considered a great concern in many fields, especially in medical devices, hospital furniture and equipment surfaces, and food packaging *(138)*. Additionally, the appearance of antibiotics resistant strains and increasing occurrence of community-acquired outbreaks worsen this problem. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a

multiple antibiotics resistant pathogen causing hospital-acquired and community-acquired infections *(68)*. A food-related acute gastroenteritis transmission of MRSA has occurred due to contamination of foods by an asymptomatic food worker *(94).* ). In addition to MRSA, *Acinetobacter baumannii* is another important multidrug-resistant microorganisms in hospitals worldwide, as well as in Iraq and Afghanistan battlefields, which become an increasingly significant and demanding species in nosocomial pneumonia and bloodstream infections *(148)*. *C. albicans* is opportunistic pathogen but the most common fungal species leading to bloodstream infections in human with mortality rates of 38-49% *(132)*.

The need to develop novel effective antimicrobial systems becomes urgent to combat these infections and improve human health. Antimicrobial polymers have been extensively and efficiently employed in all of these fields because of their intrinsic properties. Additionally, it is also a new approach to be applied as coatings used for surfaces in food processing or healthcare environments, or as elements in food packaging to prevent the contamination of pathogenic microorganisms. The vegetable oil- based biorenewable coatings appear promising in such fields for the antimicrobial functionalization of surfaces.

### 3. Antimicrobial susceptibility testing methods

Different kinds of antimicrobial susceptibility testing are performed in the clinical microbiology laboratory to choose antibiotics or other drugs effective to pathogenic microbiology or detect resistance of individual bacterial isolate to those agents *(95)*, which is of significance to guide therapy and epidemiological monitoring of resistance. Meanwhile, those in-vitro antimicrobial susceptibility tests also play an important role in determining antimicrobial property against the tested microorganisms by examining the minimum inhibitory concentration

(MIC) and zone of inhibition diameter. The most commonly used susceptibility testing methods are disk diffusion test, agar dilution test, broth microdilution test *(69, 95)*. They have been recently used by microbiologists to develop antimicrobials applied in food, cosmetics and antimicrobial materials. Some of those methods are also documented as standard methods for aerobes, anaerobes and fungi by Clinical and Laboratory Standards Institute *(179)*. Choosing appropriate and effective antimicrobial susceptibility methods is always the key for researchers to develop novel and active antimicrobials applied for food.

## 3.1 Disk diffusion test

The disk diffusion test is a simple, practical and well-standardized susceptibility method *(89)*. It is performed by applying a bacterial or yeast fresh culture to the surface of Mueller-Hinton agar plate and placing paper disks containing antibiotics or other antimicrobials on the surface. The zone of inhibition that is clear zone around disks is measured after one16-24h incubation. This test is easily to perform, does not need special equipment and relatively cheap, and results are easily interpreted. The disadvantages are lack of automation and special media or incubation conditions needed for testing *Streptococci* spp., *H. influenza* and *N. meningitidis(89)*.

## 3.2 Agar dilution test

Agar dilution testing is regarded as the golden standard for all other antimicrobial susceptibility testing methods. To perform this test, a series of plates containing two-fold serial dilutions of the antimicrobial agent are prepared. The surfaces of plates are spread with a standardized suspension of the microorganism. After 18h to 24h incubation, the results are examined and the lowest antimicrobial concentration that inhibits bacterial growth MIC is determined *(40)*.

### 3.3 Broth microdilution

Macrodilution or tube dilution method was one of the earliest antimicrobial susceptibility methods. The protocol was to prepare serial two-fold dilutions of antimicrobials in a liquid medium. After overnight incubation, the turbidity of the media was examined and recorded. The lowest concentration of antimicrobial that prevented the bacterial growth was MIC. This test was easy but very tedious and requires a lot of media and samples. The broth microdilution testing method solves this problem, which is performed in a standard disposable plate containing 96 or 100 wells. Each well only requires 200 µl liquid. In addition, two-fold dilutions can be performed by multi-channel pipette, which help to conduct the test much faster. Following incubation MIC is determined using an automated plate reader for determination the growth for each well. In our lab, we use Bioscreen C microbiology reader, which is a unique system capable to measure optical density for at most 200 wells simultaneously and continuously. The advantages of microdiluttion test are *(95)*: 1. It is a quantitative method that could generate MIC; 2. The test is reproductive and very convenient to have prepared panels; 3. It is inexpensive and saves money when using small amount reagents, samples and space; 4. It also assists to produce computerized results if an automated equipment and corresponding software is available. The main disadvantage might be a need for good water solubility in water of tested antibiotics or other antimicrobials.

### 3.4 Antimicrobial gradient method-E test

The principle of antimicrobial gradient method is to establish an antimicrobial concentration gradient in an agar medium plate to determine susceptibility. A commercial product Etest® from bioMérieux is a well-established antimicrobial gradient method used for

antimicrobial resistance testing in worldwide microbiology laboratories. A predefined stable gradient of antibiotic concentrations is prepared along the length of a plastic trip *(69)*. Five or six strips can be placed on the plate surface in a radial fashion. After overnight incubation, results can be ready by viewing the strips. The MIC is determined as the concentration of antibiotics connecting to the lower part of the eclipse shaped growth inhibition area with the strip.   This technique provides a means for determination of MIC for slow-growing and fastidious microorganisms that require special growth conditions and cannot be tested by automated methods. It is a generally very convenient, but relatively expensive antimicrobial susceptibility method.

### 3.5 Descriptive methods

The methods discussed above are all endpoint methods, which provides little information about the antimicrobial effects except MIC value and requires the concentration of an antimicrobial to completely inhibit the growth of microbiology. Oftentimes food products do not need total inhibition of pathogens or spoilage microorganisms. If all conditions are under control, an extended lag phase is enough to meet the need of consumers. Therefore, a descriptive method is needed to determine antimicrobial effects of the food compounds on the growth kinetics of a microbe. The most commonly used descriptive method includes an inhibition curve together with plating count procedure, also called "time-kill" protocol. The idea is to use non-selective broth medium mixed with a certain concentration of this antimicrobial in determining the survival microorganism either by continuously recording OD values or obtaining survival colony forming units after plating. This concentration is usually obtained based on the endpoint method. The medium is incubated at optimum growth temperature for 24 hours for bacteria and for up to 5

days for yeast. The levels of antimicrobial effects (no effects, insufficient effects, microbiostasis, sufficient effect and disinfection) will be determined based on the growth curve or inhibition curve produced *(120)*. Overall, both endpoint and descriptive method should be used together to determine antimicrobial effects of each compound. The endpoint technique helps to obtain effective concentration and descriptive procedure further examines the antimicrobial effects over time.

**3.6 Methods for combined antimicrobial system**

A single antimicrobial compound is commonly used in developing food products, however combination system with two or three antimicrobial agents becomes increasingly popular especially for those that have synergy when mixed with others. A simple endpoint and descriptive method is not applicable for this circumstance. The checkerboard method is the technique to evaluate the interaction (addition, synergism and antagonism) among those agents for several reasons such as easily understandable principle, easy calculation and easy to perform. The checkerboard is the term referring as pattern of tubes or microtiter wells. A series of dilutions of the two antimicrobial agents are being tested at concentrations equal to, below and above their MICs. Three different interactions will be calculated based on Fractional Inhibitory Concentration (FIC) (defined by MIC of each compound when used together) of each ingredient. If the sum of FICs is equal to 1.0, that is called additive antimicrobial effect meaning the effect combination is equal to the sum of effects when used separately. When the sum of FICs is below 1.0, there is an antagonistic effect. Similarly, when the sum of FICs is above 1.0, there is synergistic effect meaning that the antimicrobial effect of combination is significantly greater than the additive response *(120)*.

## 4. Novel methods for determining the antimicrobial mode of action

Besides commonly used methods discussed above, food microbiologists become more
interested in other novel indirect methods to evaluate the antimicrobial effects and discover their
mode of action against microorganisms. Antimicrobial abilities can also be evaluated by
determining changes of cell morphology and physiology as well as some virulence factors
through using microscopy and flow cytometry, and measuring intracellular leakage,
identification and quantification of important protein, quantification of specific DNA as well as
ATP analysis.

### 4.1 Fluorescence microscopy

It is an important imaging technique to monitor cell physiology, which has been used in
an enormous number of published scientific papers. Microscopy has contributed to determining
the activity of cells, from visualization of microorganisms by Antonie van Leeuwenhoek to
cellular activities with advanced sophisticated imaging systems *(160)*. Fluorescence microscopy
incorporates fluorescent indicators that target with proteins, lipids or ions of microbes to allow us
observe cell physiology to greater extent. Application of fluorescence microscopy has been used
to evaluate antimicrobial activities by food microbiologists *(14)*, which provided direct
visualization of the antimicrobial effects based on percentage of live cells or dead cells.

Some cells have intrinsic fluorescence and can be used for imaging purposes without the
addition of fluorophores. But oftentimes fluorescent dyes, also called fluorophores, are needed to
stain particular cellular components, from which we are able to know their location. In principle,
fluorescence has a characteristic excitation in a short wavelength followed by emission in a

longer wavelength. The difference between excitation and emission wavelengths, called Strokes shift, is an important factor in choosing fluorochromes in fluorescence imaging *(13, 108)*. Fluorophores were initially some simple chemical stains such as 4′,6-diamidino-2- phenylindole (DAPI), which has strong ability to bind to DNA with an increase in fluorescence *(161)*. More recently, researchers have developed a number of fluorescent probes, and discovered green fluorescent protein (GFP) and subsequent cloning of GFP genes, both of which could monitor dynamic processes of live cells *(164)*. Some commercial live/dead stain kits are available and being used in many studies. For example, LIVE/DEAD BacLight Bacterial Viability Kit from Life Technologies has two compounds Syto-9 and Propidium Iodide (PI). Syto-9 is a green-fluorescent nucleic acid dye that is able to stain both live and damaged cells, while PI is not able to penetrate healthy cells. When both dyes are present, emission of Syto-9 is decreased because of the displacement of PI and quenching by Förster resonance energy transfer *(168)*, so damaged cells only have PI red fluorescence, from which health cells and damaged cells can be distinguished.

Most widely used fluorescence microscopes in common laboratory are widefield epifluorescence microscope with an objective lens above. It has been know as a very efficient, cost effective and simple to use method. A mercury lamp is often used to illuminate the sample and an excitation filter is needed between the light source and the sample to narrow the wavelength of light and match the excitation peak of the fluorophore *(78)*. However, there area also some advantages of epifluorescence microscope. First, the entire sample is simultaneously illuminated, so the fluorescence from all the excited fluorophores were detected, which makes those outside the focal plane appear indistinct. Second, for some fluorophore such as

isothiocyanate, the intensity of light from a mercury lamp is reduced at its maximum excitation wavelength, resulting in decreased relative amount of emitted light. Third, the overlapping spectra in the samples containing multiple fluorescent labels could cause bleed-through of signals *(78)*.

To overcome the weakness of epifluorescence microscope, the confocal laser scanning microscope (CLSM) was developed, which is a big improvement in optical microscopy field. CLSM is able to capture high-resolution images from selected depths. The confocal microscope has a monochromatic laser that can excite the sample. An illumination pinhole is placed between the laser light source and the sample, lighting only one point of specimen and eliminating light interference from outside the focal plane. The capability of an imaging system to observe detail of the sample is called resolution, defined as the minimum distance between two points that can be distinguished in a field of view. Some factors affect resolution fluorescence microscopy for both epifluorescence and confocal microscopes, such as the numerical aperture (NA), the wavelength of the light. The pinpole size also influenced the resolution of CLSM. The greater NA, the shorter wavelength, the smaller pinhole contribute to getting a higher solution *(78)*. CLSM is more expensive than epifluorescent microscope and requires the operator to have specific training and lots of experience.

## 4.2 Flow Cytometry

Another essential technique using fluorescence in exploring antimicrobial activity is flow cytometry (FCM), a rapid method to provide antimicrobial activities by studying cell membrane integrity and the heterogeneity of individual cells *(130)*. FCM is used to as a valuable and powerful tool to effectively evaluate the physical and chemical characteristics of microbiology

populations such as cell integrity, cell size and cell granularity, based on light scattering and fluorescence intensity(129). Some fluorochromes label cells directly such as PI, some are used to bind cells via carrier such as antibody. The basic laws of physics such as fluidics, optics and electronics are very important in FCM system that senses moving cells in a liquid with a laser pass through. Lasers with specific wavelength provide the energy to excite the fluorochromes bound to cells and emit the fluorescent light. The computer will collect the data of Forward Scatter (FSC), Side Scatter (SSC) and Fluorescence Intensity. FSC intensity is proportional to cell size, while SSC intensity is proportional to the optical homogeneity of cells. The appropriate detectors and computer software convert those signals into electronic digital data, allowing researchers to analyze the changes of these parameters and get useful information of cell changes. Determining cell membrane permeability caused by antimicrobials is one significant application of flow cytometry(17).

Among all the kinds of flow cytometers, the Accuri C6 Flow Cyometer is an affordable and compact system requiring relatively less training, which becomes commercially available and popular flow cytometer(157). In terms of fluorochromes, LIVE/DEAD BacLight staining Kit from Life Technologies is the one commonly used in flow cytometry permeability analysis. The use of Accuri C6 Flow Cyometer and commercial LIVE/DEAD BacLight kit to estimate antimicrobial effects by studying FCM permeability is a quick affordable approach, which has been used in many studies (10, 14, 65).

### 4.3 The Fluorescent Outer Membrane Probe NPN

1-N-phenylnaphthylamine (NPN) is one commonly used uncharged hydrophobic fluorescence probe in various outer membrane (OM) permeabilization studies for gram negative

bacteria *(85, 119, 185)*. NPN has low fluorescence intensity in aqueous environment, but will become strongly fluorescent once in a hydrophobic environment. OM is a well-studied effective permeability barrier for antimicrobials to function against gram negative bacteria. NPN enables to enter into the hydrophobic membrane when OM and cytoplasmic membrane is damaged by antimicrobials or antibiotics *(186)* and increases fluorescence intensity of cells. The kinetics of an increase in fluorescence caused by partitioning of NPN into the OM was recorded by fluorescence spectrophotometer with excitation and emission wavelengths set at 350 and 420nm respectively *(92, 119)*.

## 4.4 Electron Microscopy

Driven by advances in biological and materials sciences, electron microscopy has been developed and become an important tool in multiple disciplines. Unlike light microscope, the electron microscope use electron beams to create an image. A source of high energy primary electrons is required to produce an electron beam with precisely controlled energy. Several different interactions occur between electrons and specimen when a primary electron impinges a sample *(1)*. The transmitted electrons are used by the Transmission Electron Microscopy (TEM) uses, while the Scanning Electron Microscopy (SEM) captures secondary or backscattered electrons. Sample preparation for TEM is relatively difficult and complicated. Specimen needs to be thinly sliced prior to complex following steps such as fixation, washing, dehydration and embedding, which is laborious and requires the use of various agents sequentially*(1)*. On the other hand, SEM overcomes the disadvantages of STM. Sample preparation is easier and includes fixing (not required), dehydration and coating. It also provides a 3D image of sample surface. TEM has been used to analyze food protein structures and characterize food samples. In

recent years it has been used to reveal subcellular structures of bacteria and provide detailed information of structure change caused by antimicrobial interventions *(27, 121)*. SEM has even wider application in food studies, which allows adequate magnification for thick specimens in a 3D like image. Microbiologists have recently used it as a tool to study microbial attachment, colonization and survival in food, biofilms *(17)*.

### 4.5 Image Analysis

Microscopy provides visual information by looking through microscope or taking pictures. But more advanced digital images allow us to extract quantitative data and explore more useful information. Those sophisticated processing methods include conversion to gray images, intensity adjustments, background subtraction, image calibrate, pixel analysis etc. *(17)*. It would be tedious and time consuming if done manually. Some free image analysis software is currently available such as ImageJ and CellProfiler that support automated image analysis. ImageJ is a Java-based image processing program developed by the National Institutes of Health, which can run on Mac, Windows and Linux computers with a Java 1.1 or later. It supports the processing of stacks of images, angels and distances measuring and area and pixel statistics calculation for user defined area. Besides standard image processing such as edge detection and contrast manipulation, ImageJ also supports geometric transformations, spatial calibration and density/gray scale calibration. As open source software, ImageJ has 400 free plugins available that adds more functions including acquisition, processing and analysis *(17, 41)*. This extends the application of ImageJ to a large range of fields in science and engineering including microscopy, medical imaging and material science. Like ImageJ, CellProfiler is another open source imaging processing program that can be used in high-throughput cell image analysis for Mac, Windows

and Unix. It has ability to address biological questions quantitatively, either for standard assay such as measuring the cell shape, size, texture simultaneously, or for complex morphological assays such as DNA pattern and protein staining *(29)*. This program is user friendly and requires no knowledge of imaging analysis. It includes many individual modules including MeasureObjectIntesntiy, ColorToGray, EnhanceEdges and ClassifyObjects, which can be chosen to build "pipelines" during image analysis. All this together shows that CellProfiler and ImageJ are two flexible, highly accessible image analysis program.

## 4.6 Protein and gene identification

In an effort to discover antimicrobial mechanism of action at the protein and DNA level, some advanced analysis such as protein identification and quantification, RNA extraction and quantitative real time PCR have been used in recent studies. Through those analyses, the changes of either important virulence factors or important enzymes caused by antimicrobials can be addressed, which contributes to extending application of those antimicrobials in food. Reddy and others investigated the response physiological and putative mode of action of a natural plant-derived plumbagin on *B. subtilis* using two complementary proteomics techniques: two-dimensional electrophoresis and isobaric tag for relative and absolute quantification *(156)*. They found plumbagin repressed some of enzymes in TCA cycle and indicated the decrease in energy generation. In another study of antibacterial essential oil against *E. coli* O157:H7, carvacrol was found to induce the stress protein-heat shock protein 60 and inhibited the synthesis of flagellin-an important virulence factor, which was analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and west blotting tests *(25)*. Similarly, with western blotting analysis, Qiu et al. found another essential oil eugenol reduces the production of several virulence-related

exoproteins in *Staphylococcus aureus* including staphylococcal enterotoxin A, B and tosci shock syndrome toxin 1*(152)*. In the same study, the suppression at the transcriptional level was also evaluated by real-time (RT)-PCR analysis. Their corresponding virulent genes were also expressed. With those essential novel approaches, new antimicrobials have been found and their mode of actions is suggested, which contributes to optimizing condition to maintain their antimicrobial activities and extending their applications in food.

**4.7 Metal ion chelation assay**

As discussed in 1.5, polyphenolics and polyP exhibit antimicrobial activities due to its metal ion chelation properties. Iron chelation property has been assayed by a universal and convenient colorimetric method called Chrome azurol S (CAS) *(163, 182)*. Tested agents that have ability to bind iron would remove ferric ion from blue chrome zurol sufonate ferric complex and change color. More conveniently, a quick colorimetric modified CAS method SideroTec assay from Emergen Bio has been used to determine iron-chelation activity of tetracycline *(61)*.

**4.8 Other methods**

The microbial cell membrane is damaged during antimicrobial treatment resulting in the release of intracellular compounds such as DNA and protein, which could be determined by UV spectrophotometer at 260 and 280nm *(68, 175)*. Gill and Holley *(70)* determined extracellular and intracellular ATP of *L. monocytogenes* and *L. sakei* to investigate mechanism of bactericidal action of cinnamaldehyde and eugenol. The results indicated exposure to these antimicrobial

spice oil compounds caused a rapid inhibition of the energy metabolism of *L. monocytogenes* and *L. sakei*, suggesting that membrane permeability might be related with the inhibition of glucose uptake and utilization. A continuous light output luciferase reaction was used to measure ATP contents. The relative light units of light output were determined using luminometer. In another study of the same group *(71)*, motility of *E. coli* and *L. monocytogenes* due to the treatment of aromatic compounds were determined, which suggested the important virulence factor flagella might be damaged by antimicrobial compounds. The presence of flagella specifically flagellin contributes to activating the host immune response *(84)* and cells without flagella were less able to adhere to epithelial cells and less invasive*(25)*. Those antimicrobial essential oils could be used as additives for human food or animal feed because of their ability to suppress flagella.

Some other specific methods related with virulence factors are also incorporated as a means to determine the antimicrobial ability. For example, some yeast such as *C. albicans* enables to switch morphologically between round cellular forms and hyphal forms, which is now recognized as one of its virulent factors playing a key role in exhibiting pathogenicity during infection process*(47, 169)*.  Hyphal suppression is one of physiological responses *C. albicans* make to environmental stress such as antimicrobial treatment. Agar invasion assay and cell invasive growth assay are two methods to study the hyphal suppression for *C. albicans* and *Saccharomyces cerevisiae (46, 196)*. Agar invasion assay, also called plate-washing assay, is a simple well-known method to study microbial invasive growth into the media, which is usually due to the filamentous hyphae. The microbial colonies still can be seen under the surface of solid media after carefully washing with saline or buffer water *(16, 196)*. Individual microcolonies on

the surface of media and filamentous hyphae or individual cells underneath can be observed under microscope in cell invasive growth assay, which is a direct approach to show the change of morphological change of cells caused by treatments.

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# CHAPTER 3. ANTIBACTERIAL SOYBEAN-OIL-BASED CATIONIC POLYURETHANE COATINGS PREPARED FROM DIFFFERENT AMINO POLYOLS

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**Abstract**

Antibacterial soybean oil-based cationic polyurethane (PU) coatings have been

successfully prepared from five different amino polyols. The structure and hydroxyl functionality

of these amino polyols affected the particle morphology, mechanical properties, thermal

stabilities, and antibacterial properties of the resulting coatings. An increase in the hydroxyl

functionality of the amino polyols increased the cross-link density, resulting in an increased glass

transition temperature and improved mechanical properties. Both the cross-link density and the amount of ammonium cations incorporated into the PU backbone affect the thermal stability of PU films. PUs with the lowest ammonium cation content and highest cross-link density exhibit the best thermal stability. With some strain-specific exceptions, these PUs showed good antibacterial properties toward a panel of bacterial pathogens comprised of *Listeria monocytogenes* NADC 2045, *Salmonella* Typhimurium ATCC 13311 and *Salmonella* Minnesota (*S.* Minnesota) R613. *S.* Minnesota is a "deep rough" mutant lacking a full outer membrane (OM) layer, an important barrier structure in gram-negative bacteria. With wild-type strains, the PU coatings exhibited better antibacterial properties toward the gram-positive *L. monocytogenes* than the gram-negative *S.* Typhimurium. However, the coatings had excellent activity against *S.* Minnesota R613, suggesting a protective role for an intact OM against the action of these PUs.

**Introduction**

Plant-based, renewable polymeric materials are receiving increased attention due to economic and environmental concerns. [1,2] Plant oils have been extensively studied during the last decade as potential starting materials for biorenewable polymers due to their readily availability, "green" origin, inherent biodegradability, and the versatility of their applications.[3,4] A variety of polymers have been prepared from plant oils by various polymerization methods, including free-radical, [5] cationic, [6] metathesis,[3,7] and addition polymerization.8 The polymeric materials obtained exhibit a wide range of thermophysical and mechanical properties, ranging from soft and flexible rubbers to hard and rigid plastics, suggesting their utility as replacements for petroleum-based polymers.

Polyurethanes (PUs), one of the most versatile polymer classes, possess a wide range of industrial applications.[9] One of the most rapidly developing areas of PU research involves waterborne polyurethane dispersions (PUDs), which exhibit low viscosity at high molecular weight and good applicability.[10] They are also environmentally friendly due to their low emission of hazardous air pollutants (HAPs) and volatile organic chemicals (VOCs).[11] Plant oil-based waterborne PUDs have been successfully prepared in our group by incorporating dimethylol propionic acid (DMPA)[12,13] or *N*-methyldiethanolamine (MDEA)[14,15] into the PU backbone. The resulting PU backbone is treated with either triethylamine or acetic acid and then dispersed in water to give plant-oil-based anionic or cationic PUDs, respectively. These PUDs may have wide ranging applications in adhesives and protective/decorative coatings, representing a promising use of plant oils in biorenewable polymeric materials.

Antimicrobial polymers have applications in many fields where killing of pathogens or prevention of surface colonization are essential, including self-sanitizing medical devices, environmental surfaces, food packaging, *etc*. [16] Polymers containing quaternary phosphonium and ammonium salts exhibit high antimicrobial activity, with the former generally being more effective.[17,18] Silver has been introduced into PUs by coordinating $Ag^+$ with a carboxylate-containg PU.[19] The resulting silver-containing polymers have been shown to inhibit the growth of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* for at least one month.[19] Polymers containing organotin reagents [20] and polymer anionomers containing $Cu^{2+}$, $Zn^{2+}$, and $Fe^{3+}$ also show satisfactory antimicrobial properties.[21] Hydantoin[22] and *N*-halamine[23,24] have been incorporated into polymers to confer antimicrobial properties as well. Paints containing

polymeric *N*-halamine exhibit antimicrobial activity against gram-negative and gram-positive bacteria, including drug-resistant strains, fungi, viruses, and molds.[23]

In the current study, the antibacterial properties of cationic plant-oil-based PUDs and PU coatings have been evaluated for the first time. The cationic plant-oil-based PUDs were prepared from five different amino polyols: MDEA, *N*-ethyldiethanolamine (EDEA), 1,4-piperazinediethanol (PDE), triethanolamine (TEA), and 2,2',2'',2'''-(ethylenedinitrilo)tetraethanol (EDTE) (Figure 1). Herein, we provide an extensive study of the effects of amino polyol structure and hydroxyl functionality on particle morphology, mechanical properties, thermal stabilities, and antibacterial properties of polyol-based coatings. The plant oil-based biorenewable coatings reported here appear promising in numerous industrial applications for the antibacterial functionalization of surfaces.

**Results and discussion**

<u>Structure and morphology</u>

Five different amino polyols have been incorporated into PUs to generate polymer chains bearing tertiary amine groups (Figure 1). The tertiary amine groups have then been treated with acid to form water soluble, cationic ammonium ions and thus form stable cationic PUDs. After polymerization, all of the hydroxyl groups from the amino polyols are consumed. Structure analysis, such as FTIR spectroscopy, of similar polymers has been conducted in our previous paper[15]. PUs prepared from MDEA and EDEA have relatively small side chains, namely methyl and ethyl groups, attached to the nitrogen atoms of the polymer backbone, but PUs prepared from PDE, TEA, and EDTE had a large cross-linked networks connected to the nitrogen atoms. The different groups attached to the nitrogen groups and the number of hydroxyl groups per

amino polyol not only affect the morphology of the particles in the cationic dispersions, but also affect the properties of the resulting films.

Figure 2 illustrates the negative-stained TEM images of all of the PUDs prepared from the different amino polyols. It was found previously that several factors, including hydrophilicity, the prepolymer viscosity, chain rigidity, ionic group position, chemical structure of the soft segment, and the cross-link density, affect the particle size and morphology in PUDs.[15] In this work, the particle size and morphology were mainly controlled by using different amino polyols. PU-MDEA and PU-EDEA dispersions have particle sizes around 60 nm and they also have relatively uniform particle-size distributions relative to the other three samples. The PU-PDE dispersion has a much smaller particle size relative to the PU-MDEA and PU-EDEA dispersions because PUs prepared from PDE have twice as many water soluble ammonium ions as those prepared from MDEA and EDEA. In fact, PU-PDE is completely soluble in water, whereas the other four samples yielded opaque dispersions. PU-MDEA-TEA and PU-EDTE dispersions exhibit particle aggregation, caused by the higher hydroxyl functionality of the TEA and EDTE, resulting in PUs with higher cross-link densities relative to PUs prepared from the other three amino polyols. Relative to the other four amino polyols, the PU-MDEA-TEA dispersion has the lowest amount of ammonium cations, which also contributes to its large particle size and particle aggregation.

Thermal and mechanical properties

From differential scanning calorimetry (DSC) curves for all of the PU coatings prepared from different amino polyols (Figure 3), the glass transition temperature ($T_g$) was taken from the midpoint of the heat-capacity change (Table 1).  Only one glass transition was observed for all of

the PU films, indicating the noncrystalline amorphous nature of all samples. Relative to PU-MDEA, PU-EDEA has a lower $T_g$ because the ethyl groups attached to the nitrogen atoms in EDEA are more flexible than the methyl groups in MDEA. The PU-MDEA-TEA coating has a higher $T_g$ than that of PU-MDEA because half of the MDEA has been replaced by TEA with higher hydroxyl functionality, resulting in PU films with a higher cross-link density. The PU-EDTE coating has a much higher $T_g$ than the other four PU films because the tetrahydroxyl functionality in EDTE results in the most cross-linked network for PU-EDTE. The heat-capacity change at $T_g$ for PU-EDTE is much smaller than the other four samples (Figure 3), which also demonstrates that the incorporation of EDTE increases the cross-link density substantially.[25]

The mechanical properties of all PU films have been investigated by dynamic mechanical analysis (DMA). The resulting storage modulus (E')–temperature curves are shown in Figure 4. All cationic PU films are in the glassy state at low temperatures, whereas their storage moduli decrease slightly when the temperature is increased. A rapid decrease in storage modulus has been observed for all PU films in the temperature range 20-60°C and the onset temperatures are also recorded as $T_g$ (Table 1). All $T_g$ values obtained by DMA are higher than those obtained using DSC due to the different nature of the two methods.[26] The $T_g$ values obtained by DMA follow a trend similar to those obtained by DSC analyses, except for PU-PDE. Relative to PU-MDEA, the $T_g$ for PU-PDE obtained by DMA is higher, but the $T_g$ obtained by DSC analysis was lower. PDE has a rigid ring structure (Figure 1), a feature that generally leads to an increased $T_g$; the small side chain methyl groups in MDEA are also expected to raise $T_g$. Our results may stem from the different nature of the DMA and DSC methods: DMA measures the polymer's mechanical response, whereas DSC measures the heat-capacity change from frozen to unfrozen

chains.[26] The room-temperature storage moduli of all PU films are also summarized in Table 1. Relative to PU-MDEA, PU-EDEA has a lower storage modulus because of the flexible side chain ethyl groups. PU-PDE has a higher storage modulus due to the rigid ring structure, and PU-MDEA-TEA/PU-EDTE have higher storage moduli due to their higher cross-link densities.

Figure 5 shows both the weight-loss and derivative weight- loss curves as a function of temperature. From derivative weight-loss–temperature data, three decomposition stages are observed. The first stage below 200°C can be ascribed to de- composition of the ammonium groups. The second stage from 200 to 320°C corresponds to dissociation of the urethane bonds in the polymer backbone because urethane bonds are thermally labile and generally begin to decompose at about 200°C.[27] Notably, stage one for PU films with high cross-link densities, such as PU-MDEA-TEA and PU-EDTE, is not clearly observed in the derivative weight-loss– temperature curves, and decomposition of the ammonium and urethane groups is combined at temperatures above 200°C. The third decomposition stage above 320°C is due to decomposition of the fatty acid chains. $T_5$, $T_{50}$, and $T_{max}$ values for all of the PU films are summarized in Table 1. $T_5$, the temperature at which PU films lose 5 wt % of their mass, is generally the onset decomposition temperature. The amount of ammonium groups in the polymer and the cross-link density of the polymer both affect the thermal stability of these cationic PU coatings. According to $T_5$ values, PU-PDE (highest ammonium ion content) has the lowest thermal stability because ammonium groups are thermally unstable. PU-EDTE (highest cross-link density) has the second-best thermal stability. PU-MDEA-TEA (lowest ammonium ion content and a relatively high cross-link density) has the greatest thermal stability. In contrast to $T_5$ values, $T_{50}$ and $T_{max}$ values of all PU coatings cover a much narrower range because all ammonium cations decompose at

these temperatures. PU-MDEA-TEA also has the highest $T_{50}$ and $T_{max}$ values, indicating that the amount of ammonium cations in the polymer backbone plays an important role in thermal decomposition processes of all cationic PU coatings.

<u>Antibacterial properties</u>

Cationic chemical species, including ammonium cations, have been long recognized for their antibacterial activity because they readily interact with bacterial cells, which typically have a net negative charge.[16] This work represents the first report of the antibacterial activity of soybean-oil-based cationic PU coatings. The antibacterial activity of both liquid dispersions and films of PU-MDEA against *Listeria monocytogenes* (*L. monocytogenes*) and *Salmonella* Typhimurium (*S.* Typhimurium) is summarized in Table 2. Our results indicate that although both PU dispersions and films have good antibacterial properties, dispersions show slightly better activity than films ($p<0.05$). Possible explanations include higher diffusional mobility of the liquid phase and/or decreases in the availability or display of hydrophilic ammonium sites occurring during the dispersion- to-film transition. PU-MDEA and other PUs exhibit better anti-bacterial action toward *L. monocytogenes* than *S.* Typhimurium ($p<0.05$).

The generally accepted mechanism for the antibacterial activity of ammonium species involves destructive interaction with the cell wall and/or cytoplasmic membranes.[17,18] As a gram-positive bacterium, *L. monocytogenes* has a thick, but relatively porous peptidoglycan sacculus, through which polymer or free ionic species can easily diffuse.[16] In contrast, wild type gram-negative bacteria, such as *S.* Typhimurium, possess an additional outer-membrane (OM)

layer, an important diffusional barrier that confers increased resistance to a wide variety of exogenous substances.[16]

Because the PUDs described herein are intended for practical use as dried film coatings, comparative activities of all PU films against *L. monocytogenes*, *S*. Typhimurium, and *Salmonella* Minnesota (*S*. Minnesota) are summarized in Table 3. Although structure–activity trends are not completely parallel for all three strains, PU-MDEA and PU-EDEA exhibit the best antibacterial activity overall ($p < 0.05$), possibly because the smaller methyl and ethyl side chains present on the nitrogen atoms in these two structures allow greater penetration of these materials into the cells. The replacement of half of MDEA residues with TEA results in a much more hindered polymer with lower antibacterial activity (PU-MDEA-TEA). Complete replacement of methyl/ethyl groups by bulkier alkyl substituents yields the more hindered PU-PDE, which is not inhibitory to *L. monocytogenes*, and PU-EDTE, which has no activity against *S*. Typhimurium. The reasons for the differential susceptibility seen between these two bacterial types for PU-PDE and PU-EDTE are unknown and require further investigation. The "deep rough" mutant of *S*. Minnesota is susceptible to all PU films, yielding the largest zones of inhibition (ZOIs) of all strains tested ($p < 0.05$). This can be explained by the severely truncated lipopolysaccharide present in the OM of this structural mutant, which considerably reduces the barrier properties of the OM.[28] Another important barrier layer, the cell wall, is intrinsically thinner in gram-negative bacteria than in gram-positives. Together, the lack of extensive OM and cell-wall barriers results in a hypersusceptible phenotype for this strain (Figure 6), suggesting the importance of the cell membrane or other internal cell structures as targets for the antibacterial action of the polymers tested herein.[29–31]

**Conclusions**

Soybean-oil-based cationic polyurethane dispersions (PUDs) have been prepared from five different amino polyols. The cross-link densities of PU films, which are controlled by using amino polyols with different hydroxyl contents, play an important role in controlling the particle morphologies of dispersions and the mechanical properties of films. With an increase in cross-link density, particle aggregation is observed in dispersions. The glass transition temperatures and room-temperature storage moduli both increase with an increase in cross-link density. The thermal stability of PU films is dominated by both cross-link density and the amount of ammonium cations incorporated into the PU backbone. PU-MDEA-TEA has the lowest ammonium ion content and a relatively high cross-link density and thus exhibits the best thermal stability. With some strain- specific exceptions, these PUs, as either dispersions or films, exhibit good antibacterial properties, particularly towards the gram-positive bacterium *Listeria monocytogenes*. These PUs show the best antibacterial activity against a structural mutant of *Salmonella* Minnesota possessing a severely truncated OM structure. This work provides a promising and effective route to prepare biorenewable antibacterial coatings. As with other types of cationic polymers reported in the literature, our coatings may also be effective against other types of microorganisms, including yeasts, molds, and viruses, which will be tested in future experiments.

**Experimental Section**

Materials

Wesson soybean oil was purchased at the local supermarket and used directly without further purification. Methoxylated soybean- oil polyols (MSOLs) with a hydroxyl number of 169 mg of KOH per g of oil were prepared according to our previous procedure.[12] Isophorone

diisocyanate (IPDI), dibutyltin dilaurate (DBTDL), MDEA, EDEA, 1,4-piperazinediethanol

(PDE), and TEA were purchased from Sigma–Aldrich (Milwaukee, WI). EDTE was purchased

from Acros (Geel, Belgium). Acetic acid and methyl ethyl ketone (MEK) were purchased from

Fisher Scientific (Fair Lawn, NJ). *S*. Typhimurium ATCC 13311 and *L. monocytogenes* NADC

2045 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

*S*. Minnesota R613 was obtained from the *Salmonella* Genetic Stock Center (Calgary, Alberta,

Canada). Stock cultures were maintained as frozen stocks at □-75°C in a tryptic soy broth (TSB,

BD Diagnostics, Sparks, MD) containing 20 % (v/v) glycerol. Working cultures of the three

strains were maintained on tryptic soy agar (TSA) plates at 4°C. Prior to each experiment,

cultures were grown in TSB for 18h overnight at 35°C and cell concentration was adjusted with

fresh TSB to an optical density (OD) of 0.1 at 600 nm, which corresponded to a cell count of

about $10^8$ colony-forming units (CFU) per mL.

Synthesis of the soybean-oil-based cationic PUDs

The synthesis of soybean-oil-based cationic PUDs was carried out using the amino

polyols MDEA, EDEA, PDE, TEA, and EDTE (Figure1). The detailed experimental procedure

is shown in Scheme 1. MSOL (5 g), IPDI (4.6 g), amino polyol [MDEA (1.53 g) is provided as

an example in Scheme 1], and one drop of DBTDL was added to a four-necked flask equipped

with a mechanical stirrer, nitrogen inlet, thermometer, and condenser. The molar ratio of OH

groups from MSOL, NCO groups from IPDI, and OH groups from the amino polyol was kept as

1.0:2.75:1.7. The viscosity of the reactants increased quickly and MEK (25 mL) was added after

reacting for approximately 10 min to prevent gelation. The reaction was kept for another 2h at 78

°C and was then cooled to RT. The tertiary amine groups in the PU chains were allowed to react

with acetic acid (1.53 g, 2 equiv) to form the cationic PUs, which were dispersed in distilled water (100 g) to form the cationic PUDs with a solid content of about 15wt% after removal of MEK under vacuum. The solid content of 15 wt % was selected to ensure the good stability of PUDs. The nomenclature used for the resulting soybean-oil-based cationic PUDs is as follows: the PUD prepared from MDEA was designated as PU-MDEA. The PU prepared from TEA did not form a stable dispersion because the final dispersion from only TEA has less hydrophilic ammonium cations, so a 1:1 OH molar ratio of MDEA and TEA were mixed to afford a stable dispersion designated as PU-MDEA-TEA. The corresponding PU films were obtained by drying the resulting cationic PUDs in a polytetrafluoroethylene mold at RT.

Characterization

The morphology of cationic PUDs was observed by means of TEM analysis (JEOL 1200EX). Dispersions were diluted to about 0.5 wt %, and then the dispersion (3 mL) was placed on a carbon film grid and negatively stained by an aqueous uranium acetate solution (2 wt %). The samples were characterized after drying.

The dynamic mechanical properties of the PU films were characterized by means of a dynamic mechanical analyzer (TA Instruments DMA Q800, New Castle, DE) using a film tension mode of 1 Hz in the temperature range from -40 to 150°C with a heating rate of 5 ºC min$^{-1}$. Rectangular samples with dimensions of 12 mm × 8 mm □× 0.4 mm were used for the analysis.

DSC analysis of PU films was performed by means of a thermal analyzer (TA Instruments Q2000). PU samples (≈5 mg) were cut from the film and heated from RT to 100ºC to erase the thermal history. The samples were then equilibrated at -70°C and heated to 150 ºC at

a heating rate of 20 °C min$^{-1}$. The $T_g$ of PU films was determined from the midpoint in the heat-capacity change in the second DSC scan.

The weight loss of PU films under a $N_2$ atmosphere was measured by using a thermogravimeter (TA Instruments Q50). Samples ($\approx$5mg) were heated from 50 to 600°C at a heating rate of 20 °C min$^{-1}$.

Antibacterial test methods (dispersions and films)

PU dispersions and films were tested against three pathogenic bacteria using a ZOI assay on agar overlay plates. TSA base agar plates were prepared by pouring sterile molten TSA (15 mL) onto petri dishes and allowing to solidify at room temperature. TSA overlay agar (9 mL, 0.7 % agar, tempered to 50 °C) and cell suspension (1 mL) were added to sterile 15 mL polypropylene tubes, vortexed, and the mixture was poured onto the hardened TSA base. After seeded overlays were allowed to harden, PU dispersions (20 mL) were added to paper discs (6 mm, BD Diagnostics). As a control, aqueous acetic acid (20 mL, 0.19 % w/v) was added on a paper disc. This concentration of acid was chosen to control for the possible presence of residual acetic acid from the preparation of the polymers and to control for simple pH effects as a possible confounding mode of antimicrobial action. The pH of the acetic acid solution was 3.66, similar to the pH of PUDs. The plates were kept at 4 ºC for 2 h to allow diffusion of the dispersions through the agar and then incubated at 35 °C for 24 h to allow growth of the test cultures. The diameter of the ZOI formed around the disc was measured in mm by means of a digital caliper (Hoteche, Ningbo, China). Each sample was examined in duplicate and experiments were repeated three times. Therefore, the values reported represent the average of six separate measurements.

For the testing of films, paper discs were saturated with each PU dispersion (20 µL), placed on a nonstick paper surface, and allowed to dry in a biosafety cabinet for 18 h, forming a film–disc composite. This approach enabled reproducible film dosage, facilitating accurate comparison across film types.

Statistical analyses were conducted by using the SAS 9.2 software (SAS Institute, Inc., Cary, NC). The MIXED procedure with contrast analysis was used to analyze data from the split-plot design to identify significant differences between treatments ($p<0.05$). All results were expressed as the mean standard deviation of six measurements.

## Acknowledgements

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**Table 1**. DSC, DMA, and TGA data for the PU films prepared from different amino polyols.

| PU Samples | $T_g$[a] (ºC) | $T_g$[b] (ºC) | $E^{,}$ at 25 ºC (MPa) | TGA data (ºC) | | |
|---|---|---|---|---|---|---|
| | | | | $T_5$[c] | $T_{50}$[d] | $T_{max}$[e] |
| PU-MDEA | 18.6 | 24.9 | 627 | 191 | 293 | 286 |
| PU-EDEA | 11.7 | 19.7 | 248 | 188 | 295 | 289 |
| PU-PDE | 13.7 | 27.4 | 739 | 174 | 290 | 285 |
| PU-MDEA-TEA | 19.1 | 29.4 | 641 | 223 | 299 | 291 |
| PU-EDTE | 46.9 | 54.8 | 1235 | 212 | 290 | 281 |

[a]  Glass transition temperature obtained from DSC.
[b]  Glass transition temperature obtained from DMA.
[c]  5 % Weight loss temperature.
[d]  50 % Weight loss temperature.
[e]  Temperature of maximum thermal degradation.

**Table 2**. Zone of inhibition (diameter) against two bacterial strains for PU-MDEA film and dispersion samples.

| PU-MDEA | LM[a] (mm) | ST [b] (mm) |
|---|---|---|
| Film | 12.52±0.14 | 9.77±0.08 |
| Dispersion | 12.66±0.22 | 10.92±0.03 |

[a] LM = *L. monocytogenes*. [b] ST = *S*. Typhimurium.

**Table 3**. Zone of inhibition (diameter) against three bacterial strains for all the PU films prepared from different amino polyols.

| PU Samples | LM[a] | ST[b] (wild type) | SM[c] (deep rough) |
|---|---|---|---|
| PU-MDEA | 12.52±0.14 | 9.77±0.08 | 17.39±1.22 |
| PU-EDEA | 13.11±0.08 | 9.49±0.18 | 17.63±0.18 |
| PU-MDEA-TEA | 10.65±0.14 | 7.58±0.14 | 15.86±0.19 |
| PU-PDE | _[d] | 8.88±0.09 | 14.64±0.09 |
| PU-EDTE | 8.55±0.17 | None | 12.28±0.20 |

[a]LM=*L. monocytogenes*. [b]ST=*S*. Typhimurium. [c]SM=*S*. Minnesota. [d] Only slight inhibition, resulting in a small diffuse and unmeasurable zone.
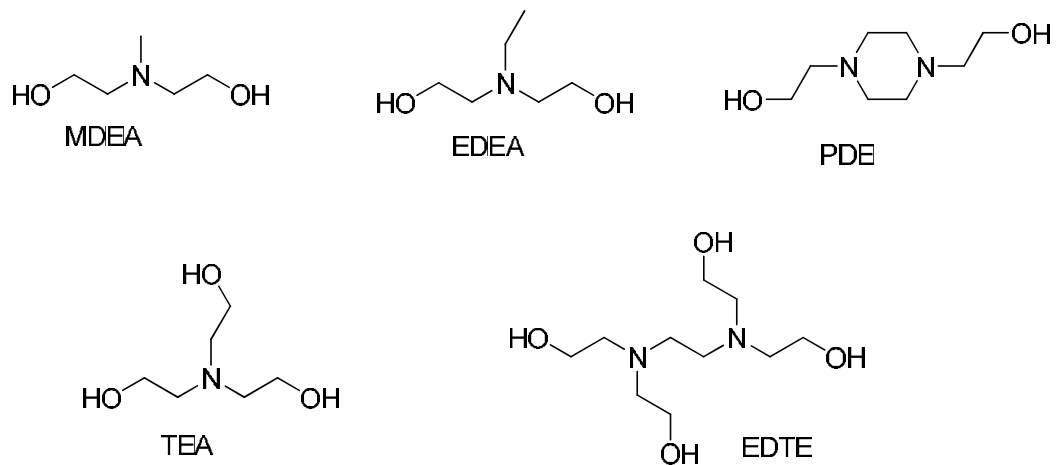
**Figure 1**. Structure of amino polyols used in the preparation of the PUDs.
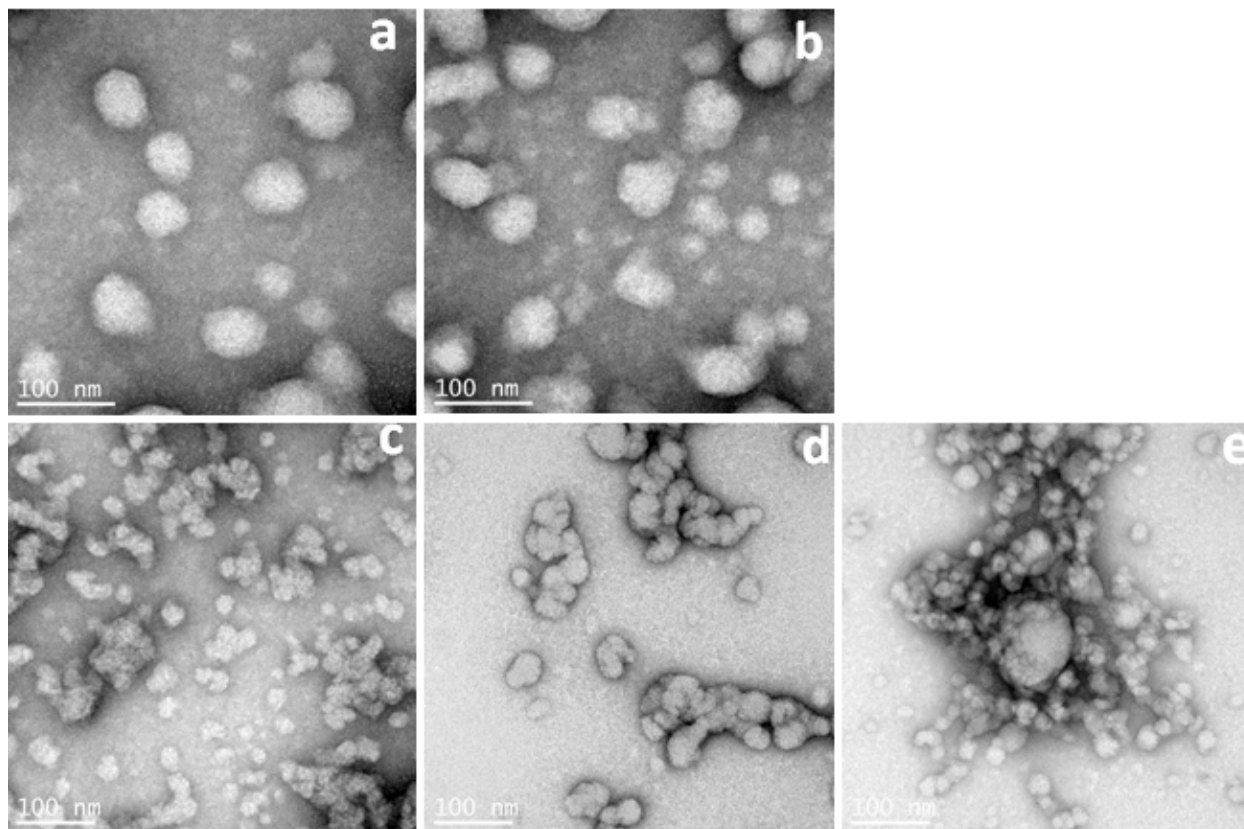
**Figure 2**. Negative-stained TEM images of a) PU-MDEA, b) PU-EDEA, c) PU-PDE, d) PU-MDEA-TEA, and e)

PU-EDTE (all scale bars are 100nm)
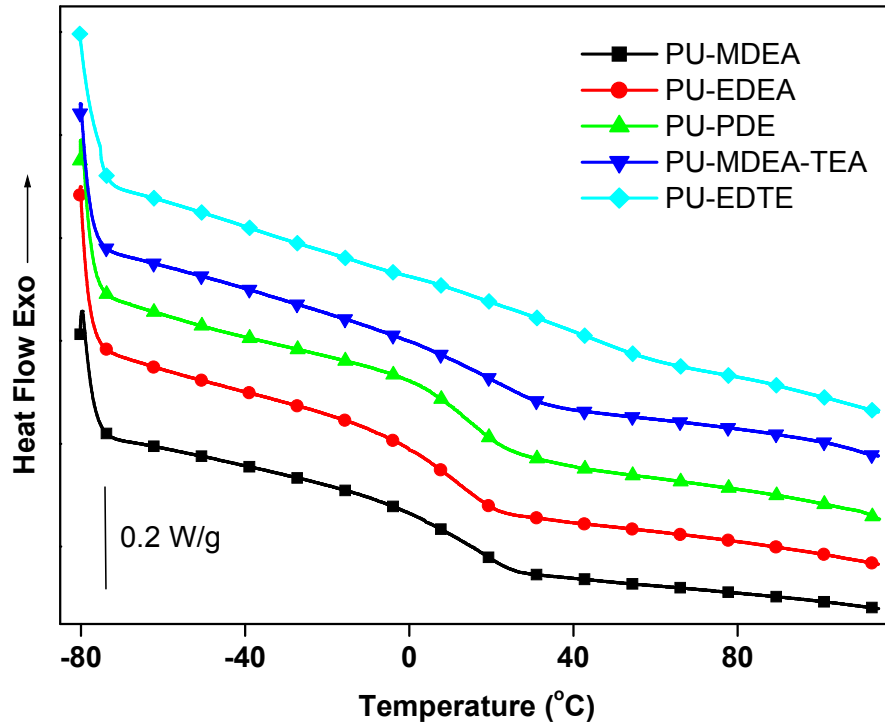
**Figure 3**. DSC curves of the PU films prepared from different amino polyols
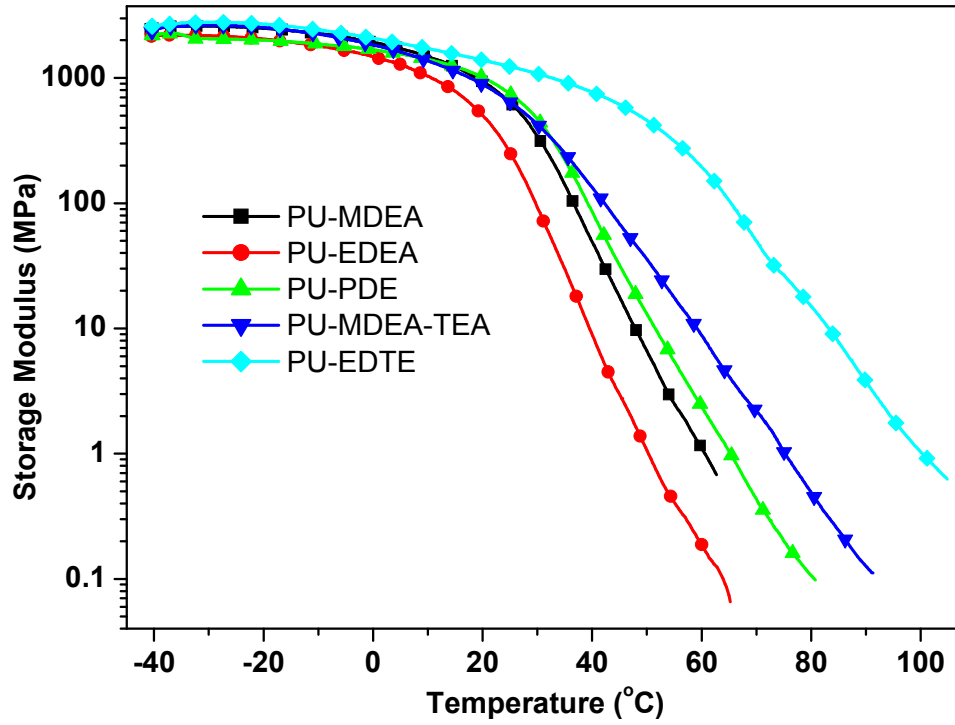
**Figure 4**. The storage-modulus-versus-temperature curves for all the PU films prepared from different amino polyols.
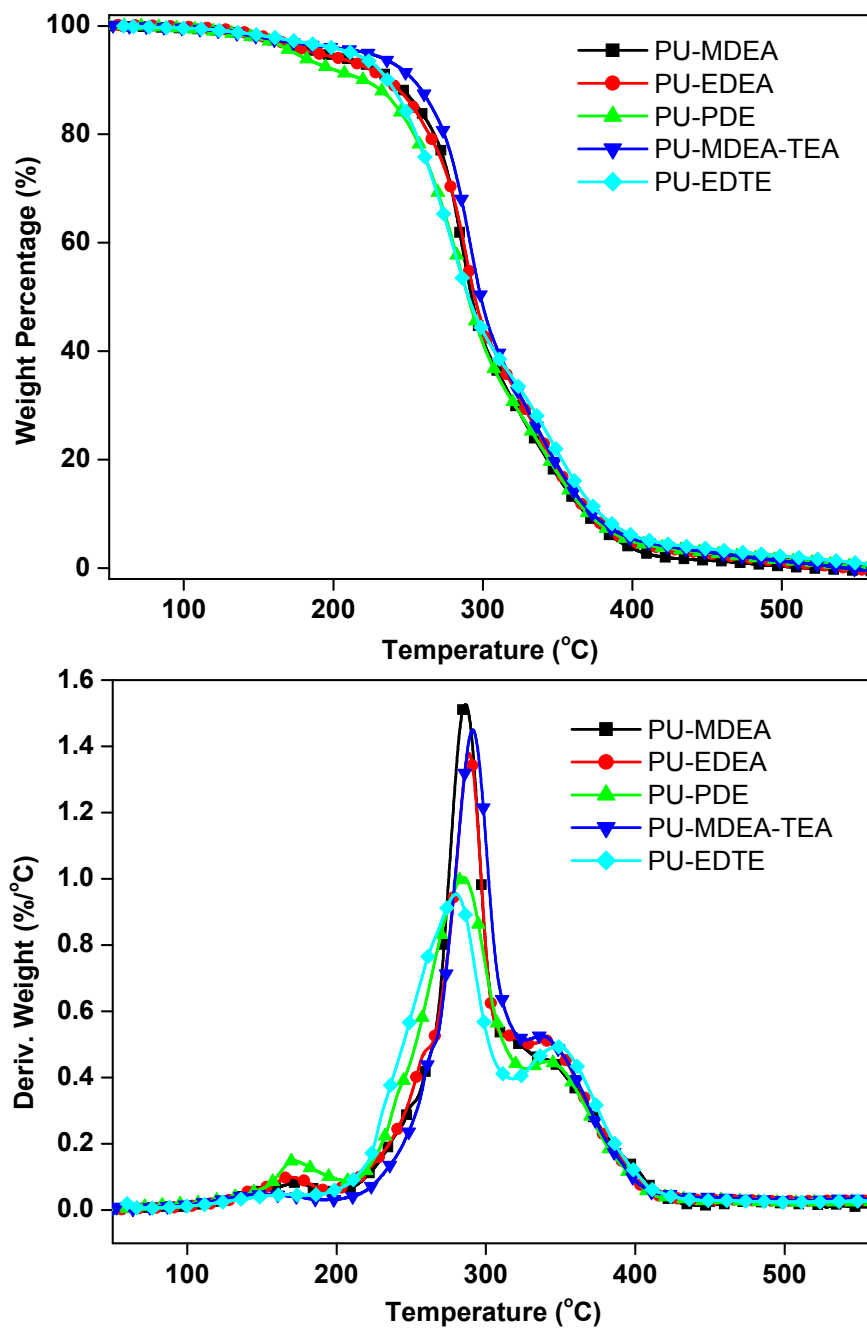
**Figure 5**. a) Thermogravimetric analysis (TGA) curves and b) their derivative curves for all PU films.
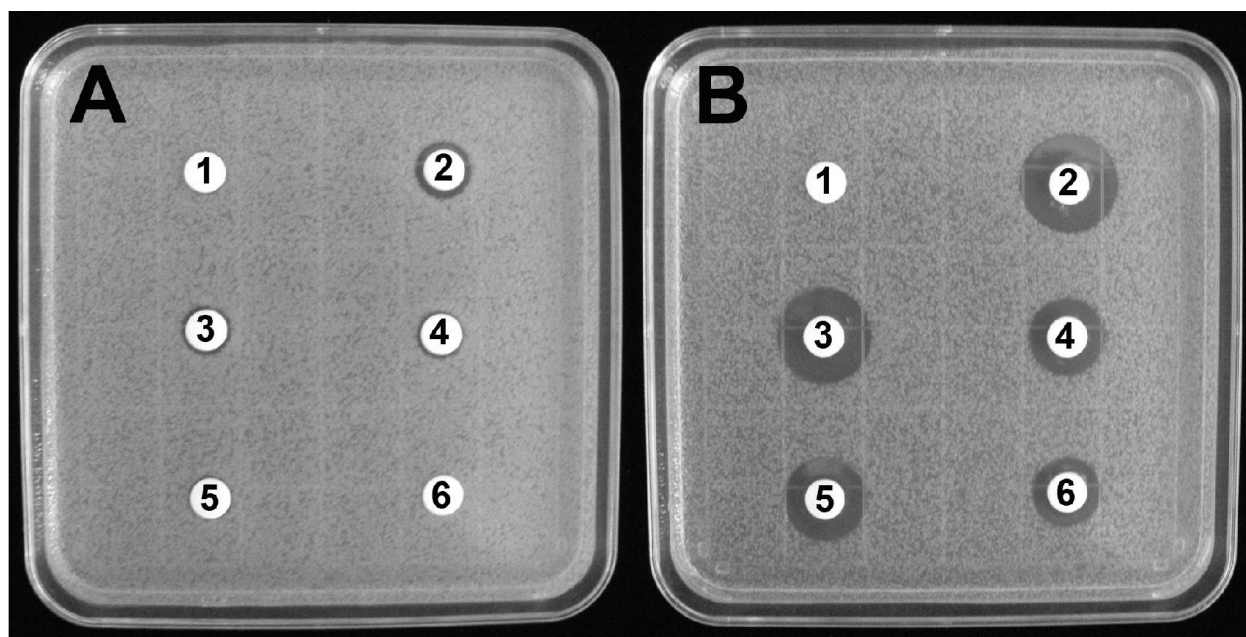
**Figure 6**. Comparative activities of PU films against wild-type and deep-rough *Salmonella* strains. Panel A: *Salmonella* Typhimurium ATCC 13311 (wild type, intact outer membrane); Panel B: *Salmonella* Minnesota R613 (deep-rough mutant, truncated outer membrane). Although the acetic acid control was not inhibitory to either strain, *S.* Minnesota was markedly more susceptible to all PU films tested. These data highlight the importance of an intact outer membrane in exclusion of these antimicrobials from their cell membrane or intracellular targets. Legend: 1) acetic acid control, 2) PU-MDEA, 3) PU-EDEA, 4) PU-PDE, 5) PU-TEA, 6) PU-EDTE. See Scheme 1 for PU structures.

**Scheme 1**. Preparation of the soybean-oil-based cationic PUDs

# CHAPTER 4. THERMO-MECHANICAL AND ANTIBACTERIAL PROPERTIES OF SOYBEAN OIL-BASED CATIONIC POLYURETHANE COATINGS: EFFECTS OF AMINE RATIO AND DEGREE OF CROSSLINKING

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## Abstract

Soybean oil-based cationic polyurethane coatings with antibacterial properties have been prepared with a range of different molar ratios of hydroxyl groups from an amine diol. A second series of polyurethane coatings were prepared from soy polyols with different hydroxyl numbers. All of the cationic PU dispersions and films exhibit inhibitory activity against three foodborne pathogens: *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus*. It is generally observed that increases in the ratio of ammonium cations improve the antibacterial

performance. Reduction of the crosslink density by decreasing the hydroxyl number of the soy polyol also results in slightly improved antibacterial properties. Higher glass transition temperatures and improved mechanical properties are observed with corresponding increases in the molar ratios of the amine diol and the diisocyanate. These results show that the mechanical properties of these coatings can be tuned, while maintaining good antibacterial activity.

## 1. Introduction

In the past few years, there has been an increased interest in the use of various agricultural commodities as biorenewable alternatives to petroleum for the synthesis of plastic materials.[1, 2] Long-term concerns about the cost and availability of petroleum supplies have been driving factors behind this push for sustainability.[3] In particular, plant-based oils are commonly used as renewable raw materials as these are readily available, generally inexpensive, are biodegradable and have low toxicities, among other advantages.[4,5]

Polyurethanes are especially amenable to use of biorenewable plant-based oils as raw materials.[6] Polyurethanes comprise an important class of polymers and are used widely throughout industry in foams, coatings, adhesives and cast elastomers.[7] In particular, waterborne polyurethane dispersions (PUDs) have garnered substantial interest as environmentally-friendly coatings.[8-10] Waterborne PUDs have significant advantages over PUDs manufactured using traditional organic solvents. Specifically, aqueous systems are more cost effective than organic-based approaches and the use of water as a solvent reduces or even eliminates the emission of harmful volatile organic compounds.[11,12] More recently, the development of waterborne PUDs made with biorenewable oils has become the focus of research into environmentally-friendly protective and/or decorative coatings.[13-15]

To date, anionic waterborne PUDs are more prevalent, both in industry and in the literature, than cationic PUDs.[11]   Typically, anionic PUDs are prepared by incorporating a diol or polyol that also contains a carboxylic acid group, such as dimethylolpropionic acid (DMPA), into the polymer backbone.   The PU is then neutralized with a tertiary amine such as triethylamine (TEA) and dispersed in water.   Anionic polyurethane dispersions have been successfully prepared using 50-60 wt % vegetable oil polyols.[14]   However, a key disadvantage of anionic PUDs is their lack of antimicrobial activity.

In contrast, cationic PUDs, which are prepared by incorporating a tertiary amine diol or polyol followed by treatment with an acid, are seldom used commercially.   One potential advantage of cationic PUDs is that they have been found to exhibit excellent adhesion properties.[11,16-18]. However, the antimicrobial properties of cationic PUDs are of much greater interest.[18] Cationic compounds are able to bind to bacteria and other microbes and disrupt cell structure, resulting in permeabilization and death. For example, other polymer coatings that contain amine groups, certain cationic peptides, and chitosan, which is a positively charged carbohydrate polymer derived from crustacean shells, are all known to be antimicrobial.[19] Recently, an aqueous coating using chitosan was reported; however, drawbacks of this technique include its complexity, the need for electrografting, and the limited solubility of chitosan.[20]

Our group has previously reported novel cationic polyurethane dispersions from vegetable oil-based polyols.[16] A further study from our group examined the effects of the hydroxyl functionality of the soybean oil-based polyols on the thermal and mechanical properties of cationic polyurethanes.[17] We recently reported the first study on the effect of different polyols on the antibacterial properties of cationic plant-oil based PUDs and PU coatings.[21]  In our previous work, cationic PUDs were prepared using different amine polyols, while the molar

ratios hydroxyl (OH) groups from the amine polyols, OH groups from the soy polyol and the isocyanate (NCO) groups from the isocyanate were held constant.[21] In the current work, environmentally-friendly, plant oil-based, cationic PUDs with excellent coating properties have been successfully synthesized from soybean oil-based polyols. In this study, two series of cationic PUDs were examined. The first series of PUDs were synthesized by varying the molar ratios of OH groups from the tertiary amine diol *N*-methyl diethanolamine (MDEA). The second series of PUDs were synthesized using methoxylated soy polyols with different hydroxyl numbers while holding the molar ratios of OH groups and NCO groups constant. We examined the effects of the different molar ratios of hydroxyls groups from MDEA and the effects of crosslink density on the thermo-mechanical properties and antibacterial properties of PUDs and PU films. The antibacterial properties of these materials have been evaluated using the disk diffusion assay against *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S*. Typhimurium), the two most common causative agents of bacterial foodborne illnesses.[22, 23] We have also performed disk diffusion tests using methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to antibiotics commonly used to treat staphylococcal infections. MRSA infection may occur after invasive surgical procedures, such as the implantation of a medical device (hospital-acquired MRSA) or as a topical infection through person-to-person transmission among those in close physical contact, such as wrestlers or care providers (community-acquired MRSA). A food-related route for transmission of MRSA has also been reported - a community-acquired outbreak of acute gastroenteritis that was traced to contamination of foods by an asymptomatic food worker.[24] Further, MRSA has been detected in raw meat samples (beef, pork, chicken, turkey) at levels ranging from 0.7% - 35.5%.[25]

Together, these data indicate that food workers or the food supply could represent additional reservoirs for the transmission of MRSA.

The goal of this study was to investigate the antibacterial properties of cationic plant oil based PUDs and PU films so that their potential value as coatings for surfaces in food processing or healthcare environments, or as elements in food packaging for pathogen control could be assessed. We also sought to determine if the thermal and mechanical properties of these novel cationic PU films could be tuned, while maintaining their antibacterial properties.

## 2. Experimental Section

### 2.1. Materials

Soybean oil was purchased at the local supermarket and used directly. Methoxylated soybean polyols (MSOL) with hydroxyl numbers of 140, 155, and 174 mg KOH/g were synthesized as previously reported.[14] DMPA, *N*-methyl diethanolamine (MDEA), isophorone diisocyanate (IPDI), and dibutyltin dilaurate (DBTDL) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Glacial acetic acid and methyl ethyl ketone (MEK) were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). All materials were used as received.

### 2.2 Synthesis of the Soybean Oil-based Cationic PUDs

The IPDI, MSOL, and MDEA were added to a three-necked flask equipped with a mechanical stirrer, condenser, and thermometer. The molar ratio of NCO groups from IPDI was varied from 2.0 to 2.75. The molar ratio of OH groups from MSOL was kept constant at 1.0, while the molar ratio of OH groups from MDEA varied from 0.95 to 1.7 (corresponding to the NCO molar ratio of IPDI). One drop of DBTDL was added to the reaction mixture. The reaction

was first carried out at 80 °C for 10 minutes and then MEK (50 wt % based on the reactant) was added to reduce the viscosity.  After 2 h reaction, the reactants were then cooled to room temperature and neutralized by the addition of 1.5 equivalents of acetic acid, followed by dispersion at high speed with distilled water to produce the cationic PUD with a solid content of about 12.5 wt% after removal of the MEK under vacuum.  The corresponding PU films were obtained by drying the resulting dispersions at room temperature in polystyrene petri dishes.  As a control to confirm the role of cationic charge in the PUD antibacterial activity, a plant oil-based anionic dispersion was prepared using DMPA as previously reported.[14]  The molar ratios for the anionic PUD control were 2.0 NCO from IPDI, 1.0 OH from MSOL, and 0.95 OH from DMPA. The chemical structure of the starting materials and a representative polymer structure are shown in **Scheme 1**.  The nomenclature used for the various PUD samples and their composition is listed in **Table 1,** where the letter (A, B, or C) corresponds to the MSOL hydroxyl number and the number (1, 2, or 3) corresponds to different molar ratios of IPDI and MDEA.

### 2.3    Thermal and Mechanical Analysis

The dynamic mechanical behavior of the PUDs was characterized using a dynamic mechanical analyzer (TA Instruments DMA Q800, New Castle, DE) in tensile mode at 1 Hz. The samples were heated from −70 to 100 °C at a rate of 5 °C/min.  For this study, the glass transition temperatures ($T_g$) were determined from the onset temperatures of the decrease in storage modulus.  Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter (TA Instruments DSC Q20, USA). The samples were heated from 25 to 80 °C at a rate of 20 °C/min to erase their thermal history, cooled to −70 °C, and heated again to 100 °C at a heating rate of 20 °C/min. The sample mass was approximately 4 mg. A

thermogravimetric analyzer (TA Instruments TGA Q50, USA) was used to measure the weight loss of the PUDs in an air atmosphere. The samples were heated from 30 to 650 °C at a heating rate of 20 °C/min. The mass of the samples used for TGA analysis was approximately 8 mg.

## 2.4 Bacterial Strains and Growth Conditions

The test cultures, *Salmonella* Typhimurium ATCC 13311 and *Staphylococcus aureus* ATCC BAA-44 (methicillin-resistant) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Listeria monocytogenes* NADC 2045 was from the USDA National Animal Disease Center (NADC, Ames, IA). Cultures were maintained as frozen stocks at -80 °C in Tryptic Soy Broth (TSB, BD Diagnostics Systems, Sparks, MD) containing 20% (v/v) glycerol. Working cultures of these bacteria were maintained on Tryptic Soy Agar (TSA) plates. Strains were grown in TSB for 18 h at 35 °C and the optical densities of the resulting bacterial cultures were measured spectrophotometrically (model DU720, Beckman Coulter). The relationship between absorbance at 600 nm ($A_{600}$) and plate counts was determined for cultures adjusted to $A_{600}$ values of 0.1 ($10^8$ CFU mL$^{-1}$) or 1.0 ($10^9$ CFU mL$^{-1}$) using fresh TSB. This relationship was used in subsequent experiments to prepare working bacterial suspensions in TSB or 0.85% saline containing $10^6$ CFU mL$^{-1}$ (for disk diffusion tests) or $10^5$ CFU mL$^{-1}$ (for Bioscreen and PU film leaching tests).

## 2.5 Antibacterial Testing

### 2.5.1 Disk diffusion tests

The antibacterial properties of both PU dispersions and dried films cast onto sterile paper disks (polymer-fiber composites) were examined against all three test organisms, as described

previously, using Clinical and Laboratory Standards Institute (CLSI) methods. [21] Statistical analyses were conducted using the SAS 9.3 software (SAS Institute, Inc., Cary, NC). Significant differences between treatments ($p<0.05$) were determined by the MIXED procedure with Tukey adjusted pairwise comparisons based on the data from the split-plot design. All the results in disk diffusion tests were expressed as mean and standard deviations of six individual measurements.

### 2.5.2 Additional testing with *S.* Typhimurium

To obtain more detailed information on the relative efficacy and mechanism of action for select PU films, further testing was performed beyond the initial disk diffusion work using *S.* Typhimurium, an organism we found in previous work to have higher intrinsic resistance to PUDs and PU films.[21] Specifically, the two most active PU films from the disk diffusion work, along with the negative control polymer A1-anionic (abbreviated hereafter as "A1-AN"), were examined for their potential to leach antibacterial components into liquid media. We also sought to examine the impact of PU films on the physical integrity and viability of *S.* Typhimurium suspensions. These experiments are described in greater detail below.

### 2.5.2.1 Leaching of antibacterial components from dried films

To determine whether or not substantial leaching of antibacterial material from cast films could occur, the following experiment was performed. Two hundred microliters of each test PUD (A1-AN, B2 and B3) were added to separate 8 mL sterile screwcap tubes and allowed to air dry in a sterile biological safety cabinet for 2 d, yielding tubes containing dried films at the bottom. The abilities of these films to affect the growth of *S.* Typhimurium in media added to these tubes was examined visually using a resazurin-based test. Briefly, 3 mL of TSB inoculated with *S.* Typhimurium ($10^5$ CFU mL$^{-1}$) was added to tubes containing each film type. As a

positive control, the same amount of cell culture was also added to an unmodified sterile tube (no PUD film). An uninoculated tube of sterile TSB served as a negative control. After incubation at 35 °C for 20 h, the oxidation-reduction indicator resazurin (alamarBlue®, Trek Diagnostic Systems, Cleveland, OH) was added into each tube to visually test the inhibitory effects of leaching components from PU films.[26] Briefly, when live, metabolically active cells were present, the dye underwent a color change from blue to pink. In media to which no cells were added or where cell viability was reduced or eliminated, the dye remained blue.

As another means for examining the degree of leaching of active PU film components into liquids, we exposed sterile bacterial growth media to the three types of PU films, then examined the subsequent ability of each medium to support growth of *S*. Typhimurium. Briefly, 3 mL of sterile TSB was added to tubes containing each type of film, prepared as described above. As a control, the same volume of TSB was added to an unmodified sterile tube (no PUD film). All tubes were incubated for 3 days at 35 °C, allowing any diffusible components of the film to partition into the TSB medium. PUD-exposed and control media were collected and dispensed into individual wells of a Bioscreen C Microbiological Reader plate (Growth Curves, USA, Piscawatay, NJ). Wells were inoculated with *S*. Typhimurium ($10^5$ CFU mL$^{-1}$) and the plates were incubated for 24 h at 35 °C in the Bioscreen, with absorbance readings taken every 15 min at 600 nm. To examine the potential impact of heat exposure on the nutrient content of TSB during the 3-d leaching period, fresh TSB was also tested in parallel with the other treatments.

2.5.2.2 Assay for intracellular leakage from PUD-exposed *Salmonella*

The impact of PUD exposure on the physical integrity of *S.* Typhimurium was examined using a spectrophotometric assay for leakage of intracellular components. Briefly, an overnight culture of *S.* Typhimurium was harvested and cells were suspended in 0.85% saline at a concentration of $10^9$ CFU mL$^{-1}$. One milliliter aliquots of this suspension were added to screwcap tubes coated with A1-AN, B2 or B3 PUD films or, as a control for the effects of saline alone, to an unmodified sterile tube (no PUD film). These were incubated for up to 24 h at 35 °C. After 1, 3, 5, 7 or 24 hours exposure, 100 µL portions were removed for analysis. These were diluted in 200 µL 0.85% saline and centrifuged for 6 min at 16,600 x *g* to ensure that the supernatant contained only macromolecular material released during PU film exposure. Two hundred microliters of this clarified supernatant were removed to a new centrifuge tube and the absorbance read at 260 nm using a BioMate 3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).[27] Appropriate blanks were used to control for any potential contribution of PUD material to UV absorbance. Viability testing was also carried out in parallel at each timepoint. Briefly, PUD-containing tubes were vortexed to suspend cells and aliquots were taken, serially diluted into 0.85% saline and viable cells quantified using the track dilution method.[28] The relationship between the rate of leakage of UV-absorbing intracellular material and the death rate for *S.* Typhimurium was plotted using the combined spectrophotometric and plate count data from the 3 h, 5 h and 7 h timepoints, during which OD increased in a linear fashion. Briefly, the slope for each OD curve over this initial 4 h period was calculated for the three PU films, and these data were plotted against the death rate ($\log_{10}$ reduction hr$^{-1}$).[29] This

plot provides another means for ordering the PU films in terms of their efficacy against *S.* Typhimurium.

## 3. Results and Discussion

### 3.1. Thermal and Mechanical Properties

Dynamic mechanical analysis (DMA) was used to elucidate the thermal and mechanical properties of the PU films. The results are summarized in Table 2 and Table 3. As the relative molar ratio of MDEA increases, the $T_g$ increases from 14.3 to 20.0 °C (see Table 1). Likewise, the storage modulus at room temperature (25 °C) increased from 126.1 to 406.8 MPa. The PU films with higher amounts of MDEA, a chain extender, have improved mechanical properties because the increases also correspond to increases in overall hard segment content supplied by the IPDI.[14] Likewise, increasing the hydroxyl numbers of the soy polyol while keeping the overall molar ratios between NCO groups and OH groups constant, raises the crosslink density of the PU films.[14] This is demonstrated by the increase in the $T_g$ from 3.0 to 17.8 °C as the hydroxyl number changes from 140 to 174 mg KOH/g (see Table 2). The storage modulus at 25 °C increased from 21.0 to 211.6 MPa. The trends from increasing MSOL hydroxyl number and from increasing MDEA and IPDI molar ratios are illustrated together for the storage modulus in Figure 1 and for the glass transition temperature in Figure 2. Additionally, as a reference, one anionic PU film with DMPA instead of MDEA was evaluated. The anionic PU had a much higher $T_g$ (20.6 vs 3.0 °C) and storage modulus at 25 °C (305.3 vs 21.0 MPa) compared to the cationic PU sample with the same IPDI molar ratios due to the physical differences between DMPA and MDEA. Differential scanning calorimetery (DSC) was also used to examine the thermal properties of the PU films (data not shown). The thermograms for the

films showed single, but broad glass transition temperatures that are consistent with amorphous (non-crystalline) polymers.

Figure 3 displays the weight-loss and derivative weight-loss curves as a function of temperature for PU films with different ratios of OH groups from MDEA. The simultaneous increase in molar ratios of NCO groups from IPDI and of OH groups from MDEA increases the amount of thermally labile ammonium and urethane groups. The temperature at which 5% ($T_5$) and 50% ($T_{50}$) degradation occur decreases as the MDEA content increases, as listed in Table 2. However, there is a minimal change in the temperature of the maximum rate of weight loss, $T_{max}$. The samples with the highest relative ratios of ammonium groups had more pronounced derivative peaks for ammonium decomposition (below 200 °C) and dissociation of urethane bonds (200 to 320 °C).[21] Figure 4 displays the weight-loss and derivative weight-loss curves as a function of temperature for PU films prepared with different MSOL hydroxyl numbers. Although higher MSOL OH numbers result in higher crosslinking, as reflected by increases in the $T_g$, there is also a higher content of IPDI.[14]  This results in the incorporation of more labile urethane groups, thereby decreasing the thermal stability, which is reflected by the decreases in $T_5$, $T_{50}$ and $T_{max}$ listed in Table 3.

### 3.2 Antibacterial Properties

Based on the results of disk diffusion tests for PU dispersions and films, as the MDEA OH group molar ratio increased from 0.95 to 1.45 (samples B1 to B2), zones of inhibition (ZOI) also increased significantly ($p < 0.05$) (see Table 4). These results indicate that antibacterial properties increase as the relative concentration of quaternary ammonium groups in the polymer backbone increases (from 8.8 wt.-% to 12.0 wt.-% MDEA for B1 and B2, respectively).

However, there was no significant difference between B2 (MDEA OH group molar ratio of 1.45) and B3 (MDEA molar ratio of 1.7) in this assay. This can be attributed to the smaller change in the concentration of quaternary ammonium groups from B2 to B3 (12 wt.-% MDEA to 13.2 wt.-% MDEA).

The polyurethanes made from MSOL with larger hydroxyl numbers have an increased crosslink density as a result of the higher hydroxyl functionality. By changing the MSOL hydroxyl functionality while keeping the molar ratio of hydroxyl groups from MDEA constant, samples prepared from MSOL with larger hydroxyl numbers have slightly higher quaternary ammonium concentrations. However, PU sample A1, which had the lowest hydroxyl number (140 mg KOH/g) and the lowest quaternary ammonium concentration (8.3 wt.-% MDEA), showed significantly better antibacterial properties than the corresponding samples B1 and C1 with higher hydroxyl numbers and higher quaternary ammonium concentrations ($p < 0.05$). This trend indicates that the polymers with lower crosslink densities have better antimicrobial properties. The higher molecular mobility of these less crosslinked polymers likely enhances effective physical interaction with target bacteria, resulting in a net increase in antibacterial activity.

The same relative trend of antibacterial activity was observed for all three strains tested in our disk diffusion assay. *L. monocytogenes* was most susceptible to the PUDs and PU films tested, and *S.* Typhimurium was the least susceptible ($p < 0.05$). The greater resistance of *Salmonella* to PUD activity can be explained by the outer membrane (OM) of this Gram-negative bacterium, which serves as an additional permeability barrier to exogenous compounds. The absence of an OM in Gram-positive bacteria such as *L. monocytogenes* and *S. aureus* allows

more ready uptake of antimicrobials by these bacteria and is consistent with earlier findings by Xia et al.[21]

By their very nature, the disk diffusion tests indicated that dried films were capable of releasing diffusible antibacterial material into the agar medium. In practical use, the mobility of material from films could be problematic for food-related applications, where chemical residues are not desirable. Therefore, we sought to examine the potential importance of leaching from cast films in more detail. In these tests, the most active PUs (B2 and B3) were examined further against *S.* Typhimurium in liquid systems using both qualitative and quantitative assays. Figure 5 shows that PU film B3 could effectively inhibit the growth of this pathogen inoculated at $10^5$ CFU ml$^{-1}$ into TSB. Although PU B2 did not prevent growth of *S.* Typhimurium under these conditions, it did possess good antibacterial activity, as determined by the disk diffusion and cell leakage assays. This underlines the importance of using multiple types of analyses when evaluating and ranking antimicrobial activity. The negatively-charged A1-AN did not show antibacterial activity, as expected given the known relationship between cationic charge and antimicrobial efficacy. While the results of this assay suggest that diffusible polymer elements could be responsible for the growth inhibition seen, it is also possible that inactivation of cells could have resulted through direct contact with the film at the bottom of the tubes. The test for leaching (see Figure 6) shows that antibacterial components were able to diffuse from films into TSB held for 3 days at 35°C and exert their activities in solution. This assay also provides clear corroboration that A1-AN is not inhibitory, and that both B2 and B3 are antibacterial, with B3 being the most active. The greater activity of B3 indicates that the molar ratio of MDEA had an impact on antibacterial activity.

Based on the known action of other cationic antimicrobials, we expected that the PUDs and their films might exert their antibacterial activities by causing gross physiological damage to bacterial cells. We tested this hypothesis by assaying for leakage of UV-absorbent materials (i.e. DNA, proteins, and other macromolecules) from bacterial cells and into the surrounding medium. Our results from the intracellular leakage assay agreed with those of the film leaching assay, with A1-AN yielding little more damage to cells than did exposure to saline alone, and polymers B2 and B3 causing a steady increase in loss of intracellular contents to the medium, plotted in Figure 7(A). These results confirm that antibacterial components capable of permeabilizing this Gram-negative bacterial pathogen are able to leach into liquids from these PU films. Figure 7(B) shows that over the 24 h period, exposure to B2 and B3 films resulted in a ≈4-log reduction in viable cells. Conversely, exposure to the A1-AN film, as with saline alone, did not cause cell death. These results mirror those of the UV leakage assay, demonstrating that increasing exposure of S. Typhimurium cells to diffusible elements from the cationic PU films results in increased permeability, loss of cellular contents and cell death. The relationship between the rate of leakage of UV-absorbent material and the cell death rate over the period extending from 3 h to 7 h exposure is plotted in Figure 7(C). This plot demonstrates that B3 was the most effective antibacterial film, followed by B2, and finally by A1-AN, which was not antibacterial.

## 4. Conclusions

Soybean-oil based cationic PUDs have been prepared with three different relative compositions of ammonium cations. Additionally, cationic PUDs were prepared using methoxylated soy polyols with three different hydroxyl numbers. The glass transition temperatures and room-temperature storage moduli both increased with the increase in MDEA

and IPDI molar ratios, as well as with increases in the hydroxyl number of the soy polyol. All of the cationic PUDs were found to have inhibitory activity against three foodborne pathogens, *Salmonella* Typhimurium, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus*. Increasing the relative ratio of ammonium cations improved the antibacterial properties, however the results show that this effect may level out around 12 wt.-% MDEA. This observed trend was consistent across the different bacterial strains. This investigation shows promising results in that the mechanical properties of environmentally friendly, soy-based cationic PUDs can be tuned while maintaining their antibacterial activity. Because we observed diffusive loss of antibacterial material from PU films, additional work is required to determine the suitability of these materials for applications involving direct contact with foods. However, additional non-contact uses are possible, such as coating of shipping pallets used in food storage or shipping operations, which have been implicated as points of harborage for human pathogens.

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**Table 1**. Sample nomenclature and composition

| Sample | Polyol OH Number[a] | Molar Ratios of Functional Groups | | |
|---|---|---|---|---|
| | | OH Groups (Polyol) | OH Groups (MDEA) | NCO Groups (IPDI) |
| A1-AN[b] | 140 | 1.00 | 0.95[c] | 2.00 |
| A1 | 140 | 1.00 | 0.95 | 2.00 |
| B1 | 155 | 1.00 | 0.95 | 2.00 |
| B2 | 155 | 1.00 | 1.45 | 2.47 |
| B3 | 155 | 1.00 | 1.70 | 2.75 |
| C1 | 174 | 1.00 | 0.95 | 2.00 |

[a] mg KOH / g polyol [b] A1-AN: an anionic version of PUD A1, used as a control for the cationic nature of the PU antibacterial activity [c] DMPA was used instead of MDEA

**Table 2**. Comparison of DMA and TGA data of PU films with different ratios of MDEA.

| Sample[a] | OH Ratio from MDEA | wt% MDEA | $T_g$[a] (°C) | E' at 25 °C (MPa) | TGA Data | | |
|---|---|---|---|---|---|---|---|
| | | | | | $T_5$ (°C) | $T_{50}$ (°C) | $T_{max}$ (°C) |
| B1 | 0.95 | 8.8 | 14.3 | 126 | 248 | 360 | 350 |
| B2 | 1.45 | 12.0 | 16.7 | 220 | 196 | 349 | 346 |
| B3 | 1.7 | 13.2 | 20.0 | 407 | 193 | 348 | 349 |

[a] $T_g$ based on onset of storage modulus

**Table 3**.  Comparison of DMA and TGA data of PU films with different hydroxyl numbers.

| Sample[a] | OH Number (mg KOH/g) | Wt.-% MDEA | $T_g$[a] (°C) | E' at 25 °C (MPa) | TGA Data | | |
|---|---|---|---|---|---|---|---|
| | | | | | $T_5$ (°C) | $T_{50}$ (°C) | $T_{max}$ (°C) |
| A1-AN[b] | 140 | n/a[C] | 20.6 | 305 | 170 | 352 | 384 |
| A1 | 140 | 8.3 | 3.0 | 21.0 | 246 | 355 | 342 |
| B1 | 155 | 8.8 | 14.3 | 126 | 248 | 360 | 350 |
| C1 | 174 | 9.4 | 17.8 | 212 | 216 | 306 | 285 |

[a] $T_g$ based on onset of storage modulus [b] A1-AN: an anionic version of PUD A1, used as a control for the cationic nature of the PU antibacterial activity [c] DMPA was used instead of MDEA for sample A1-AN.

**Table 4**.   Summary of disk diffusion testing

| Sample Name | *L. monocytogenes* NADC 2045 | | *S.* Typhimurium  ATCC 13311 | | *S. aureus* ATCC BAA-44 | |
|---|---|---|---|---|---|---|
| | ZOI [a] (mm) | | ZOI [a] (mm) | | ZOI [a] (mm) | |
| | Dispersion | Film | Dispersion | Film | Dispersion | Film |
| A1-AN[b] | None | None | None | None | None | None |
| A1 | 11.65 ± 0.22 | 11.12 ± 0.12 | 9.75 ± 0.16 | 9.04 ± 0.21 | 9.74 ± 0.16 | 9.28 ± 0.16 |
| B1 | 10.79 ± 0.17 | 10.48 ± 0.27 | 9.15 ± 0.17 | 8.18 ± 0.23 | 9.18 ± 0.12 | 8.35 ± 0.13 |
| B2 | 11.94 ± 0.15 | 11.43 ± 0.13 | 10.48 ± 0.22 | 9.57 ± 0.21 | 10.68 ± 0.16 | 10.23 ± 0.28 |
| B3 | 11.92 ± 0.32 | 11.33 ± 0.17 | 10.29 ± 0.12 | 9.56 ± 0.29 | 10.74 ± 0.18 | 10.00 ± 0.06 |
| C1 | 10.93 ± 0.27 | 10.23 ± 0.19 | 9.11 ± 0.11 | 8.02 ± 0.16 | 9.63 ± 0.13 | 8.56 ± 0.11 |

[a] Zone of Inhibition; [b] A1-AN: an anionic version of PUD A1, used as a control for the cationic nature of the PU antibacterial activity
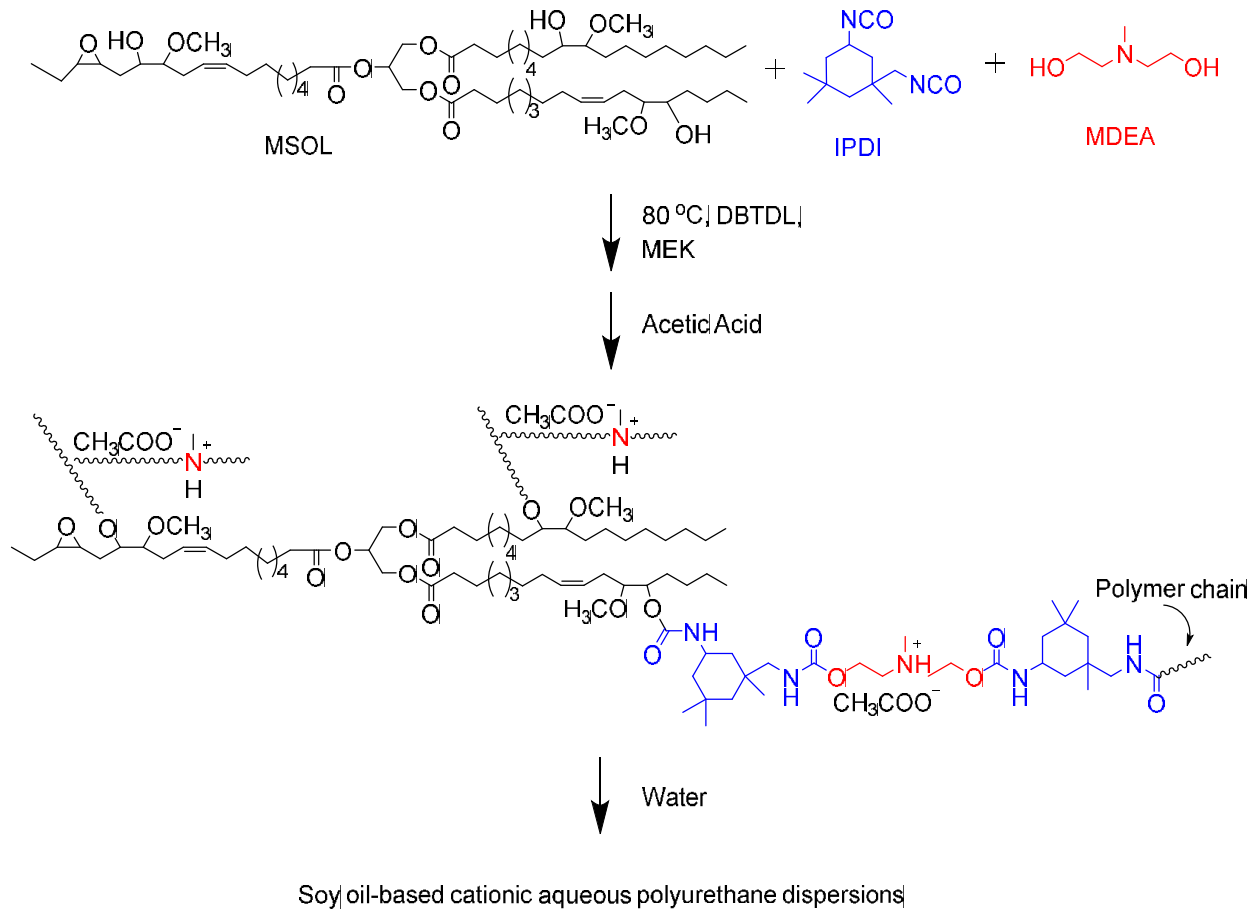
**Scheme 1**.  Reaction scheme of the cationic plant-oil based polyurethanes.
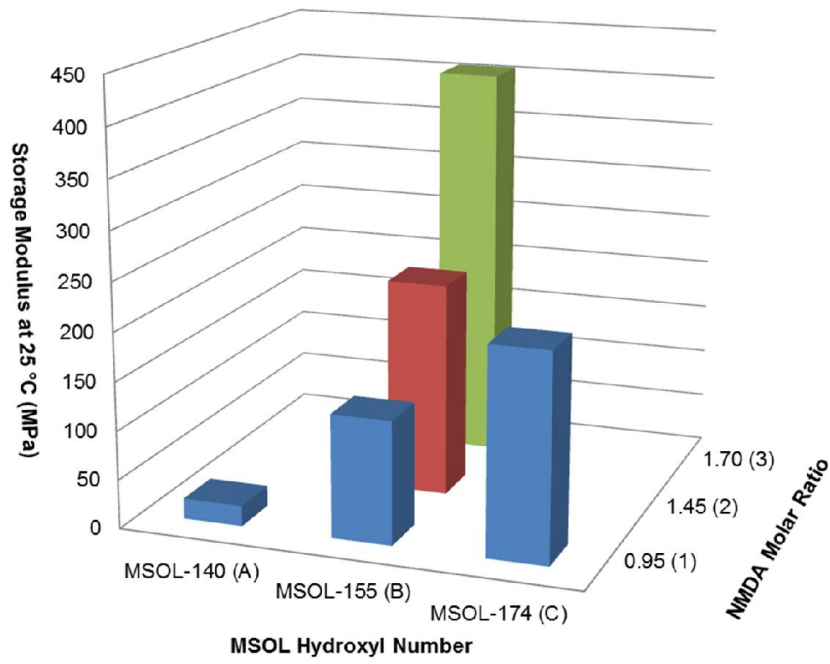
**Figure 1**. Storage modulus at 25 °C for PU films with different compositions.
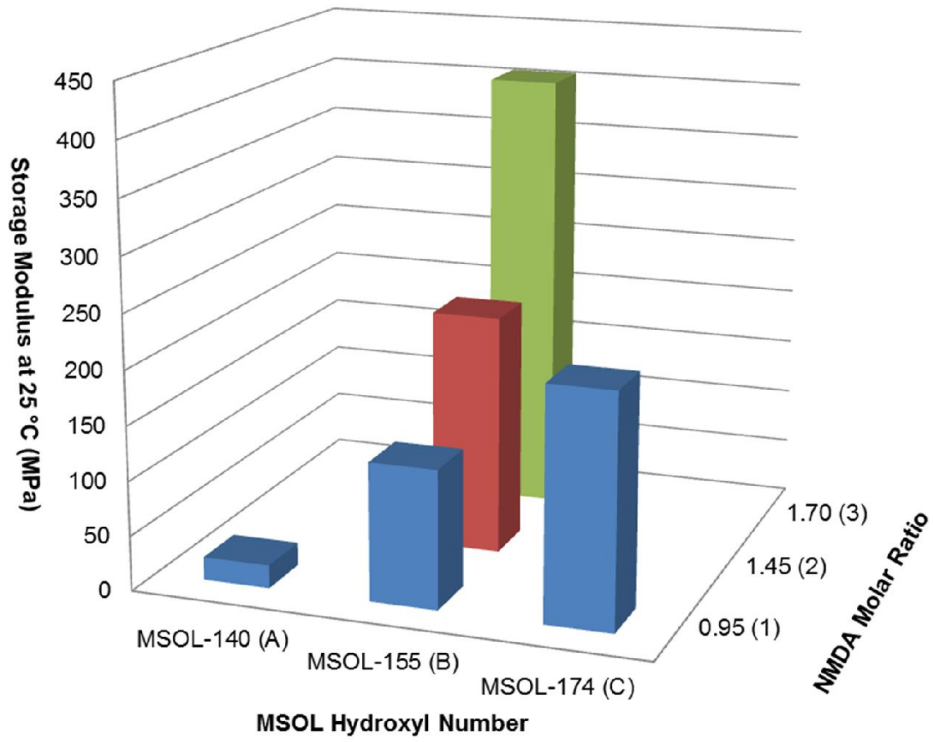
**Figure 2**. Glass transition temperatures for PU films with different compositions

**Figure 3**. Thermograymetric analysis (TGA) curves and their derivative curves for PU flims with different molar ratio of MDEA
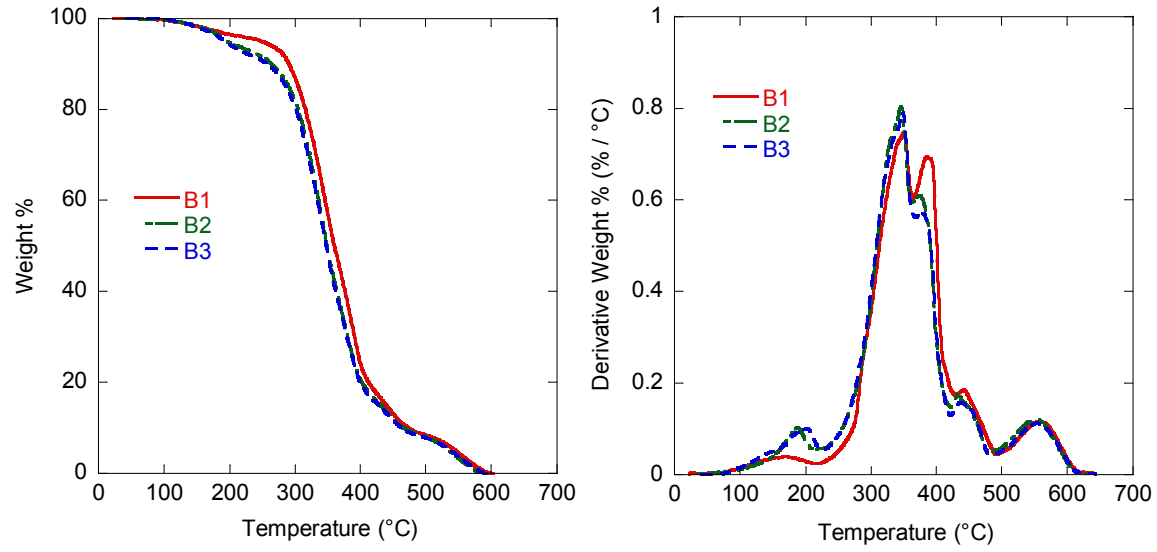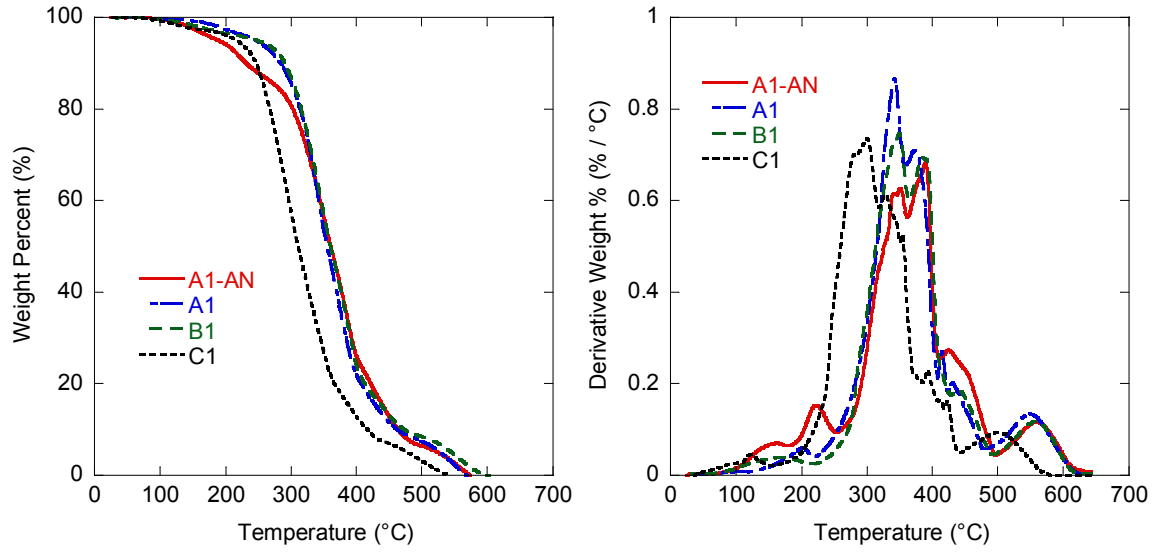
**Figure 4**. Thermogravimeteric analysis (TGA) curves and their derivative curves for PU films with different hydroxyl numbers
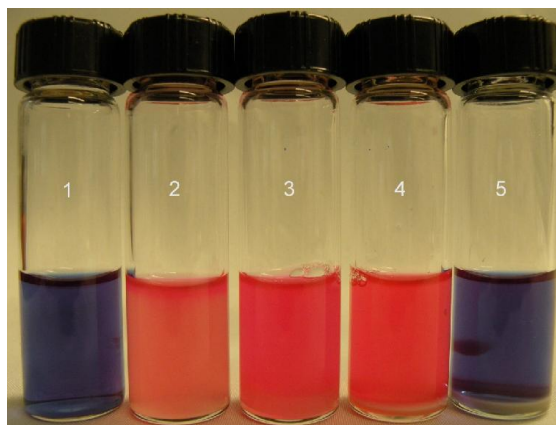
**Figure 5**. Visual demonstration of bacterial inhibition in tubes containing PU films. The following treatments were examined: 1. negative control (uninoculated TSB, no PU coatings), 2. Positive control (no PU coatings), 3. A1-AN coating (negative control), 4. B2 coating, 5. B3 coating. Treatments 2-5 were inoculated with *S.* Typhimurium ATCC 13311 at $10^5$ CFU mL$^{-1}$.
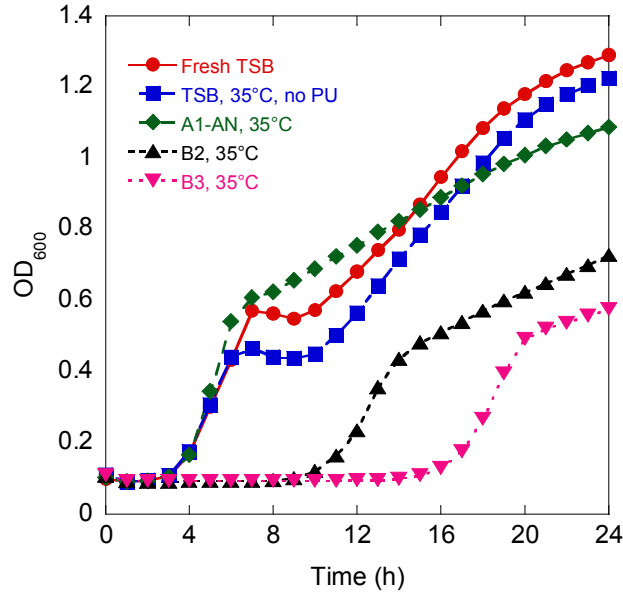
**Figure 6**. Results of antibacterial leaching assay. Effects of the following treatments on growth of *S.* Typhimurium ATCC 13311 in TSB were examined in a Bioscreen C automated turbidimeter: fresh, untreated TSB; TSB pre-incubated for 3 days at 35 °C; and TSB incubated for 3 days at 35 °C in the presence of the films A1-AN, B2 and B3, respectively. Absorbance was measured at 600 nm. Decreased absorbance indicates suppression of bacterial growth. Representative results from three independent experiments are shown.
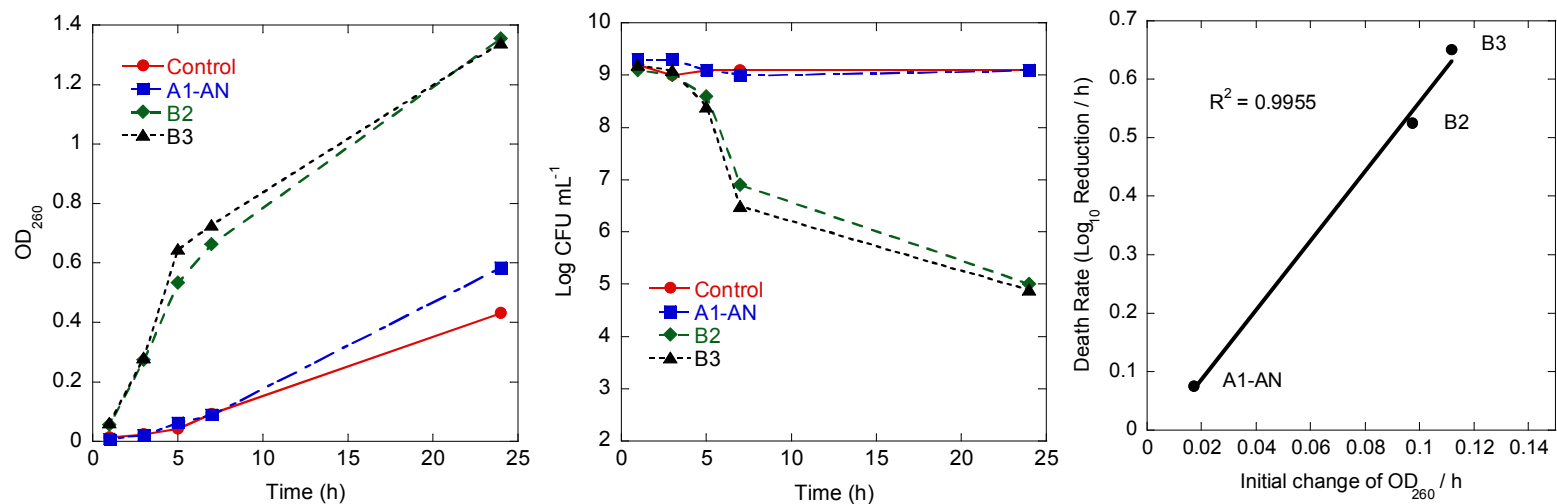
**Figure 7**. PU film-mediated leakage of intracellular components from *S.* Typhimurium ATCC 13311 and parallel plating analysis. Increases in UV-absorbance indicated physical permeabilization of cells and leakage of cellular macromolecules. Loss of cell integrity was accompanied by decreases in viability, as determined by plating. Representative results from three independent experiments are shown.

(A) Absorbance vs. time for cell suspensions exposed to saline alone (control) or tubes containing PU films       A1-AN, B2 or B3.
(B) Viability vs. time for cell suspensions analyzed in Fig 7(A).
(C) Relationship between rate of leakage at 260 nm and death rate during initial period of linear OD increase (hours 3-7).

## CHAPTER 5.  EFFECTS OF COUNTERANION ON THE PHYSICAL AND ANTIMICROBIAL PROPERTIES OF CASTOR OIL-BASED CATIONIC POLYURETHANE COATINGS

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## 1. Introduction

Aqueous polyurethane dispersions (PUDs) are highly useful coating materials that have found widespread applications in coatings, adhesives, paper sizings, and textiles.[1, 2] The resulting polyurethane films are known for their toughness, tensile strength, abrasion resistance, and excellent film forming properties. [3,4] Furthermore, waterborne coatings, including polyurethane dispersions, have significant advantages over conventional solvent-borne coatings as a result of regulations restricting the emissions of volatile organic compounds from organic solvents. [3, 5, 6]

Conventional polyurethanes are hydrophobic and require either the use of external emulsifiers or large shear forces to disperse in water. [3, 7] However, polyurethanes can be readily dispersed or dissolved in water by incorporating hydrophilic copolymers, called ionomers, into the polyurethane chains. Ionomers contain functional groups, such as acids or tertiary nitrogen groups, that can be neutralized or quaternized, respectively, to form salts.[8] The extent of neutralization or quaternation, which can range from partial to complete neutralization, has been shown to impact the stability and particle size of the PUDs.[9, 10] A higher degree of neutralization has been found to increase the tensile strength, modulus and glass transition temperature due to increased interchain interactions and hydrogen bonding.[11] Ionomers can be classified by the charges incorporated in the main polymer chain: anioniomers (negatively charged), cationomers (positively charged), and zwitterionomers (both positive and negatively charged).[3, 7, 12] The majority of commercial PUDs use anionic ionomers.[7, 13] In particular, dimethylolpropanoic acid (DMPA) has been one of the most widely used anionomers for PUDs.[10, 13]

Anionic PUDs have been extensively studied, including a number of investigations focused on the role of the counterion.[14] The studies have shown that the counterion does influence the thermal and mechanical properties of the polyurethane films, particularly the thermal stabilities.[14] A variety of bases have been used as countercations in anionic PUDs, including amines and metal hydroxides.[15, 16] Increased ionization of the carboxylic acid groups has been shown to decrease the onset temperature of decomposition in anionic PUDs.[16] Neutralization of anionomers with metal hydroxides has yielded films with increased initial thermal stabilities due to the involatility of the metal carboxylate products.[14, 16, 17] Degradation of thermally labile ionomers, such as phosphate groups, has

been shown to be dependent on the neutralizing base.[14, 17]  Increases in the ionic potential of countercations has corresponded to increased tensile strength, decreased elongation at break, and decreased particle size when anionic PUDs were neutralized with different metal hydroxides and amines.[15]  The glass transition temperature has also been shown to be impacted by counterions.[12, 18]

More recently, cationic PUDs are gaining attention because of the antimicrobial activity of quaternary amines.[19-21]  In particular, antimicrobial cationic polyurethane coatings are well suited for paper coating applications since cationic polymers strongly adhere to fiber surfaces.[22]  Cationic PUDs are typically synthesized by reacting diisocyanates with nitrogen-containing alkyl diols, such as *N*-methyldiethanolamine (MDEA).[14, 23, 24]  The nitrogen atoms are subsequently quaternized, either by protonation with acids or by an $S_N2$ reaction with alkyl halides, to produce the ionic groups.[19, 24, 25]  Previously, our group has examined the effect of different amino polyols on the thermal, mechanical, and antimicrobial properties of vegetable oil-based cationic PUDs.[20]  Our group has also investigated the effects of MDEA molar ratios and different hydroxyl numbers of soybean oil-based polyols on vegetable oil-based cationic PUDs.[26]

In the current study, the effect of the counteranion on the thermal, mechanical, and antimicrobial properties of cationic castor oil-based PUDs and their films has been evaluated for the first time.  Previous studies on the effect of counterions have been largely limited to countercations of anionic ionomers.  The cationic castor oil-based PUDs have been prepared from seven different carboxylic acids: formic acid, acetic acid, propanoic acid, butanoic acid, isobutyric acid, acrylic acid, and lactic acid.  In this study, the effect of carboxylic acid chain length has been examined as the carboxylic acids range in size from

one to four carbons (formic acid < acetic acid < propanoic acid < butanoic acid). Furthermore, the effect of substitutents (hydrogen, hydroxyl, or methyl group) and unsaturation has been examined as shown in Figure 1.

The need to develop new antimicrobial agents is greater than ever because of the emergence of bacterial multidrug resistance as well as emerging opportunistic yeast infections. The rise of antibiotic-resistant microbes poses a serious threat to public health and the world economy. A new improved strategy in researching the new drugs and preserving the efficiency of existing drugs is required to combat antibiotic resistance. [27] The biorenewable castor oil-based coatings reported in this study appear promising and effective for applications requiring antimicrobial functionalized surfaces and controlling antibiotics resistance. We had conducted antimicrobial evaluations of these PUDs against a fungus *Candida albicans*, two antibiotics-resistance bacterial pathogens *Acinetobacter baumannii* and *Staphylococcus* aureus. *C. albicans* is opportunistic pathogen but the most common fungal species leading to bloodstream infections in human with mortality rates of 38-49% . [28, 29] *Acinetobacter baumannii* is one of the most important multidrug-resistant microorganisms in hospitals worldwide, as well as in wounded soldiers serving in Iraq and Afghanistan, which become an increasingly important and demanding species in nosocomial pneumonia and bloodstream infections. [30, 31] Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multiple antibiotics resistant pathogen causing hospital-acquired and community-acquired infections [26]. Mode of action and mechanism of the most effective PU-Acrylic acid (PAA) against *C. albicans* were studied further on effects of cell membrane permeability as well as invasive growth of filamentous form-hyphae that was reported to play an important role in overall virulence of *C. albicans* [28, 32].

## 2. Experimental Section

### 2.1 Materials

Castor oil, *N*-methyl diethanolamine (MDEA), isophorone diisocyanate (IPDI), dibutyltin dilaurate (DBTDL), acrylic acid, butanoic acid, isobutyric acid, and lactic acid were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Methyl ethyl ketone (MEK), acetic acid, formic acid, and propanoic acid were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). All materials were used as received.

### 2.2 Synthesis of the Castor Oil-based Cationic PUDs.

The synthesis of the castor oil-based cationic PUDs was carried out using different organic acids (formic, acetic, propanoic, butanoic, acrylic, lactic, and isobutyric acids) to neutralize the amino groups. The detailed experimental procedure shown in Scheme 1 follows. Castor oil (5.00 g), IPDI (4.43 g), MDEA (1.47 g), and one drop of DBTDL were added to a three-necked flask equipped with a mechanical stirrer, thermometer, and condenser. The molar ratio of the OH groups from the castor oil, the NCO groups from the IPDI, and the OH groups from the MDEA was kept as 1.0 : 2.75 : 1.7. The reaction was first carried out at 80 °C and, after approximately 10 min, MEK (25 mL) was added to reduce the viscosity. After 2 h of reaction, the reactants were then cooled to room temperature and neutralized by the addition of 2.0 equivalents of acid, followed by dispersing at high speed with distilled water to produce the cationic PUD with a solid content of about 13 wt% after removal of the MEK under vacuum. The corresponding PU films were obtained by drying the resulting dispersions at room temperature in a polytetrafluoroethylene mold. The nomenclature used for the resulting castor oil-based

cationic PUDs is as follows: the PUD quaternized with butanoic acid was designated as PU-Butanoic.

## 2.3 Characterization.

The weight loss of the PU films under an air atmosphere was measured by using a thermogravimeter (TA Instruments Q50).  Samples ($\approx$ 8 mg) were heated from room temperature to 650 °C at a heating rate of 20 °C/min.  One representative PU film sample (PU-Butanoic) was analyzed by evolved gas analysis using a thermogravimeter coupled with a mass spectrometer (TA Instruments Q5000IR TGA interfaced to a Pfeiffer Thermostar mass spectrometer by means of a heated capillary transfer line).  The sample was kept isothermal at room temperature for 30 minutes and then heated from room temperature to 1000 °C at 20 °C/min. The capillary transfer line was heated to 195 °C.  The Thermostar unit is based on a quadrapole design and the masses scanned ranged from 1-200 amu.  The sample gas from the TGA was ionized at 70 eV. The purge gas was 25 mL min$^{-1}$ nitrogen through the furnace and 10 mL min$^{-1}$ nitrogen through the balance.  The dynamic mechanical properties of the PU films were characterized by means of a dynamic mechanical analyzer (TA Instruments DMA Q800, New Castle, DE) using a film tension mode of 1 Hz in the temperature range from - 60 to 100 °C with a heating rate of 5 °C / min. Rectangular samples with dimensions of 10 mm $\times$ 8 mm $\times$ 0.5 mm were used for the analysis.  DSC analysis of the PU films was performed by means of a thermal analyzer (TA Instruments Q2000). PU samples ($\approx$ 5 mg) were cut from the film and heated from room temperature to 100 °C to erase the thermal history. The samples were then equilibrated at -70 °C and heated to 150 °C at a heating rate of 20 °C/min. The $T_g$ of PU films was determined from the midpoint in the heat-capacity change in the second DSC scan.

## 2.4 Microorganism Strains and Growth Conditions

*C. albicans* ATCC 90028 and *Staphylococcus aureus* ATCC BAA-44 (methicillin-resistant) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Acinetobacter baumannii* ATCC BAA-747 were acquired from Microbiologics (St. Cloud, MN, USA). Cultures were maintained as frozen stocks at 75°C in Tryptic Soy Broth (TSB, BD Diagnostics Systems, Sparks, MD) containing 20% (v/v) glycerol. Working cultures of the three strains were maintained on Tryptic Soy Agar (TSA) plates. Strains were grown in TSB for 18 h at 35°C and the optical densities of the bacterial cultures were measured spectrophotometrically (model DU720, Beckman Coulter). Prior to each experiment, cultures were grown in TSB for 18h overnight at 35°C and cell concentration was adjusted with PBS to an optical density (OD) of 1 at 600 nm, which corresponded to a cell count of about $10^9$ colony-forming units (CFU) per mL for MRSA, $10^8$ CFU mL$^{-1}$ for *Acinetobacter*, $10^7$ CFU mL$^{-1}$ for *Candida*.

## 2.5 Antimicrobial Testing Methods

### 2.5.1 Disk diffusion test for all PU coatings

As described previously [20, 26], the antimicrobial properties of PU coatings cast onto sterile paper disks (polymer–fiber composites) were examined against all three organisms using Clinical and Laboratory Standards Institute (CLSI) methods. As controls, 20 mL aqueous formic acid, acetic acid, lactic acid, propanoic acid, butanoic acid and isobutyric acid (pH was adjusted as similar to pH of its corresponding PUD) were added on a paper disc separately and their zone of inhibition was determined. These controls were chosen for the possible presence of residual acids in PU systems as confounding mode of antimicrobial

action. Statistical analyses were conducted by using the SAS 9.4 software (SAS Institute, Inc., Cary, NC). MIXED procedure with Tukey adjusted pairwise comparisons was used to determine significant differences between treatments ($p<0.05$). All the results in disk diffusion tests were expressed as mean and standard deviations of six individual measurements.

### 2.5.2 Time course plating method

To test effects of PU films on viability of the three microorganisms, time course plating assay were performed on the most effective PU-Acrylic acid (PAA) shown in disk diffusion test. Washed cells of tested strains were suspended in 0.85% saline to obtain approximately $10^8$ CFU mL$^{-1}$ for *Acinetobacter*, $10^7$ CFU mL$^{-1}$ for *C. albicans*. Paper discs with a diameter of 6mm were saturated with PAA or PU-Anionic dispersion (20 mL), placed on a nonstick paper surface, and allowed to dry in a biosafety cabinet for 18 h, forming a film–disc composite. Aliquots of 1mL of cell suspension and a film-disk composite were placed into 1.5-mL microcentrifuge tubes. Tubes were incubated at room temperature statically. At 4, 8, 12, 24 hours, 100µl samples were taken and serially diluted (1:10) in 0.85% saline water, and plated on TSA or YM plates. Plates were incubated at 35°C for 24 h, and colonies were counted. Three independent replications of the experiment were conducted.

### 2.5.3 Additional tests against *C. albicans*

### 2.5.3.1 Flow Cytometry Permeability Testing Method

Flow cytometry experiments were performed to obtain detailed information of cell membrane permeability on the anticandidal activities of PAA. The reason to choose *C*

*albicans* ATCC 90028 was, as an opportunistic pathogen, *C albicans* live cells did not

represent an aerosol hazard as multiple antibiotics resistant MRSA or *A. baumannii* during

experiments would. Aliquots of 1 mL ($10^7$ CFU mL$^{-1}$ cells) of washed *Candida* cells and a

PAA film-disc composite were placed in a centrifuge tube as previously described. A PU-

Anionic film-disk exposed to 1 mL ($10^7$ CFU mL$^{-1}$ cells) of washed *Candida* cells as well as

cells without film-disk composite in separated tubes were used as controls. One hundred

microliter of samples were taken at 2-, 4-, 8-, 10-, 12-, 14, 24-hour intervals and centrifuged

at 7000 ×g, washed once in fresh saline, resuspended in 0.5 mL saline, and submitted to

fluorescent staining as well as plating as described in Time Course Plating Method. The

membrane integrity probe propidium iodide (PI), component B from L13152 Live/Dead

BacLight Kit (Invitrogen Corporation, Carlsbad, CA), was prepared by dissolving one

applicator in 5 mL of filtered distilled water to form 2× working solution. Fifty microliters

of this working stock was mixed with 50 µl sample，incubated in the dark for 15 min, and

submitted to cytometric analysis using a FACSCanto flow cytometer (BD Biosciences, San

Jose, CA). For each sample, data on cell scatter and PI fluorescence (488 nm excitation and

670 nm long-pass emission) were collected for 20,000 events at a flow rate of 10 mL min$^{-1}$.[33] Controls included live cells with or without PI, cells treated with PAA coating for 10

min without PI staining, both stained and unstained isopropanol-killed cells.

### 2.5.3.2 Agar Invasion Assay

Agar invasion assay was performed to determine whether or not the ability of *C.

albicans* to invade into agar media was affected by PAA. Ten milliliter of melted YM

(Yeast Mold Agar, BD, Diagnostics Systems, Sparks, MD)(50°C) was mixed with different

levels of PAA dispersion to make YM agar with PUD (Final concentrations of PUD were 0,

0.062, 0.125, 0.25, 0.5, 1% respectively). Overnight *C. albicans* culture were collected and washed as previously described in time course plating method. Twenty microliter $10^3$ CFU mL$^{-1}$ diluted *C. albicans* culture were spread onto the plates. Three days later invasion into YM agar media was observed after the colonies and cells that did not invade the agar were washed off from the plates by sterile loops.

### 2.5.3.3 Microscopic microcolony Assay

A mini-slab agar media system was set up with a simple cassette made of two sterile microscope slides and tape. Two parallel slides were upright to be adhered onto the tape. The space between two slides was left 2-3mm. Melted YM agar media (control) or melted YM agar with 1% PAA dispersion at 60°C were carefully filled into the system without making any bubbles, leaving 2-3mm space on the top. Once the media became hardened, 1µl of $10^5$ CFU mL$^{-1}$ *C. albicans* overnight culture was placed on different spots of media surface. After 18-hour incubation at 37°C, colonies on the surface were observed under Axio Zoom.V16 Fluorescence Stereo Zoom Microscope (Carl Zeiss Microscopy, Thornwood, NY)

### 2.5.4 Enhancement of antibiotics effectiveness by PAA

The antibiotics test discs (gentamicin 120 µg, ampicillin 10 µg, linezolid 30 µg, ciprofloxacin 5 µg, vancomycin 5 µg, erythromycin 15 µg, tetracycline 30 µg, ceftazidime 30 µg, imipenem 10 µg, clindamycin 2 µg, chloramphenicol 5 µg, fluconazole 25 µg, colistin 10 µg, blank discs) were purchased from BD Diagnostics Systems (Sparks, MD). Twenty microliter of PAA was added onto each antibiotic test disc or blank test disc and air

dried in bio-safety hood for overnight, forming PAA-antibiotic-disc composite. As controls, antibiotics test discs themselves were also air dried overnight. Disk diffusion tests (procedure as described in 2.5.1) were conducted to compare the Zone of Inhibition between PAA, antibiotic and their combination composite discs. Statistical analyses were conducted by using t-Test using the Excel 2011 14.4.1 software. Pairwise comparisons were performed to identify significant differences between three treatment groups (antibiotic, PAA and the combination). All results were expressed as the mean standard deviation of six separate measurements.

## 2.6 Metal ion chelation assay of PAA

SideroTec Assay Kit (Emergen Bio, Ireland) was used to determine metal chelation ability of PAA. This is a modified Chrome Azurol S (CAS) method.[34] The indicator was the ternary complex chrome azurol S/iron (lII) /dye with a maximum absorbance at 630 nm. A strong iron chelator such as desferoxamine would remove the iron from the dye complex and change the color from blue to pink. A serial of two-fold PAA solutions (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%) were tested for the chelation ability with iron. One hundred microliter of PAA was mixed with 100 μl premixed R1/R2 solution (iron-dye complex) on 96-well plate (Amazon). Due to considerable turbidity disturbance to absorbance of each well from PAA, the normal colorimeter failed to accurately measure absorbance for each well, so image analysis was performed by ImageJ 1.48v software (National Institutes of Health, US) to compare the color intensity among wells. The image was split into blue, green and red three channels. The image under red channel was used for final image analysis. In this test indicator dye-iron complex was in red color and tested samples capable to chelate iron would steal iron from complex and make solution become

from red to blue. The same area was selected for each well and its mean gray value under red channel was analyzed.

## 3. Results and discussion

### 3.1 Themogravimetric Analysis.

The thermal degradation of polyurethanes is a complex process and thermal stability measurements are strongly dependent on the characterization methods.[35] Polyurethanes with different structures are known to exhibit different thermal stabilities.[17] Previous research has reported three decomposition stages for vegetable oil-based cationic polyurethane dispersions: initial decomplexation of the ammonium groups, followed by decomposition of thermally labile urethane bonds, and then the decomposition of the fatty acids.[20] The initial decomposition of the ammonium groups primarily occurs below 200 °C, which was confirmed by thermogravimetric analysis combined with mass spectrometric analysis (TG-MS) of the PU film neutralized with butanoic acid. In Figure 2a, the thermogrametric spectrum shows the TGA data with a derivative weight peak at 165.9 °C (t = 37.3 min). The molecular ion peak of butanoic acid, m/z = 88, occurs in low abundance [35]. However, the base peak occurs at m/z = 60 for butanoic acid due, to the loss of an ethylene unit by McLafferty rearrangement [36, 37]. In Figure 2b, the spectral data shows a strong overlap between a sharp peak for m/z = 60 and the first derivate weight peak, which confirms the decomposition of the amine complex below temperatures of 200 °C.

TG-MS enables an estimation of the number of equivalents of acid remaining in the film. During the dispersion synthesis, 2 molar equivalents of acid per molar equivalent of amine groups were added to neutralize the amine groups. During film formation, the excess acid evaporated along with the water. However, we suspected that the larger carboxylic

acids, *i.e.* butanoic acid or isobutyric acid, might remain trapped inside the film and act as a plasticizer. Furthermore, there were concerns that the excess residual amounts of acids could interfere with the analysis of the antimicrobial activity. Organic acids are known to have antimicrobial activity against food-borne pathogens and are recognized as safe for people by the U.S Food and Drug Administration [38]. In order to address these concerns, TG-MS data has been used to estimate the wt% of excess butanoic acid retained by assuming all mass loss during the initial stage of decomposition is due to decomplexation of the acid molecules from the ammonium groups. The mass loss attributed to butanoic acid was 10.45 wt%, as shown in Figure 2. Theoretically, the minimum and maximum amounts of residual butanoic acid were 9.10 wt% and 16.7 wt%, respectively. Based on these amounts, we determined there were 1.15 equivalents of butanoic acid in the films, which corresponds to evaporation of 85% of the excess butanoic acid during film formation.

The thermograms of the polyurethane films neutralized with carboxylic acids of different chain lengths, shown in Figure 3, have decomposition profiles similar to the TG-MS thermogram of the PU-butanoic acid film. This is consistent with previous studies, which have reported the presence of oxygen does not significantly change the onset degradation temperature of ionomer polyurethanes compared to a nitrogen atmosphere.[17]

$T_5$, $T_{50}$, and $T_{max}$ for PU films neutralized with different chain length carboxylic acids are summarized in Figure 1. $T_5$, the temperature at which the samples lose 5 wt% of their mass, is a measure of the onset temperature of degradation.[20] No clear trend is observed in the $T_5$ results due to the large differences in molecular weight of the counteranions. After the initial stage of decomposition, the thermal stabilities of the different PU films are nearly identical. No significant difference is observed in the $T_{50}$ and $T_{max}$ values which is

consistent with decomplexation of the ammonium groups prior to the urethane bonds breaking.  Based on these results, as well as the TG-MS results for the PU-Butanoic film, we have used the TGA data to estimate the amount of residual acid in all of the PU films. For calculation purposes, decomposition of the amine complex was assumed to be complete prior to degradation of the urethane bonds.  The base of the weight derivative corresponding to urethane was used as the reference point for the calculations.  Increases in the molecular weight of the carboxylic acid correspond to increases in the wt% of acid in the PU films. The estimated wt % of acid and residual acid equivalents, listed in Table 1, show that the majority of the excess acid evaporated.

Substituents on the carbon chain of the carboxylic acids have a more pronounced impact on the initial thermal stabilities and residual acid content of the PU films than the chain length of the carboxylic acid chains.  The thermograms of the PU films with different substituents on the carboxylic acid carbon chains are shown in Figure 4 and the thermogravimetric data is summarized in Table 2. For this series of acids, the $T_5$ results can be used to compare onset temperatures since the range of molecular weights between propanoic acid, acrylic acid, isobutyric acid is smaller (74 g/mol for propanoic acid to 90 g/mol for lactic acid).  The initial thermal stability and onset temperature of the PU-Lactic and PU-Acrylic films are significantly lower than those of the PU-Propanoic film.  These observations are not explained by differences in molecular weight alone, as evidenced by the higher amounts of residual acid.  For the PU-Lactic film, hydrogen bonding between the hydroxyl group on the lactic acid molecules and the polymer chain is a likely factor.  Also, the sharp increase in the derivative weight peak around 100 °C indicates that the PU-Lactic film retained more water than other films.   For the PU-Acrylic film, the carbon-carbon

double bond makes acrylic acid more rigid than propanoic acid, which could explain the increased retention of acid in the corresponding PU film.

**3.2 Dynamic Mechanical Analysis.**

The mechanical properties of all of the PU films have been investigated by dynamic mechanical analysis.  For PU films neutralized with different chain length carboxylic acids, the resulting storage modulus (E') and tan delta curves are shown in Figure 5a and 4b, respectively.  At low temperatures, the cationic coatings are glassy.  The storage modulus decreases rapidly in the temperature range from 20 – 50 °C.  The tan delta peaks are recorded as $T_g$ values in Table 3.  Generally, the storage modulus at room temperature (Table 3) and $T_g$ decreases as the carboxylic acid chain length increases.

Figure 6 shows the storage modulus-temperature curves and the tan delta curves for PU films neutralized with carboxylic acids containing different substituents. The DMA data is summarized in Table 4. The values for the storage modulus at room temperature indicate that the sharp drop in the storage modulus for the PU-Lactic and PU-Acrylic films is shifted to higher temperatures.  The PU-Lactic and PU-Acrylic films also exhibit higher $T_g$ values than the PU-Propanoic film.  The increased $T_g$ value for the PU-Lactic film is consistent with the presence of hydrogen bonding by the counterion.  The $T_g$ value for the PU-Isobutyric film is slightly lower than the $T_g$ for the PU-Propanoic film, which can be attributed to steric contributions of the counterion.

## 3.3 Differential Scanning Calorimetry.

The $T_g$ values obtained by DSC analyses are lower than those obtained from the DMA tan delta peaks due to inherent differences in the two techniques [39]. The $T_g$ values obtained from DSC follow the same general trends as observed by DMA for all of the PU films. The $T_g$ values for PU films neutralized with different chain length carboxylic acids and for PU films neutralized with carboxylic acids containing different substituents are listed in Table 3 and 4 respectively. The DSC curves for all of the PU films are shown in Figure 7.

## 3.4 Antimicrobial properties

### 3.4.1 Disk Diffusion Test

From the results of disk diffusion tests for PU coatings, the order of overall antimicrobial activities of PU coatings against three pathogens was (from most effective to least effective): PU-Butanoic acid>PU-Propanoic acid>PU-Acetic acid>PU-Formic acid ($p < 0.05$) (Table 5). The longer chain length of carboxylic acid, the stronger antimicrobial effects against tested pathogens. The order of antimicrobial activities among PU-Lactic acid, PU-Propanoic acid, PU-Isobutyric acid and acrylic acid was (from most effective to least effective): PU-Acrylic acid (unsaturation) >PU-Lactic acid (hydroxyl group) > PU-Propanoic acid (hydrogen group) > PU-Isobutyric acid (methyl group) ($p < 0.05$).

### 3.4.2 Further antimicrobial analysis on PU-Acrylic acid (PAA)

### 3.4.2.1 Time Course inactivation of *Acinetobacter*

Based on the results of disk diffusion tests, all PU films showed the least antimicrobial activities against *A. baumannii* compared with other two microorganisms. The disk diffusion tests also indicated PU films were able to release antimicrobial materials into the agar medium. It was hypothesized that a liquid medium assisted the release of more active component leaching from PU films and exhibited better antimicrobial activities. To test the hypothesis, time course plating method was used against *A. baumannii* in 0.85% saline solution after exposure to PAA films (the best antimicrobial PU films showed in disk diffusion test). After 12 hours and 24 hours exposed to PAA, *A. baumannii* ATCC BAA-747 was reduced by 5.41 and more than 6 $\log_{10}$ CFUmL$^{-1}$ respectively (shown in Figure 8), while exposure to PU-Anionic control did not inhibit viability of *A. baumannii*. This further explained that positive charge of PU films was the key to inhibit the growth of microorganisms having negative charged wall, which was consistent with our previous findings [20, 26]. This emphasized the importance of using multiple types of analyses to evaluate antimicrobial effectiveness. These results showed that, in comparison to control of PU-Anionic, PAA component leached from PU acrylic acid coating was very effective to kill multiple antibiotics resistant *A. baumannii*, which seemed promising as an approach to kill *A. baumannii* and prevent spreading of infections. *A. baumannii* strains resistant to many antibiotics have become one of the organisms threating the current antibiotic era and challenged our ability to treat serious infections. The potential applications might be to coat PAA film with fiber based clinical materials such as bandage and fiber wound dressing as solutions to cure *A. baumannii* infections.

### 3.4.2.2 Membrane permeability and cells viability of *Candida*

Flow cytometry is a powerful tool with fluorescent probe to analyze the cell population based on individual cell characteristics such as cell size, cell surface roughness and cell number. [33, 40] The most outstanding contribution of flow cytometry is used to study antimicrobial effects and susceptibility testing by analyzing the change of cell light scatters due to changes in the integrity of *C. albicans* cytoplasmic membranes and cells viability after antimicrobial treatment. A nucleic acid-staining fluorochorme PI was used in our work, which is a nonpermeable dye excluded by viable cells, but had ability to enter the damaged cell cytoplasm and bound to DNA increasing fluorescence by 20-30 times [41]. We hypothesized that combined data of cell permeability and cell survival would be able to reveal the relationship between those two key parameters, providing us further insight into the anticandidal activities of PAA. The trend of the increasing percentage of permeabilized cells along with decreased cell viability over exposure time to PAA coating were shown in Figure 9A. During exposure of 24 hours to PAA survival *Candida* were reduced by more than 5 $\log_{10}$ CFU mL$^{-1}$ when permeability increased from 2% to 98%. However, those changes were not simultaneous. From 2h to 6h, cells were permeabilized rapidly, achieving 49.8% of cells permeabilized at 6h shown in Fig. 9B (49.8% population of *Candida* cells obtained PI fluorescence due to being permeabilized), while most of cells were live with survival $\log_{10}$ CFUmL$^{-1}$ of 6.78. This explained that during this period of time exposure to PAA made half population of *Candida* damaged but still enabled to recover when plating on nutritious YM plate media. During treatment time from 6h to 10h, cells were continuously permeabilized with permeability percentage increased from 49.8% to 94.6% (shown in Fig. 9B) and *Candida* cells starts to be killed exponentially reducing about 2 $\log_{10}$ CFUmL$^{-1}$ of

population. From time of 10h to 14h, survival *Candida* was continuously reduced by 2.4 $\log_{10}$ CFUmL$^{-1}$ when more than around 95% cells were already permeabilized. The incessantly reducing survival *Candida* from 6h to 24h indicated that the increasing number of intracellular proteins or nucleonic acids were leaked or damaged and lost their functions after cell membrane become permeabilized. The set of data over time of flow cytometry permeability together with plating course inactivation of *Candida* revealed in detail the kinetics of *C. albicans* cell membrane permeability as well as cell death rate, providing more information of anticandidal process by PAA than each test alone. Our group [26] had found that soybean oil-based PU films exhibited their antibacterial activities by causing gross physiological damage to bacterial cells resulted in intracellular macromolecules leakage. Similarly, it indicated that castor oil-based PAA film inactivated *C. albcians* by physically damaging cell membrane leading to the release of intracellular macromolecules as well.

### 3.4.2.3 Suppression effects on the hyphal growth of *Candida*

The outstanding feature of *C. albicans* is its ability to grow in a diversity of morphological forms, either unicellular budding yeast, or filamentous structures such as hyphae and pseudohyphae.[32, 42] The conversion from unicellular cells to filamentous stage has been suggested to contribute to overall virulent factors in *C. albicans* and may be a target for the development of antifungal drugs. The influence of PAA on hyphae growth of *C. albicans* ATCC 90028 was investigated by agar invasion assay and cell invasive growth assay.

Agar invasion assay (plate washing assay) is a well-known methodology to study bacterial and fungal invasive growth both qualitatively and quantitatively.[42, 43] We used agar

invasion assay to investigate the effects of PAA on invasion of *Candida* cells. The results showed that after 3 days incubation at 37°C, *Candida* cells were capable to invade to YM agar. Smaller colonies and fewer invasions were observed with the increasing concentration of PAA (0%, 0.062, 0.125%, 0.25%, 0.5%). When the concentration of PAA was as high as 1%, even smaller colonies were grown and no invasion was observed. This result suggested that PAA enabled to suppress hyphal growth of *Candida* cells, which was further tested by cell invasive growth. Low magnification microscopy (20-30 times) images showed YM agar media containing 1% PAA inhibited the formation of hyphae as well as cell invasive growth compared with control YM media (Fig. 10), which was consistent with the result of agar invasion tests.

Based on the results of agar invasion assay as well as cell invasive growth assay, PAA exhibited an inhibition effect on hyphal growth of *C albicans*, which would be applied to modulate the morphological switch of *Candida* cells, a major virulent factor of infections.

### 3.4.2.4 Enhancement of antibiotics effectiveness by PAA

Disk diffusion test results were shown in Fig 11. PAA was found to significantly enhance the sensitivity of MRSA to Imipenem, Tetracycline, Clindamycin and Chloramphenicol and the sensitivity of *C. albicans* to Fluconazole ($p < 0.05$), which suggested that there were synergistic inhibitory effects against these two pathogens between these antibiotics and PAA. MRSA, as one of multidrug resistance pathogens posing a threat to human health, need alternative therapies to be controlled.

Impenem, the first clinically useful carbapenem with broad antibacterial spectrum, has been reported to act synergistically with other antibiotics fosfomycin against MRSA both in vivo and in vitro.[44, 45] However, this might eventually cause resistance of pathogens

to these antibiotics combination and bring new difficulties and challenges to combat antibiotics resistance in the long run. Our study showed promising enhanced effects between polymer and imipenem against MRSA, which could be alternative means to combat MRSA infections without generating antibiotics resistance. As an inhibitor of protein synthesis, Clindamycin is able to target virulent factor of MRSA and used as another drug of choice to treat MRSA but it was recently reported that Clindamycin resistance increased and has risk of clinical failure during therapy. [46] Our study also showed here the tested MRSA strain *S. aureus* ATCC BAA-44 was resistant to Clindamycin (no ZOI), while ZOI of the combination of PAA and Clindamycin was almost twice as ZOI of PAA alone, showing the sensitivity of MRSA to Clindamycin was increased greatly. In addition, PAA also enhanced the activities of tetracycline and chloramphenicol, which were well known inexpensive drug but with unfortunately high resistance in recent years.

Fluconazole is the most widely used antifungal antibiotic because of its high bioavailability and low toxicity, but the most prevalent fungal human pathogen *C. albicans* developed strong resistance to it. [47] Our study also showed *C. albicans* was not susceptible to fluconazole (Fig 11b). However, with the presence of PAA, fluconazole restores its antifungal ability to *C. albicans*. Fiori and Van Dijck [47] studied the synergistic antifungal activity existed between fluconazole and doxycycline and found deoxycycline enabled to convert the action of fluconazole from fungistatic to fungicidal. They also explained that iron depletion caused by metal chelation property of doxycycline led to decrease of ergosterol levels in cell membrane and an increase in membrane fluidity, which therefore enhanced passive diffusion of fluconazole into *Candida* cells. [48] To determine the mechanism of synergistic action between PAA and antibiotics and seek whether metal ion

chelation play roles in synergistic abilities between them, a further iron chelation assay was performed.

### 3.5 Metal ion chelation property of PAA

In our studies PAA was shown to have ability to permeabilize *C. albican* cells, suppress their hyphal growth, as well as enhance the effectiveness of multiple antibiotics against three pathogenic microorganisms. In an effort to determine its antimicrobial mode of mechanism of PAA, a simple metal chelation-SideroTec assay was used and pictures were taken for image analysis by ImageJ software. Fig. 12 showed the relationship between PAA concentration and red value in this test. With the increasing level of PAA, the value under red channel became bigger, meaning more red color remained in the reaction system, which explained that more iron ions were stolen from dye-iron complex by PAA. This result indicated that PAA has strong iron chelation property and its antimicrobial effects against MRSA could partially rely on that.

The iron with unique redox potential is an ideal cofactor in many biochemical reactions, which is critical for the survival and growth for almost all organisms including bacterial and fungal pathogens. *S. aureus* owning a unique highly efficient iron acquisition system can modulate metabolism and regulate protein synthesis in order to adapt to environment, probably contributing to a decrease of virulent factors. [49] The most efficient iron uptake system of *S. aureus* was to secrete small molecules siderophores staphyloferrin A and B with high affinity for iron, which could steal iron from the iron-restricted environment used for survival and growth of *S. aureus*.[49] Courcol [50] found sub-inhibitory concentrations of oxacillin and amikacin reduced significantly siderophore production under iron depletion condition. Base on this, in our study MRSA was more susceptible to

antibiotics and PAA than each alone would be due to their possible synergistic effects: Iron depleted condition caused by the iron chelation of PAA, reduced siderophore production by antibiotics, damaging cell membrane due to binding to positively charged quaternary ammonium group in PAA [20], and some other bactericidal effects caused by both antibiotics such as protein synthesis inhibition, cell wall synthesis inhibition. On the other hand, iron chelation ability of PAA might be related with synergistic antifungal activity between fluconazole and PAA. Prasad and others [48] reported a close relationship between the level of cellular iron and drug susceptibilities of *C. albicans*. The supplementation of chelator bathophenanthrolene disulfonic acid or ferrozine supplemented in medium increased the drug sensitivity of *C. albicans* cells to fluconazole and other drugs, while supplementation of the medium with iron reversed this enhancement susceptibility. In the same study they further demonstrated that iron deprivation of different kinds of *Candida* cells regulated ergosterol synthesis genes, resulting in decreased levels of ergosterol and accordingly increased membrane fluidity. Similarly, Fiori and Van Dijck [47] confirmed iron-chelating activity of doxycycline was the main reason why its synergistic combination with fluconazole against *C. albicans*. Taken together, the possible mechanism of enhancement of fluconazole ability against *C. albicans* by PAA in our study could be that the iron chelation ability of PAA decreased synthesis of ergosterol cell membrane and increased cell membrane fluidity, leading to enhanced diffusion of fluconazole into *Candida* cells and increased susceptibility of Candida to fluconazole.

Besides the possible explanation given above, PAA would also enable to chelate other metal ions such as $Mg^{2+}$ and $Ca^{2+}$ structurally essential for cell wall of microorganisms, resulting in permeabilized cell membrane. The detailed synergistic mechanisms between

PAA and antibiotics against MRSA and *C. albicans* remain unclear and need to be further investigated. Although Polyurethane (PU) has been used as a carrier for metal chelator such as protein [51] to develop metal chelation system, our study is the first report of its own metal ion chelation property. This study is of significance in providing alternative approach to combat antibiotics resistance and preserve effectiveness to serious bacterial and fungal infections.

## 4. Conclusions

Castor oil-based cationic polyurethane dispersions (PUDs) have been prepared by neutralizing the intermediate amines with seven different carboxylic acids. Increases in the chain length of the counterions slightly decrease the $T_g$ values of the PU films. Substituents on the counterion appear to have a larger impact than the carbon chain length. In particular, hydrogen bonding increased the $T_g$ value and storage modulus at room temperature of the PU-Lactic films. After initial decomposition, the thermal stabilities of all of the PU films were very similar. However, the PU-Lactic film exhibited significantly lower initial thermal stability due to excess water and residual acid in the film. The antimicrobial testing results indicate that selection of the counterion plays a role in the antimicrobial properties of the cationic PU films. PAA had the most effective antimicrobial activities against three important clinical pathogens, MRSA, antibiotics resistant *A. baumannii* as well as opportunistic yeast pathogen *C. albcians*. It was also found that PAA had an ability to permeabilize cell wall of *C. albicans* and accordingly kill the cells. Furthermore, PAA was able to suppress the hyphal growth that is major virulent factor of *C. albicans*, tested by two methods. Meanwhile, metal chelation of PAA enhanced susceptibilities of multi-drug

resistant MRSA and fluconazole resistant *C. albicans*. These together suggested that PAA film could be coated with fiber based clinical materials such as bandage and wound dressings, which would be a promising way to cure infections caused by multiple antibiotics resistant pathogens and emerging opportunistic yeasts.

## Acknowledgements

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**Table 1**. Thermogravimetric results of PU films neutralized with different chain length carboxylic acids

| PUD | $T_5$ | $T_{50}$ | $T_{max}$ | wt% Acid | Residual Acid Equivalents |
|---|---|---|---|---|---|
| PU-Formic | 170 ± 5 | 319 ± 2 | 312 ± 4 | 6.5 ± 0.5 | 1.2 |
| PU-Acetic | 206 ± 12 | 322 ± 1 | 315 ± 1 | 6.7 ± 0.5 | 1.1 |
| PU-Propanoic | 209 ± 6 | 320 ± 1 | 313 ± 1 | 8.2 ± 0.4 | 1.1 |
| PU-Butanoic | 180 ± 1 | 319 ± 4 | 312 ± 4 | 10.8 ± 0.5 | 1.2 |

**Table 2**. Thermograms of polyurethane films neutralized with carboxylic acids containing different substituents

| PUD | $T_5$ | $T_{50}$ | $T_{max}$ | wt% Acid | Residual Acid Equivalents |
|---|---|---|---|---|---|
| PU-Propanoic | 209 ± 6 | 320 ± 1 | 313 ± 1 | 8.2 ± 0.4 | 1.1 |
| PU-Acrylic | 172 ± 1 | 328 ± 3 | 323 ± 4 | 10.0 ± 0.3 | 1.3 |
| PU-Lactic | 151 ± 3 | 311 ± 1 | 323 ± 4 | 15.8 ± 0.3 | 1.5 |
| PU-Isobutyric | 180 ± 3 | 320 ± 1 | 313 ± 1 | 10.8 ± 0.2 | 1.2 |

**Table 3**. DSC and DMA data for PU films neutralized with different chain length carboxylic acids

| PU Film | $T_g^{[a]}$ (°C) | $T_g^{[b]}$ (°C) | E' at 25 °C (MPa) |
|---|---|---|---|
| PU-Formic | 39.7 | 4.4 | 132.5 |
| PU-Acetic | 40.1 | 2.4 | 242.6 |
| PU-Propanoic | 36.8 | -2.8 | 57.9 |
| PU-Butanoic | 33.7 | -2.9 | 44.6 |

[a] *Glass transition temperature obtained from DMA analysis.* [b] *Glass transition temperature obtained from DSC analysis*

**Table 4**. DSC and DMA data for PU films neutralized with carboxylic acids containing different substituents.

| PU Film | $T_g^{[a]}$ (°C) | $T_g^{[b]}$ (°C) | E' at 25 (MPa) |
|---|---|---|---|
| PU-Isobutyric | 36.32 | -3.2 | 45.9 |
| PU-Propanoic | 36.82 | -2.8 | 57.9 |
| PU-Acrylic | 48.1 | 2.0 | 139.1 |
| PU-Lactic | 44.4 | 10.5 | 517.8 |

[a] *Glass transition temperature obtained from DMA analysis.* [b] *Glass transition temperature obtained from DSC analysis*

**Table 5**. Zone of inhibition (ZOI) against three microorganisms for all PU coatings
After 24 hours incubation there were two zones of inhibition of PU-acrylic acid against *C. albicans* ATCC 90028 and *S. aureus* ATCC BAA-44. Inner zone was clear, while outer zone was full of colonies. After extended 24 hours incubation, outer zones became indistinguishable, so only clear inner zone was recorded.

| Sample | *C. alibicans* ATCC 90028 | *S.aureus* ATCC BAA-44 | *A. baumannii* BAA-747 |
|---|---|---|---|
| PU-Anionic control | None | None | None |
| PU-Formic acid | 7.54±0.26 | None | None |
| PU-Acetic acid | 7.83±0.42 | 8.30±0.26 | 7.12±0.12 |
| PU-Acrylic acid | 11.04±0.56 | 9.70±0.64 | 7.73±0.24 |
| PU-Lactic acid | 8.99±0.24 | 9.39±0.25 | 8.04±0.26 |
| PU-Propanoic acid | 8.49±0.42 | 8.48±0.25 | 7.44±0.27 |
| PU-Butanoic acid | 9.27±0.30 | 9.28±0.16 | 7.53±0.24 |
| PU-Isobutyric acid | 8.10±0.24 | 8.10±0.33 | 7.21±0.12 |
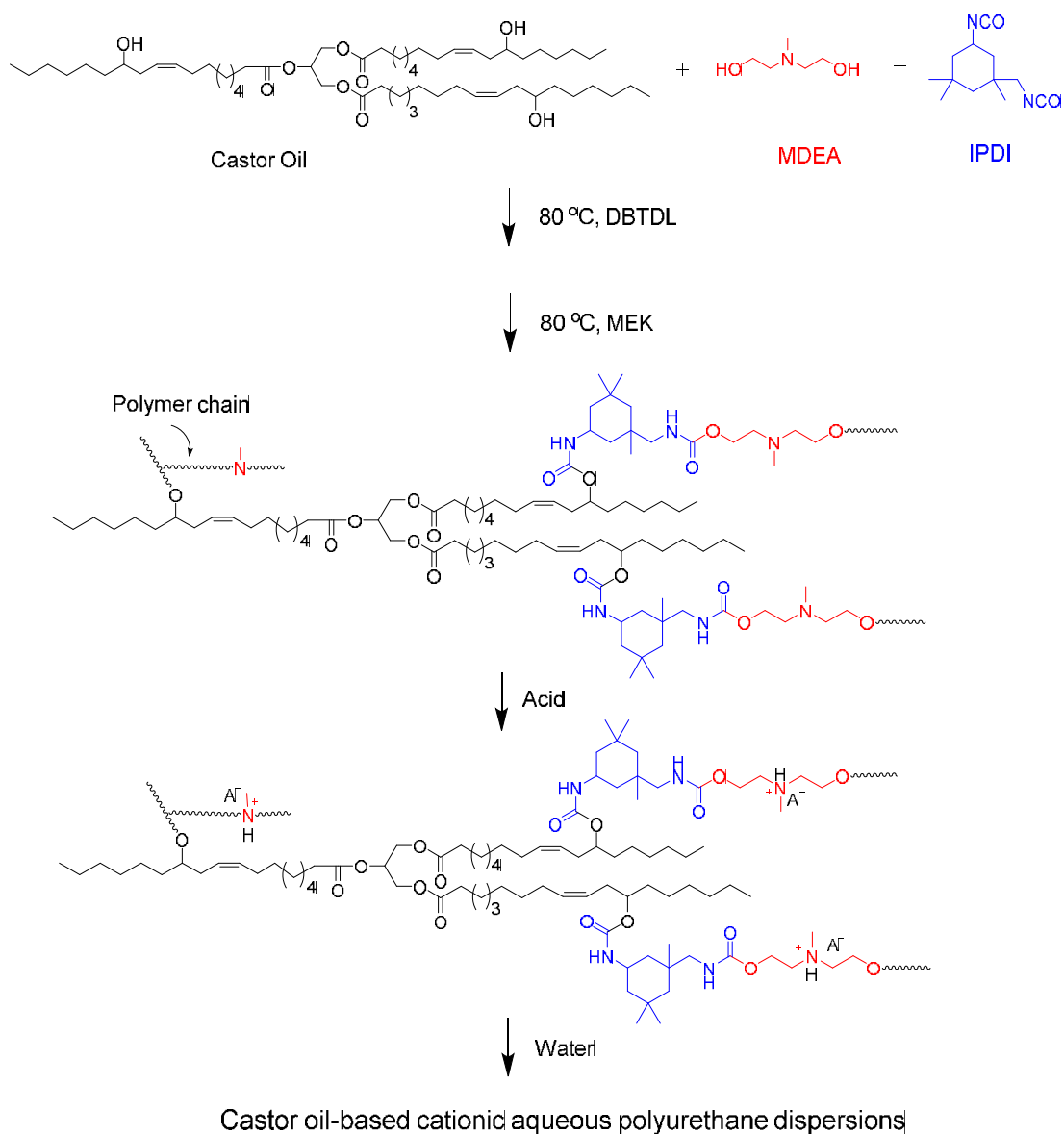
**Scheme 1**. Preparation of the castor oil-based cationic aqueous polyurethane dispersions

X = H (propanoic acid), OH (lactic acid),
or $CH_3$ (isobutanoic acid)
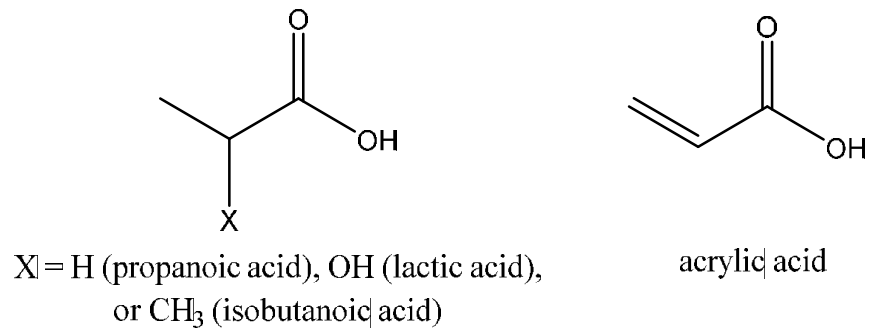
acrylic acid

**Figure 1**. Structure of various carboxylic acids used to neutralize the amino groups
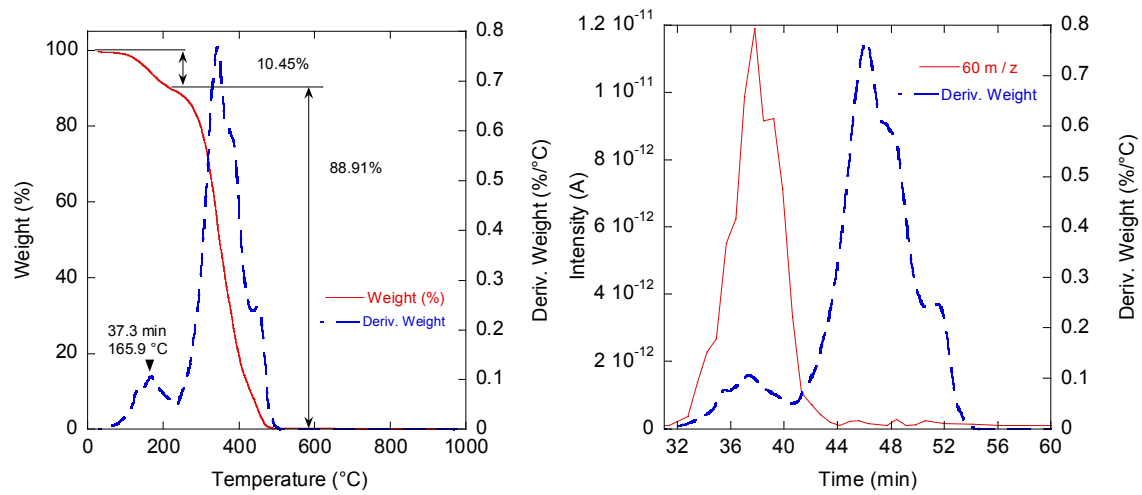
**Figure 2**. Thermogravimetric analysis with mass spectrometry (TG-MS) of a PU film neutralized with butanoic acid.  a) Thermogravimetric analysis data.  b) MS data and TGA weight derivative curve
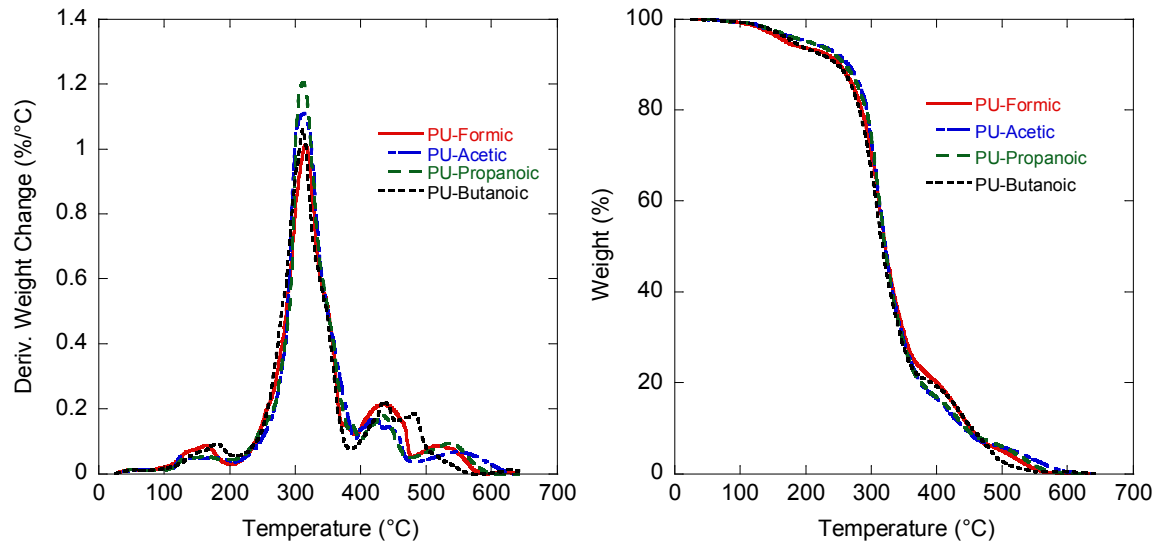
**Figure 3**. Thermograms of PU films neutralized with different chain length carboxylic acids
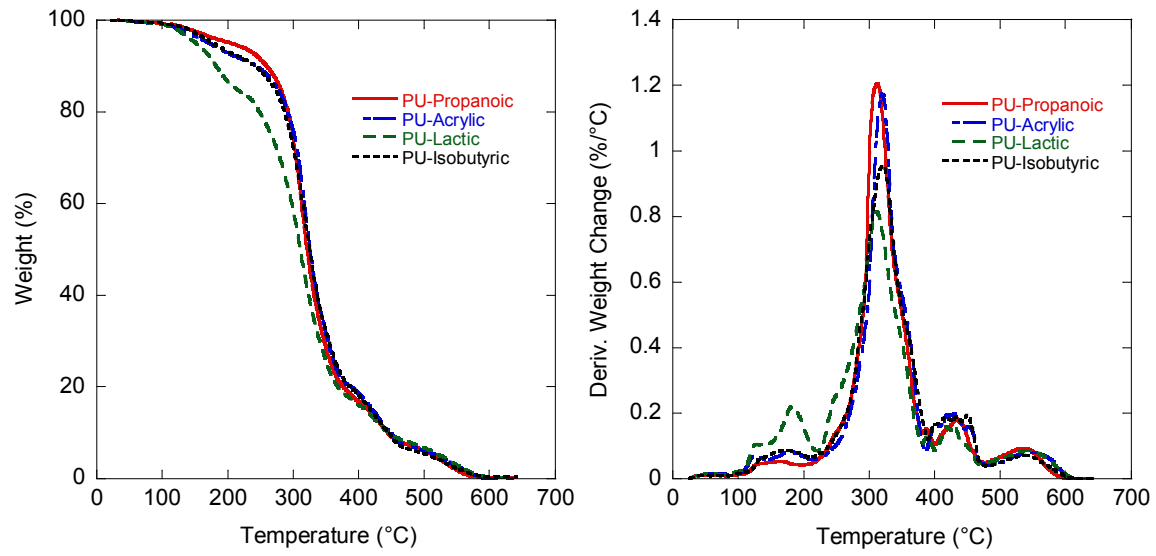
**Figure 4**. Thermograms of polyurethane films neutralized with carboxylic acids containing different substituents
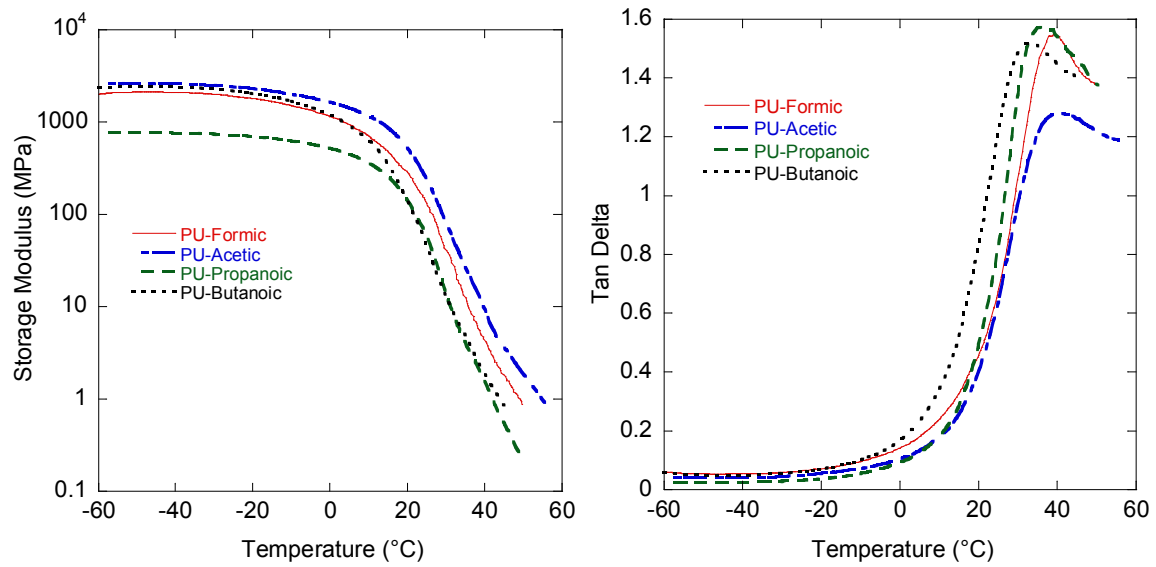
**Figure 5**. DMA curves for PU films neutralized with different chain length carboxylic acids. a) Storage modulus versus temperature, and b) Tan delta versus temperature.
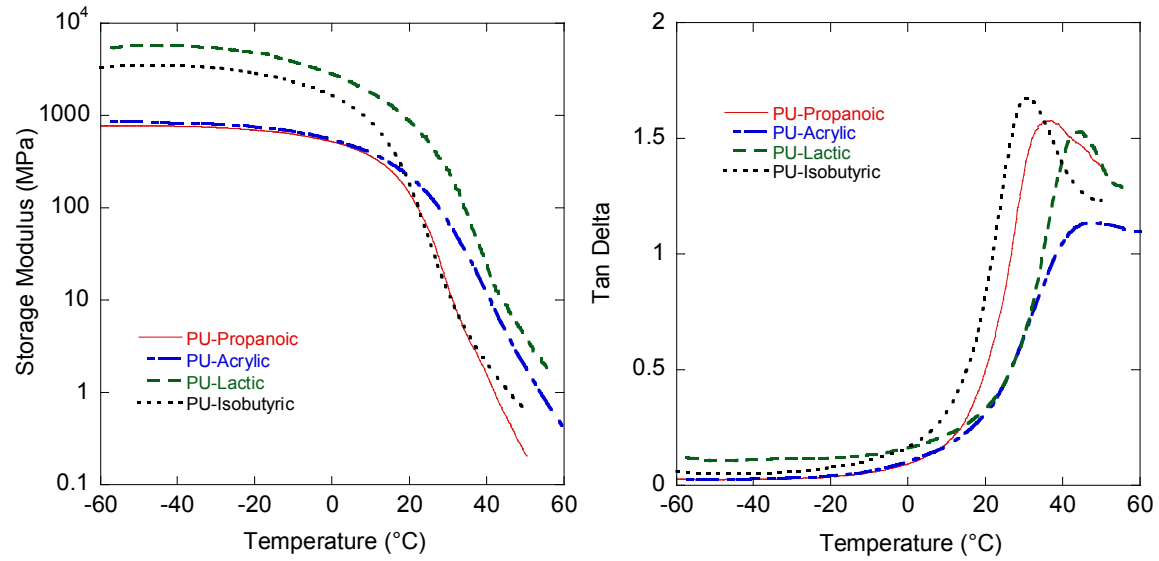
*Figure 6.* DMA curves for PU films neutralized with carboxylic acids containing different substituents. a) storage modulus versus temperature and b) Tan delta versus temperature
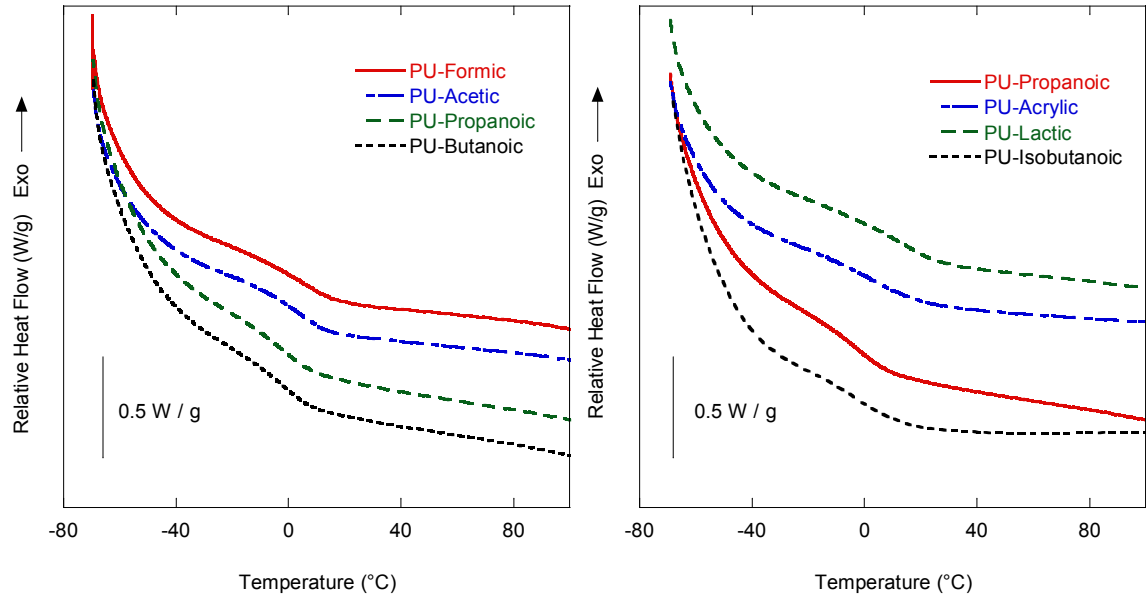
**Figure 7**. DSC curves for a) PU films neutralized with different chain length carboxylic acids and b) PU films neutralized with carboxylic acids containing different substituents
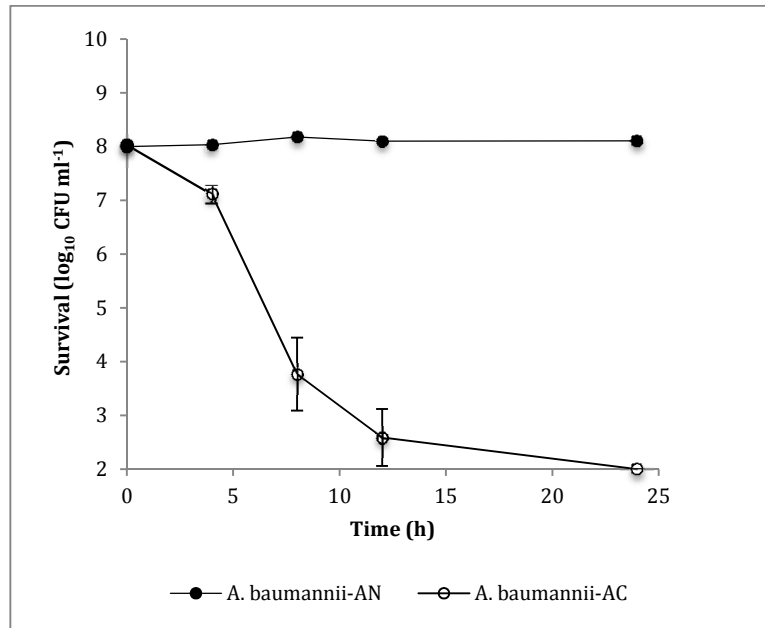
**Figure 8**. Time course inactivation of *Acinetobacter*. *A. baumannii* exposed to PAA were used as a control. Sensitivity of track plating method was 100 CFU.
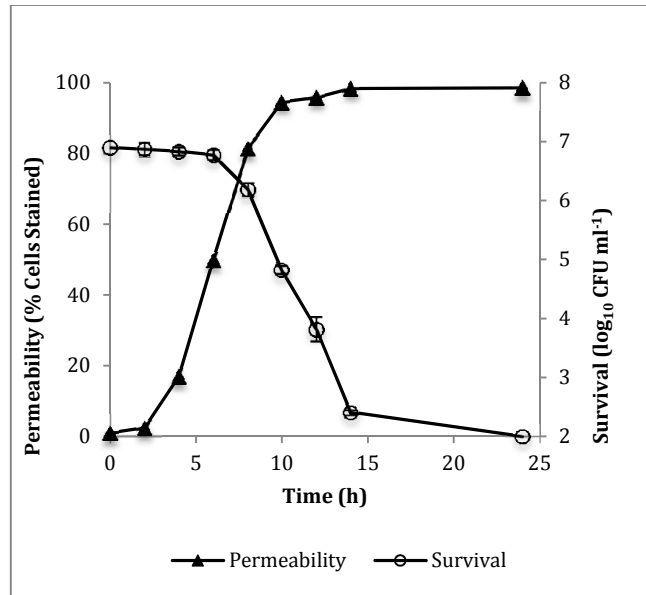
A. Cell permeability of *C. albicans* ATCC 90028 over time parallel plating analysis
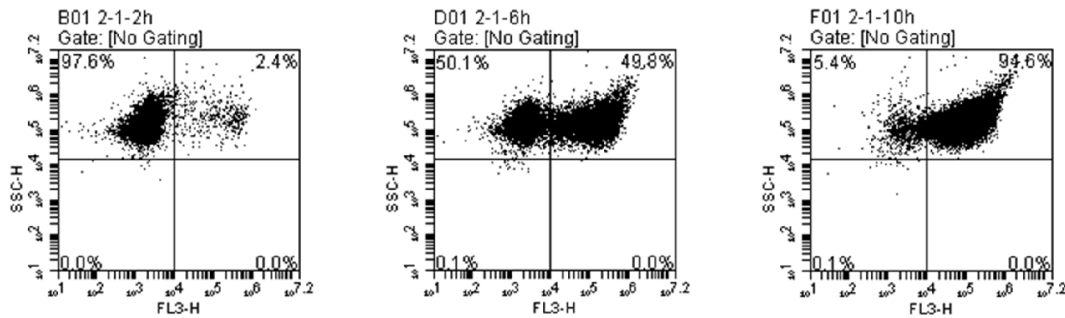


B. Flow cytometric analysis of the effects of leaching component of PU-Acyrlic acid films on live cells of *C. albcians* ATCC 90028. Samples were taken at 2h, 6h and 10h and then analyzed by flow cytometry.

**Figure 9**. Cell permeability of *C. albicans* ATCC 90028 over time parallel plating analysis. Increase permeability percentage was accompanied by decrease in viability, as determined by plating.
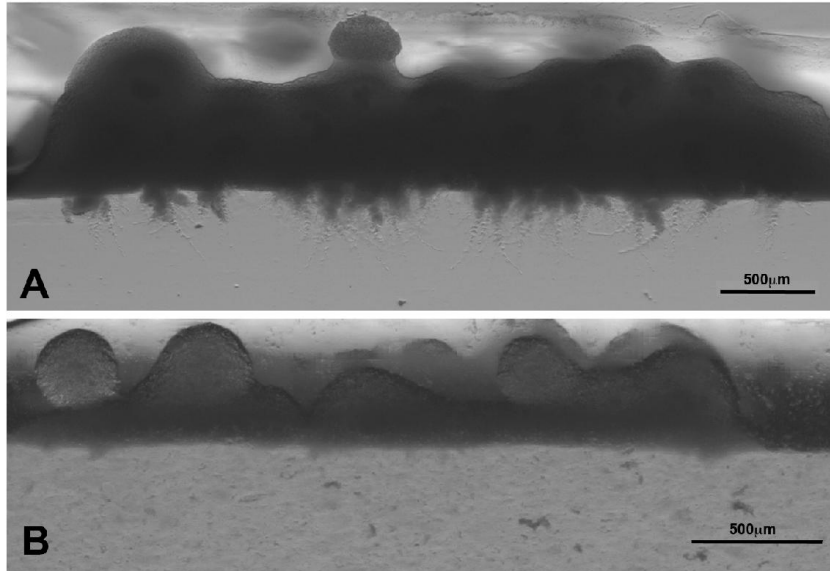
**Figure 10**. Suppression of hyphae growth of *C. albicans* ATCC 90028 by PAA Images were taken under Zeiss microscope after 18 hours incubation at 37°C. A. Colony growing on YM control agar with hyphae invaded into agar media. B. Colony on YM containing 10% PU-AA without hyphae, explaining PU-AA had ability to inhibit the formation of hyphae.
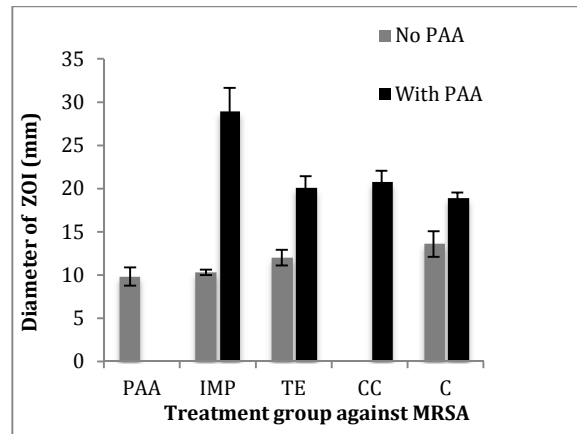
**Fig 11a**. Enhancement of antibiotics by PAA against MRSA ATCC BAA-44
IMP: Imipenem; TE: Tetracycline; CC: Clindamycin; C: Chloramphenicol. No obvious ZOI was found for CC
alone against MRSA.
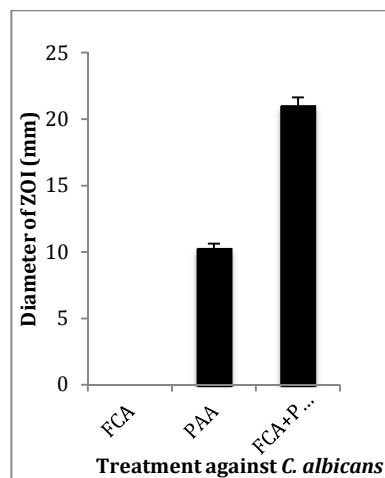


**Fig 11b**. Enhancement of Fluconazole against *C. albicans* ATCC 90028 by PAA
FCA: Fluconazole. No obvious ZOI was shown against *C. albicans* for FCA alone Similar with the results
from 3.4.1, or some treatments, there were two zones of inhibition found, inner clear zone and outer unclear
zone including colonies. After two days, outer zones became undistinguishable, so only inner zones were
measured and recorded.

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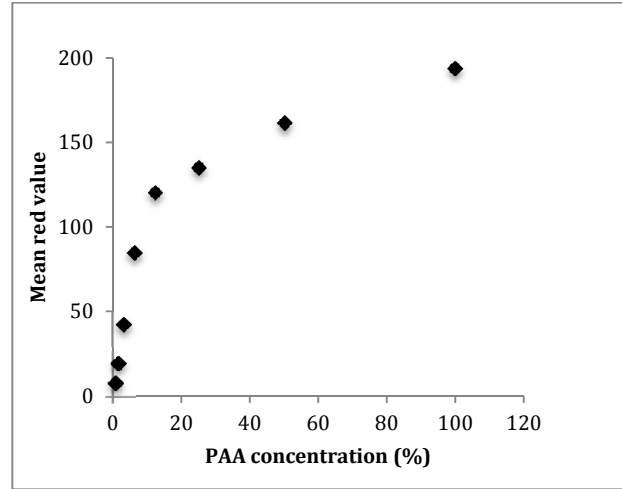


**Fig. 12**. Iron chelation of PAA

# CHAPTER 6. NATURAL ANTIMICROBIAL SYSTEMS FOR INHIBITION OF PATHOGENS ON FRESH PRODUCE

A paper to be submitted to the Journal of Food Protection

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## Abstract

*E. coli* O157:H7 and *Salmonella* Typhimurium are two prevalent pathogens that have caused fruit and vegetables outbreaks for the last three decades. Although chemical sanitizers such as chlorine and hydrogen peroxide are widely used, industry trends toward "greener" or more "natural" interventions continue to gain momentum. Natural

antimicrobial systems capable of inactivating bacterial pathogens in complex food matrices would provide attractive clean-label solutions for enhancing produce safety. The purpose of this work was to develop an effective multicomponent antimicrobial system for fresh produce based on rational combinations of interactive natural components. Antimicrobials examined included Grape Seed Extract (GSE), long-chain sodium polyphosphate (BEKAPLUS FS, polyP) and various organic acids, including citric, malic and tartaric acids. The efficacy of individual antimicrobials and their combinations against *E. coli* O157:H7 ATCC 35150 and *S.* enterica ser. Typhimurium ATCC 14028 was evaluated using a Bioscreen C Microbiological Reader. One antimicrobial system was also evaluated against *S.* Typhimurium in lettuce extract with plating after 12 and 24 h exposure onto both selective and non-selective media. A combination of GSE (0.5%), polyP (1%) and tartaric acid (0.125%) showed the best inhibitory effect against two pathogens in our Bioscreen tests. When applied in lettuce extract, this system reduced *S.* Typhimurium by ~3 logs within 12 h, and by > 6 logs after 24 h, demonstrating efficacy in the presence of an organic challenge (decompartmentalized plant constituents). Selective and non-selective plating indicated that GSE alone was capable of cellular injury. Higher levels of polyP (5%) resulted in buffering and reduced system efficacy. Our work indicates that natural antimicrobial systems composed of phenolic compounds, metal chelators and organic acids may have promise for control of *E. coli* and *Salmonella* on minimally processed fruits and vegetables. By using a systems approach, the levels of individual components required for efficacy may be determined, potentially reducing both formulation expense and negative organoleptic impact on produce.

## Introduction

Over the past decade, produce has emerged as the leading category for foodborne disease, being associated with 696 outbreaks (17% of total outbreaks) and 25,222 illnesses (24% of total illnesses)*(9)*. Over this period, the most commonly identified pathogens in or on produce products have been *E. coli* O157:H7 and *Salmonella* spp., with their presence on leafy greens (spinach and lettuce) of particular concern to industry. In 2006, the Centers for Disease Control and Prevention *(3)* reported a multinational/multistate outbreak (U.S. and Canada) of *E. coli* O157:H7 associated with spinach and causing 199 illnesses, with 102 (51%) hospitalized. Among those hospitalized, 31 (16%) developed hemolytic-uremic syndrome (HUS, a type of kidney failure), and three died *(4)*. In 2012, two hundred and sixty one persons from 24 states were infected with *Salmonella* Typhimurium and *Salmonella* Newport linked to cantaloupe. In this outbreak, 94 (36%) were hospitalized and three died.

Chlorine (as liquid chlorine or sodium hypochlorite) at recommended concentrations of 50 to 200 ppm is currently the predominant sanitizer used in the produce industry, mainly because it is both effective at killing a broad range of pathogens and is relatively inexpensive. Drawbacks to the use of chlorine include its reduced efficacy in the presence of organic matter and the production of chlorinated compounds such as trihalomethanes, which are of safety concern *(10)*. Other forms of chlorine, such as chlorine dioxide and other chemical sanitizers such as hydrogen peroxide and peroxyacetic acid are also available as alternatives to chlorine. However, each of these has it's own disadvantages, and as chemical sanitizers, they are not considered to be "green". Issues concerning the overall safety of chlorine and its fate in the environment have generated consumer and industry interest in

"greener" non-chemical sanitizing agents for use in or on produce, has grown.  Natural plant

extracts, as well as  as well as some Generally Recognized as Safe (GRAS) food

antimicrobials such as organic acids have recently attracted the attention of researchers and

consumers. Grape Seed Extract (GSE), a polyphenolic-rich by-product of grape juice and

wine processing operations, is a natural value-added antimicrobial that has been used for

inactivation of bacteria and viruses in fresh produce. Bisha et al. found that 0.125% GSE

reduced *L. monocytogenes* on tomato surfaces by 2 logs within 2 min *(2)*.  Su et al. *(21)*

reported that 1mg/ml GSE led to a 1 log PFU reduction in hepatitis A virus on lettuce and

peppers within 2 h. Both studies demonstrated the utility of GSE as a promising and natural

means for inhibiting pathogens on fresh produce.

Organic acids are widely distributed in nature and are found in high concentrations

in fruits such as citrus (citric acid), cranberry (benzoic acid), grapes (tartaric acid), apples

(malic acid) and as end products in natural microbial fermentations (lactic acid, acetic acid,

propionic acid). Stemming from their age-old association with fermentation, organic acids

have long been recognized as effective food preservatives. In modern food preservation

approaches, organic acids have been used individually or in combination with other

antimicrobials to inactivate pathogens on fresh produce. Park et al. *(19)* studied the

antimicrobial effects of organic acids against a pathogen cocktail of *Salmonella*

Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* on whole red organic

apples and lettuce. A 10 min treatment with organic acids (acetic, lactic and citric acids) at a

concentration of 2% resulted in a 2.75 - >3.42 log CFU/apple reduction in pathogen load

without significant changes in sample color. Others have combined organic acids with GSE

for effective inhibition of pathogens on fresh produce.  For example, Ganesh et al. *(11)*

found that the combination of malic acid, lactic acid and GSE (3% each) was able to reduce *E. coli* O157:H7 by 2.7 log CFU/g on spinach and 2.8 log CFU/g on iceberg lettuce during 12 days of storage.

Polyphosphates are a structurally diverse family of compounds, nearly all of which are considered GRAS in applications as food ingredients. They serve a multitude of functions in foods, ranging from emulsifying, buffering, thickening or dispersing agents, antioxidants and protein modifiers *(15)*. Another important function of polyphosphates is as a metal sequestrant/chelator, which is believed to be the principal mechanism behind their observed antimicrobial properties *(17, 25)*. Polyphosphates have have previously been reported to enhance various antimicrobial hurdles or treatments, including modified atmospheres, salt, nisin, essential oils, plant polyphenolics and antibiotics *(18, 20, 23, 24)*.

The purpose of this study was to investigate the potential for long-chain sodium polyphosphate (polyP) to enhance the antimicrobial activities of GSE-organic acid combinations against *E. coli* O157:H7 and *Salmonella* Typhimurium, *in vitro* or in a model produce system. The ability to enhance the activities of GSE-organic acid combinations may allow development of systems containing lower levels of organic acids or GSE, potentially minimizing both cost and organoleptic impacts of the treatment.

**Materials and methods**

Bacteria and growth conditions

*E. coli* O157:H7 ATCC 35150 was obtained from Microbiologics (St. Cloud, MN). *Salmonella enterica* serovar Typhimurium ATCC 14208 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Stock cultures were kept as frozen stocks

at -80°C on Microbank™ cryobeads (Pro-Lab Diagnostics, Round Rock, TX). Working cultures were maintained on Tryptic Soy Agar (TSA, BD Diagnostics Systems, Sparks, MD) plates and stored at 4°C. Before using in experiments, strains were grown overnight (18 h) in Tryptic Soy Broth (TSB, BD) at 35°C and the optical densities of the resulting bacterial cultures were measured in a spectrophotometer (model DU720, Beckman Coulter, Brea, CA) and diluted to the desired inoculum level ($10^5$ CFU/ml for Bioscreen testing and $10^8$ CFU/ml for time course plating), which was confirmed by plating on TSA plates.

**Antimicrobials**

Organic acids

Tartaric and malic acids were obtained from J. T. Baker Chemical Company (Center Valley, PA); Caprylic and Levulinic acids were from MP Biomedicals (Santa Ana, CA); lactic and propionic acids were from Sigma-Aldrich (St. Louis, MO) and citric acid was from EMD Millipore (Temecula, CA). Stock solutions of organic acids (1%) were prepared in distilled water and sterilized *via* filtration (0.22 μm).

Grape Seed Extract (GSE)

GSE (Gravinol-S) was sourced from the Kikkoman Corporation (Tokyo, Japan). A stock solution (50 mg/ml) of GSE was prepared in 10% ethanol and sterilized *via* filtration (0.22 μm).

Sodium polyphosphate (polyP)

A commercial preparation of long-chain sodium polyphosphate (BEKAPLUS FS, 68% $P_2O_5$ content) was obtained from ICL Performance Products (St. Louis, MO). A 10% stock solution was prepared in distilled water and sterilized *via* filtration (0.22 μm).

## Lettuce extract

Fresh Romaine lettuce was purchased from a local grocery store. A fresh stock of 5.3% (w/v) Romaine lettuce extract solution was prepared by blending in a sterile Waring blender for 30 s and centrifuging @ 5,200 x $g$ for 3 min to remove large particulates. Antimicrobial stock solutions or their combinations were then added to the desired concentrations for each experiment.

## Optical density-based antimicrobial assay

A Bioscreen C Microbiology Reader (Growth Curves USA, Piscataway, NJ) was used to determine the inhibitory effects of different organic acids (tartaric, caprylic, levulinic, citric, malic and propionic acids), GSE, polyP and combinations thereof against $E.$ $coli$ O157:H7 ATCC 35150 and $S.$ Typhimurium ATCC 14028. The Bioscreen is a combination incubator and automated turbidimeter. Inhibition of test organism growth by antimicrobials or their combinations is registered as lower OD readings compared to no-antimicrobial and other appropriate controls. Interference with OD readings caused by the intrinsic coloration of GSE was minimized through use of a wideband collection filter (420 to 580 nm), as described previously (2). .Bioscreen assays were carried out in Mueller-Hinton (MH) broth. Not only is MH broth the recommended medium for broth microdilution assay (7), but its use also minimizes the turbidity sometimes seen when GSE is added to liquid media (2). Test antimicrobials or their combinations combinations were added to Bioscreen wells and wells were inoculated with target pathogens to a final concentration of $10^5$ CFU/ml. The final volume in each well was 200 µL and duplicate wells were used for all treatments. OD data were collected every 15 min, with shaking before each

reading. After 24 h incubation in the Bioscreen, the resulting growth curves were accessed and analyzed using the EZ Experiment software (Growth Curves USA, Piscataway, NJ).

## Time course inactivation of *S.* Typhimurium

In addition to Bioscreen testing, inactivation of *S.* Typhimurium by individual antimicrobials or their combinations was examined using time course plating. An overnight culture of *S.* Typhimurium ATCC 14028 was washed and resuspended in 0.85% saline to obtain a cell suspension containing $10^9$ CFU/ml. One hundred microliters of this suspension were added to a sterile microcentrifuge tube, pelleted by centrifugation at 7000 x *g* for 3 min and then resuspended in a solution containing GSE, polyP, tartaric acid or combinations thereof at the desired concentrations of each antimicrobial. After incubation at ambient temperature (~25°C) for 12h or 24 h, serial dilutions were made in 0.85% saline and plated using the abbreviated track dilution technique (*20*, *23*). Treatments were plated onto both selective (XLT-4) and non-selective (TSA) media in order to investigate the ability of antimicrobials or their combinations to cause sublethal injury. Plates were incubated at 35°C for 24 h and the resulting colonies were counted. Statistical analyses were conducted by using t-Test using Excel 2011 v.14.4.1 software only for some treatment groups (5 mg/ml GSE, 0.125% tartaric acid, and the combination of 0.125% tartaric acid, 5 mg/ml GSE and 1% polyP). Pairwise comparisons were performed to identify statistically significant differences between treatment groups.

**Results and discussion**

Enhancement of GSE activity by polyP

The Minimum Inhibitory Concentration (MIC) of an antimicrobial is a fundamental metric that allows direct comparison with other inhibitory compounds. Once measured, the MIC can also guide further studies, allowing calibrated application of compound at various concentrations related to this value (1x MIC, 2x MIC, etc.). The MIC is defined as the lowest concentration of an antimicrobial at which growth of a test organism does not occur under standardized conditions of inoculum level and growth. Because MIC is an optical density (OD)-based value, artifacts that interfere with OD measurement may confound determination of MIC. Example issues include intrinsic and sometimes intense coloration of plant-derived compounds and haze in or precipitation of the test medium caused by chemical or charge-based interactions between media components and an antimicrobial. When such issues occur, it may be possible to modify an assay in order to avoid or minimize such artifacts. In earlier work with GSE, our group used ethanol as a carrier to increase the solubility and dispersion of GSE into the growth medium, switched from Brain Heart Infusion (BHI) broth to Mueller-Hinton (MH) broth to minimize precipitation of media components and collected scattered light using the "wideband" filter on the Bioscreen C (420 – 580 nm) *(2)*, Although the same assay parameters were used here, the MIC of GSE in the current work could not be determined due interference from turbidity of the growth medium at higher concentrations of added GSE. Reported MIC values for GSE from the literature are highly variable. For example, one study reported MIC values between 0.85 to 1.5 mg/ml for various Gram-positive and Gram-negative bacteria *(14)*, while we previously

reported MICs for a different preparation of GSE against *L. monocytogenes* NADC-2045 and *L. innocua* ATCC 33090 as 50 and 78 µg/ml *(2)*. As our group previously noted, variability in MIC values between studies can stem from a number of sources, including use of different test organisms, media, inoculum levels or methods for determining MIC (broth microdilution vs. plating, for example) *(2)*.  Additionally, plant extracts can be highly variable in their antimicrobial or other biological activities, with this variation stemming from varietal differences, conditions of cultivation or seasonal variation, methods for extraction or combinations of these factors.

In the current work, 1% polyP alone increased the lag phases of *E. coli* O157:H7 and *S.* Typhimurium, but did not decrease the endpoint OD for either pathogen (Figure 1). As hypothesized, the combination of 1% polyP with 500 µg/ml GSE resulted in cooperative inhibition of *E. coli* O157:H7 and *S.* Typhimurium, (Figure 1). Compared to treatment with GSE alone, the addition of 1% polyP extended the lag phase of *E. coli* O157:H7 from 3.5 h to 16.5 h and decreased the endpoint OD from 0.35 to 0.1. *S.* Typhimurium grew slower when treated with the combination of 500 µg/ml GSE and 1% polyP, but the endpoint OD (0.46) was similar to that for GSE alone (0.475). Polyphosphates are known to have antibacterial activity against Gram-positive bacteria *(17, 18, 16)*.  Lee et al. *(16)* found long chain phosphates alone exerted bacteriolytic effects on *Staphylococcus aureus*, releasing intracellular nucleotides and proteins. Maier et al. *(17)* reported that the activity of polyP against *Bacillus cereus* was both concentration- and growth phase-dependent. At lower levels, these authors found that polyP was bacteriostatic, but that above 0.1% it was bacteriocidal, causing preferential lysis of log-phase cells. Because sublethal concentrations of polyP inhibited septation and led to cell filamentation, Maier et al. suggested that the

cation-dependent cell division protein FtsZ may be an important target for polyP *(7)*. While Gram-negative bacteria are able to tolerate high levels of polyphosphates *(22)*, polyP is able to permeabilize the Gram-negative outer membrane (OM), facilitating entry of exogenous molecules, including hydrophobic antibiotics, essential oils and nisin into the cell *(8, 22, 23, 24)*. As a metal chelator, polyP is thought to act on structurally important cations present in microbial cells *(24)*. In Gram-negative bacteria, these include the $Ca^{2+}$ and $Mg^{2+}$ ions that are crucial for maintaining the integrity of the Gram-negative outer membrane, the key permeability barrier in these bacteria *(22)*. In our study, we expected that polyP-mediated permeabilization of the OM would affor both GSE and organic acids access to the cytoplasmic membrane and other interior targets within the cell. Our results from plating of *Salmonella* onto selective and non-selective agars suggest that GSE possesses some OM-permeabilizing activities. Further weakening of the OM through removal of divalent bridging cations is therefore expected to lead to enhanced OM permeability and greater uptake organic acids.

Inhibitory effects of tartaric acid (TA) in combination with polyP and GSE

TA is a natural component of grapes, where it is relatively abundant, compared to other fruits. TA is used in the food industry as a preservative, acidulant, flavorant, and as an anti-browning agent, a property related to it's metal chelating activities. TA from grape wine is also listed in 7 CFR §205.605 as a nonagricultural substance allowed as ingredients in or on processed products labeled as "organic". Based on these properties, we chose TA to test our hypothesis that the activity of a system comprised of GSE and an organic acid could be enhanced with the application of polyP. Figure 2 and Figure 3 show that 1% polyP (or

with 500 μg/ml GSE) enhances the inhibitory effects of 0.0195% TA or 0.078% TA against *E. coli* O157:H7 and *S.* Typhimurium. Tartaric acid alone at 0.0195% did not show inhibitory effects against either strain. With the addition of 1% polyP, the lag phase of *E. coli* O157:H7 was extended, but the endpoint OD remained the same. When both 1% polyP and 500 μg/ml GSE were combined, the lag phase of *E. coli* O 157:H7 was extended further, but the endpoint OD was not markedly changed (data not shown). However, the combination of 0.019% TA, 1% polyP and 500 μg/ml GSE substantially inhibited the growth of *E. coli* O157:H7 (Figure 2A). At a higher concentration of TA (0.078%), the addition of 1% polyP extended the lag phase to 16 h and decreased the endpoint OD from 0.53 (TA-only control) to 0.23. The combination of 0.078%TA, 1% polyP and 500 μg/ml GSE completely inhibited the growth of *E. coli* O157:H7 (Figure 2B). Figure 3 shows that while *S.* Typhimurium was inhibited to a lesser degree by this system, a similar trend for the cooperative and cumulative effects between TA, polyP and GSE were observed. These data suggest that a lack of susceptibility to TA may be responsible for the lower activity of the three-component system against *S.* Typhimurium, as growth of this organism in the presence of either level of TA was similar to the untreated control. Whether the interactions between TA acid, GSE and polyP against these two Gram-negative foodborne pathogens are synergistic or merely additive is not clear. A common method for calculating whether an antimicrobial pairing is indifferent, additive or synergistic is the fractional inhibitory concentration (FIC) method. However, this approach requires measurement of the MIC of each component used in the mixture. Weinkauf *(24)* found that both *E. coli* O157:H7 ATCC 35150 and *L. monocytogenes* F6854 were not inhibited by polyP, even at relatively high

concentrations, so use of the FIC approach would not be an appropriate means for making this determination and an alternate method would be required.

## Comparison of the inhibitory effects of additional organic acids alone or in combination with polyP and GSE

Due to both the efficacy and GRAS status of organic acids, organic acid-based produce washes have attracted recent attention from both academia and industry. Commercial systems include Ecolab's Antimicrobial Fruit & Vegetable Treatment (AFVT), which lists its active ingredients as lactic acid and sodium dodecylbenzenesulfonate) FreshRinse from Fresh Express, a combination of lactic acid and peracetic acid. In published studies, a greater than 4.95 log reduction of *Salmonella enterica* on tomato stem scars was achieved with the treatment of 5.1% total combination of acetic, lactic, and levulinic acids, or with the treatment of 6% combination of lactic acid and acetic acid *(13)*. Peroxyacetic acid *(26)*, lactic acid *(1)* and acetic acid in rice vinegar *(5)* reduced *E. coli* O 157:H7 or *S.* Typhimurium on vegetables or fruits by 1 to 3 logs. However, 0.25 g/100 g citric acid plus 0.50 g/100 g ascorbic acid treatments at 10°C for 2 min were not effective in removing biofilms comprised of *Escherichia coli* and *Listeria monocytogenes* on green leaf lettuce *(27)*. A "systems" approach for antimicrobial development is a promising strategy for development of effective, synergistic and clean-label antimicrobial systems that do not degrade the organoleptic attributes of produce. The work performed here builds on prior studies showing the promise of GSE/organic acid combinations for sanitization of fresh produce *(11, 12)*, and explores the addition of long-chain polyphosphate as a means for enhancing such combinations.

Having established that a system combining polyP, GSE and TA showed promising cooperative activity against *E. coli* O157:H7 and *S.* Typhimurium, we sought to evaluate additional organic acids that have been reported in the literature for use as antimicrobials on produce, including caprylic, levulinic, citric, malic, lactic and propionic acids. The MIC of tested organic acids against two foodborne pathogens with 500 μg/ml GSE or 1% polyP was assessed in Mueller-Hinton media using the Bioscreen broth microdilution assay. The results (Table 1) showed that the addition of 1% polyP decreased MIC of citric acid and malic acid against the two pathogens. In combination with 1% polyP and 500 μg/ml GSE, MICs of all tested organic acids were substantially decreased, with those of citric acid and malic acids decreased to less than one tenth of their original value. These data corroborate our hypothesis that systems comprised of various organic acids, GSE and polyP could show promising cooperative antimicrobial activities against *E. coli* O157:H7 and *S.* Typhimurium. Table 1 and Figures 2 and 3 also showed that all systems tested were more inhibitory against *E. coli* O 157:H7 than against *S.* Typhimurium. Further studies need to be conducted to determine whether these are general or strain-specific effects. Among the organic acids tested, the high water solubilities of citric and malic acids make them good candidates for produce sanitizing applications. After TA, citric and malic acids showed the best cooperative activities with polyP and GSE and like TA, they are also included in 7 CFR §205.605 as nonagricultural substances allowed as ingredients in or on processed products labeled as "organic". Ganesh et al. *(11)* found malic acid in combination with GSE/lactic acid solutions reduced *S.* Typhimurium by 2 to 3 logs on spinach after application by electrostatic spraying. In our tests, caprylic acid showed good inhibition against *E. coli* O157:H7 combined with polyP and GSE, but it has poor water solubility and is not listed in

7 CFR §205.605. Chen et al. *(6)* reported that levulinic acid reduced *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 by 6 to 8 logs when mixed with sodium dodecyl sulfate. However in our study, the levulinic acid solution darkened after mixing with GSE, indicating a chemical reaction between the two ingredients that could represent organoleptic or safety concerns.

<u>Time course inactivation of *Salmonella* Typhimurium in a lettuce extract system</u>

Zhang et al. *(26)* found the antimicrobial efficacy of peroxyacetic acid was reduced in the presence of an organic load introduced using an aqueous extract of lettuce. Based on their results, these authors underlined the importance of considering the effects of organic matter (soil, material released from mechanically damaged or cut produce, etc.) when validating antimicrobial treatments. We sought to challenge our antimicrobial system using lettuce extract as a simulant for the organic load that might be present in a vacuum cooler containing freshly lettuce cut freshly from the field. Instead of the lower inoculum of $10^5$ CFU/ml used in our Bioscreen tests, we inoculated *S*. Typhimurium at $10^8$ CFU/ml for time course plating. We expected that the greater abundance of organic matter present in the lettuce extract system might require a higher amount of polyP, so we examined polyP at both 1% and 5%. GSE was also incorporated at the higher level of 5 mg/ml (0.5%) than used in Bioscreen testing. As reported in Table 2, neither level of polyP alone inhibited the growth of *S*. Typhimurium. After 24 h, exposure to 0.125% TA reduced *S*. Typhimurium by ~2.5 logs. Over the same time period, exposure to 0.5% GSE resulted in a reduction of less than 1 log. Exposure of *S*. Typhimurium to the system comprised of 0.125% TA, 1% polyP and 0.5% GSE resulted in ~ 3 log reduction after 12 h and a greater than 6 log reduction

after 24 h. Instead of preserving or improving the antimicrobial efficacy of the system, the higher level of polyP resulted in reduced activity. The endpoint pH of this system was 5.5, vs. 5.0 for the system containing 1% polyP. The lower activity of the 5% polyP system is therefore probably due to the buffering effect of the higher polyP concentration, as at lower pH, a greater proportion of TA is expected to be in the antimicrobially effective undissociated form.

In an effort to investigate which combinations of system components might contribute to cellular injury, we plated *S.* Typhimurium onto both selective (XLT-4) and non-selective (TSA) media. It was clear from these results (Figure 2), that GSE is capable of causing injury to *S.* Typhimurium, possibly permeabilizing the OM. These results provide some insight into the mechanism behind GSE's contribution to the overall efficacy of this multicomponent system and support our initial rationale for the choice of antimicrobials used in the system.

Compared to the work of Ganesh et al. *(11, 12)*, who combined organic acids with GSE, we used much lower levels of organic acid (0.125% TA vs. 3% lactic or malic acids used by Ganesh et al.) and lower levels of GSE (0.5%, vs. the 2 – 3% used by Ganesh et al.). The combination of multiple, cooperatively-interactive compounds in our system should enable the use of individual components at lower concentrations, potentially reducing both cost and organoleptic impact vs. application of the individual components or their use in binary combinations.

**Conclusions**

In this study, we demonstrated that long-chain polyphosphate (polyP), a multi-use

functional food ingredient, enhanced the antimicrobial effectiveness of GSE against *E. coli* O157:H7 and *S.* Typhimurium. The combination of tartaric acid, GSE and polyP was active against these two pathogens in a lettuce extract system, demonstrating its ability to function in the presence of an organic load challenge. Because polyP-based antimicrobial activity has been shown to be rescued by the addition of metal cations in excess, future work should include samples having high divalent cation concentrations. These experiments, as well as those focused on the effects of this system on quality and sensory characteristics, would enable discovery of parameters for effective use of this system and help guide its appropriate application. . Our work suggests that natural, value-added antimicrobial systems composed of phenolic compounds, metal chelators and organic acids may have promise for control of *E. coli* and *Salmonella* on minimally processed fruits and vegetables.

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**Table 1**. **Antimicrobial effects of organic acids alone, or combined with polyP and GSE**

| Organic Acid | pKa | Water Solubility/Color | MIC against *E. coli* O157:H7 | | | MIC against *S.* Typhimurium | | |
|---|---|---|---|---|---|---|---|---|
| | | | Acid alone | Acid + 1% polyP | Acid + 1% polyP + 500µg/ml GSE | Acid alone | Acid + 1% polyP | Acid + 1% polyP + 500µg/ml GSE |
| Tartaric | 2.95, 4.25 | 133g/100m (20°C) | 0.156% | 0.156% | 0.039% | 0.312% | 0.156% | 0.156% |
| Caprylic | 4.89 | 0.068 g/100 ml, poor water solubility | 0.156% | 0.156% | 0.0195% | 0.312% | 0.156% | 0.078% |
| Levulinic | 4.59 | Solution darkened when mixed with GSE | 0.156% | 0.156% | 0.078% | 0.156% | 0.156% | 0.156% |
| Citric | 3.14, 4.75, 6.39 | 73 g/100 ml (20 °C) | 0.312% | 0.156% | 0.0195% | 0.312% | 0.156% | 0.078% |
| Malic | 3.40, 5.20 | 55.8 g/100 ml (at 20 °C) | 0.312% | 0.156% | 0.0195% | 0.312% | 0.156% | 0.078% |
| Lactic | 3.86 | High solubility 100mg/ml | 0.156% | 0.156% | 0.0195% | 0.156% | 0.312% | 0.156% |
| Propionic | 4.87 | 37 g/100 ml | 0.078% | 0.078% | 0.0195% | 0.156% | 0.078% | 0.078% |

**Table 2**. **Log CFU/ml of survival *S.* Typhimurium ATCC 14028 in lettuce extract after treatment with GSE, polyP or their combination.**

| Treatment time | Media | *Salmonella* Control | 5 mg/ml (0.5%) GSE* | 0.125% TA* | 0.125% TA+ 0.5% GSE +1% polyP * | 1% polyP | 5% polyP | 0.125% TA+5mg/ml GSE | 0.125% TA+ 5 mg/ml GSE+5% polyP |
|---|---|---|---|---|---|---|---|---|---|
| 12h | TSA | 8.38 | 7.58± 0.16 | 5.84± 0.38 | 5.20± 0.72 | 8.56 | 8.45 | 6.60 | 7.40 |
|  | XLT4 | 8.08 | <2 | 3.24± 1.12 | <2 | 8.64 | 7.60 | <2 | <2 |
| 24h | TSA | 8.31± 0.09 | 7.46± 0.15 | 5.63± 0.76 | <2 | 8.63± 0.07 | 8.45 | 5.48 | 7.11 |
|  | XLT4 | 8.02± 0.10 | <2 | 2.73± 0.75 | <2 | 7.95± 0.71 | 7.70 | <2 | <2 |
| Endpoint pH |  | < 5.0 | 5.0 | < 5.0 | 5.0 | 6.0 | 6.0 | 5.0 | 5.5 |

Initial experiments were used to focus data collection on particular experiments. Values without reported standard deviation were from experiments carried out only once, with plating in triplicate.  All other results are from three independent experiments, with plating in triplicate. Statistical analyses were conducted only for treatment groups marked with *. TA: tartaric acid; GSE: grape seed extract; polyP: sodium polyphosphate, BEKAPLUS FS.

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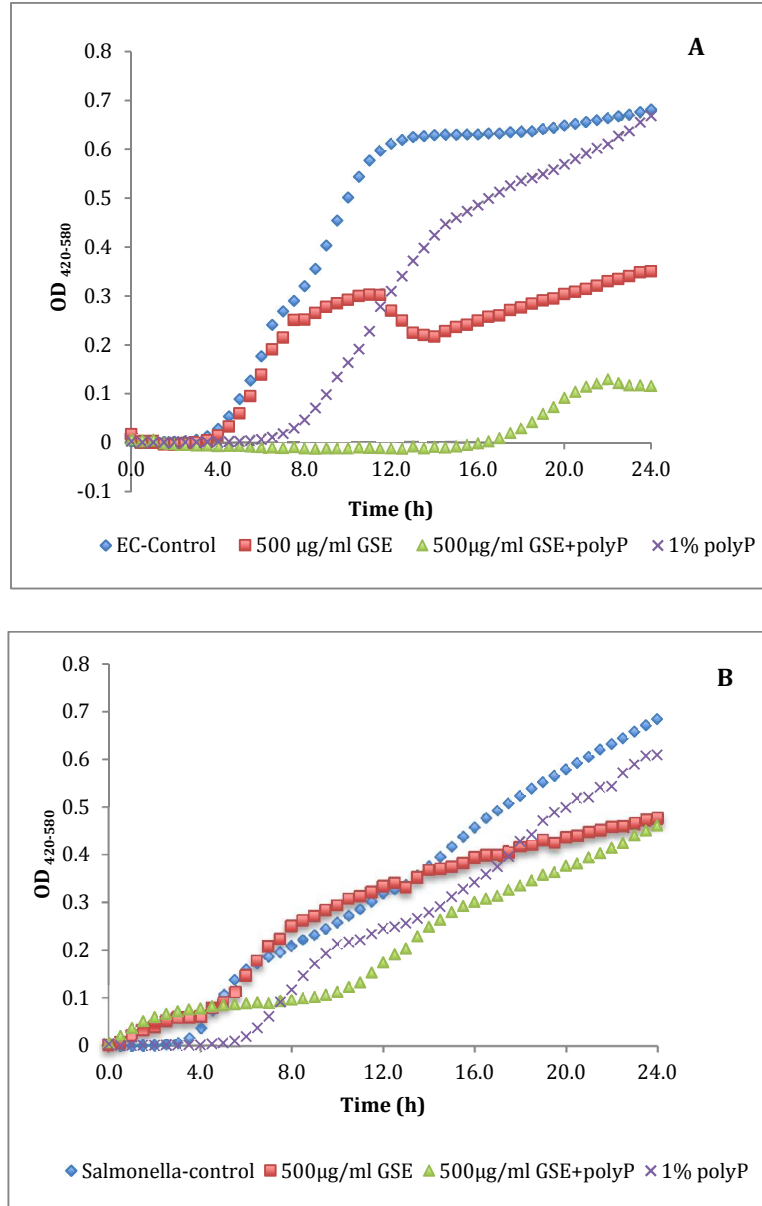


**Figure 1**. **The impact of polyP on the activity of GSE against two pathogens**
    A.   Inhibitory effects of polyP and/or GSE against *E. coli* O157:H7 ATCC 35150
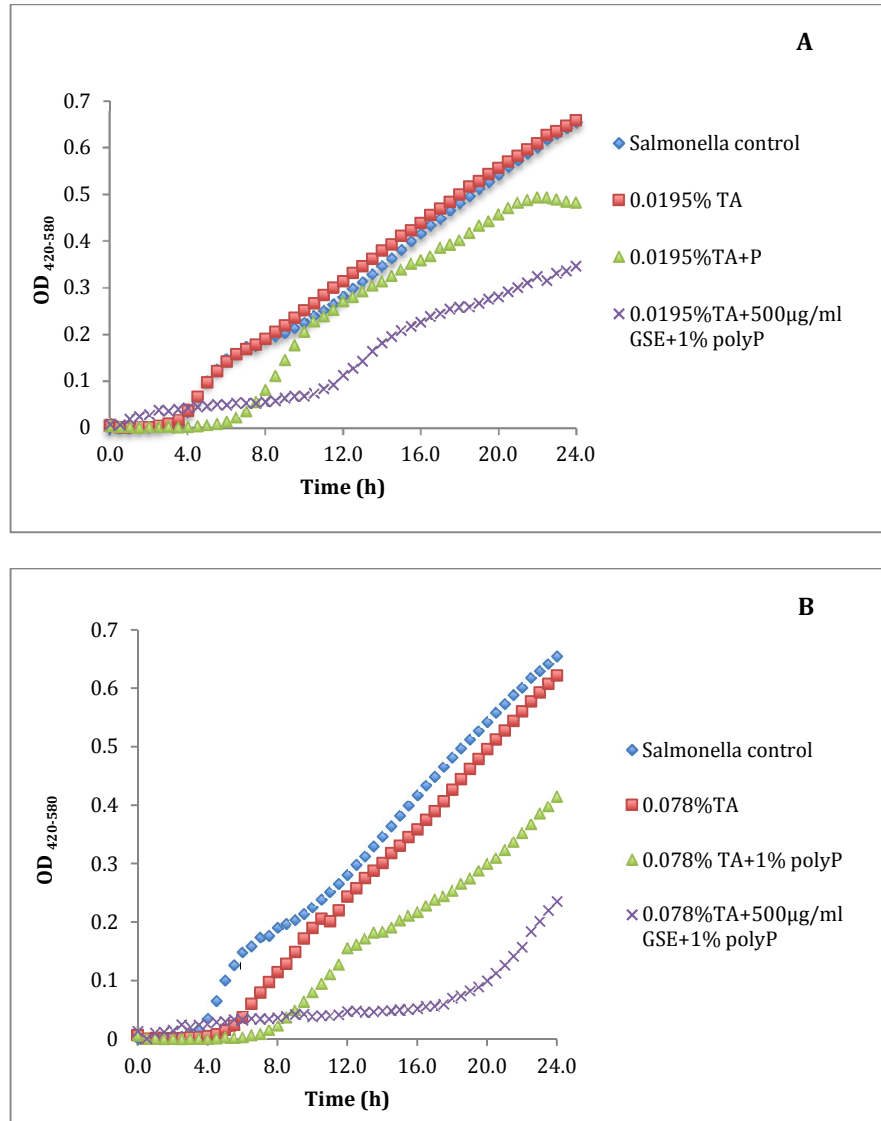    B.   Inhibitory effects of polyP and/or GSE against *S.* Typhimurium ATCC 14208
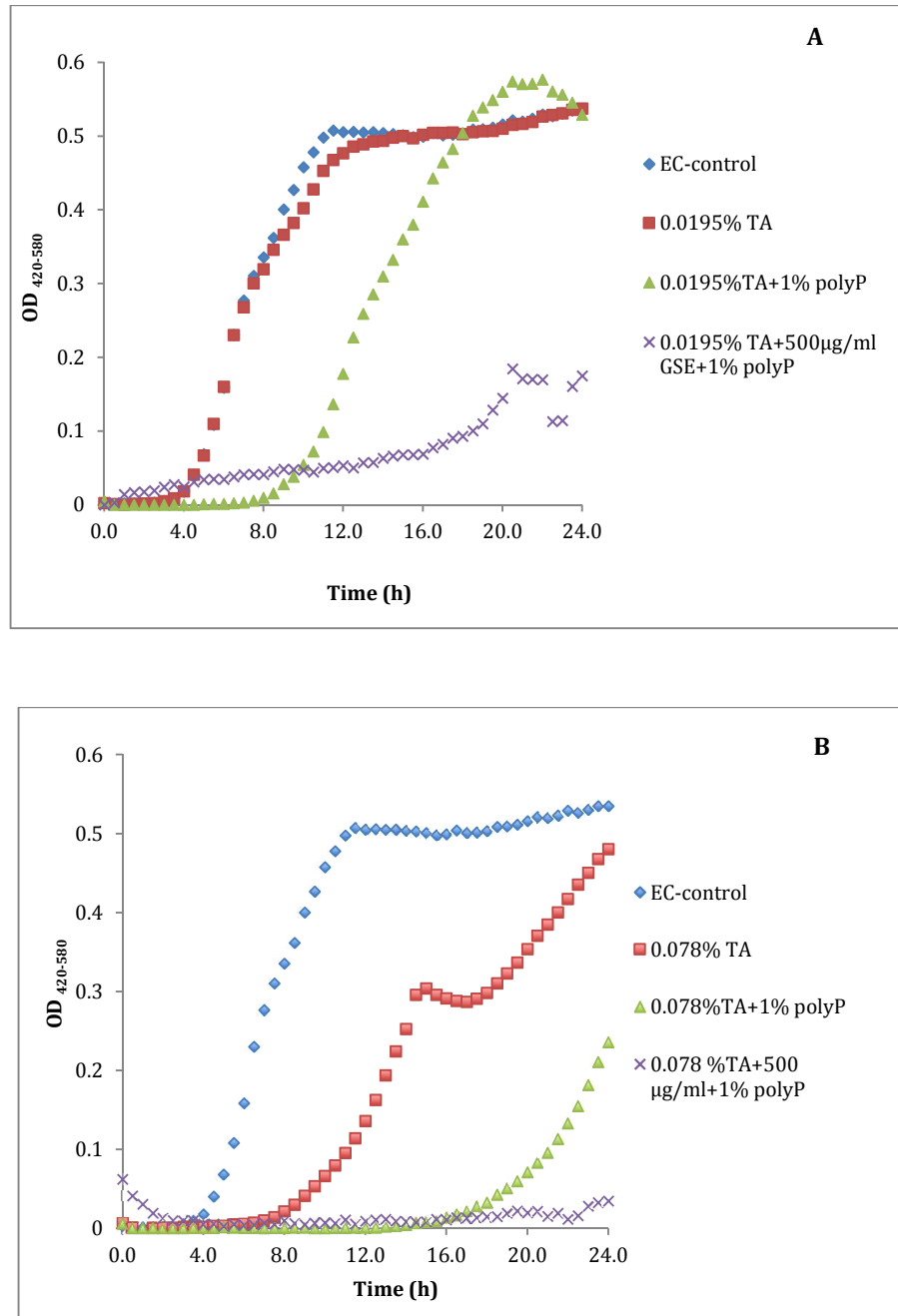
**Figure 2**. **Inhibition of *E. coli* O157:H7 ATCC 35150 with polyP, GSE and tartaric acid.**
A: polyP (1%), GSE (500 μg/ml), 0.0195% tartaric acid, alone or in combination
B. polyP (1%), GSE (500 μg/ml), 0.078% tartaric acid, alone or in combination

**Figure 3**. **Inhibition of *S.* Typhimurium ATCC 14208 with polyP, GSE and tartaric acid.**
A: polyP (1%), GSE (500 μg/ml), 0.0195% tartaric acid, alone or in combination
B. polyP (1%), GSE (500 μg/ml), 0.078% tartaric acid, alone or in combination

# CHAPTER 7. DEVELOPMENT OF A NATURAL ANTIMICROBIAL HURDLE SYSTEM FOR USE IN PRODUCE AND EXAMINATION OF ITS EFFICACY AGAINST LISTERIA MONOCYTOGENES

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## Abstract

*Listeria monocytogenese* is a foodborne pathogen that caused large number of deadly outbreaks especially associated with fresh produce. Due to health concerns about chemical sanitizers, natural source antimicrobials such as plant extracts and their synergistic combination system have gained the attention of researchers, customers and manufacturers. The mode of antimicrobial mechanism for those compounds is also of great importance to guide the researchers and industries to develop cost-effective natural source antimicrobial system. The purpose of this work is to perform multiple antimicrobial methods to study the enhancement of sodium polyphosphate on the inhibitory effects of Grape Seed Extract

against *Listeria monocytogenes*. Generally recognized as safe food ingredient sodium polyphosphate (polyP) has stronger chelation ability with $Mg^{2+}$ and $Ca^{2+}$ that are structurally essential in cell membrane than with $Fe^{3+}$, which was confirmed by using a rapid testing method of SideroTec Assay Kit. Growth curve based Bioscreen method, flow cytometry permeability testing method as well as time course plating method all showed that polyP had enhanced the antilisterial effects of GSE. In time course plating method the combination of 0.5% polyP and 0.5% GSE reduced 4 log CFU/ml of *L. monocytogenes* at 18h while each alone did not show obvious reduction. However, big variations between two types of GSE on antimicrobial system were found, so characterization of GSE polyphenolic compounds was performed by LC-MS. The major polyphenolic compounds in Kikkoman GSE were 1.5 to 2 times of those in OptiPure GSE. The possible affecting factors was also discussed in this work. Standardization and quality control of GSE is needed in order to better applied to fresh produce industry.

**Introduction**

As a life-threatening foodborne pathogen, *Listeria monocytogenes* is estimated to cause 94.0% of hospitalization and 15.9% of mortality among all foodborne illnesses *(38)*. It is a ubiquitous pathogen found in natural environment and food and has ability to survive at low pH, high salt conditions and high hydrostatic pressure *(2, 9)*. In recent years there were deadly multistate fresh produce outbreaks caused by *L. monocytogenes*. In 2011, a big multistate outbreak of listeriosis associated with cantaloupe caused 143 people hospitalized, 34 people die and one miscarriage *(26)*. In 2015 another *Listeria* outbreak linked to prepackaged caramel apples caused 34 hospitalization and 7 deaths*(6)*. The prevention of

human listeriosis caused by the consumption of contaminated vegetable and fruit becomes an important challenge in the food industry and public health.

Alternative to chemical sanitizers such as chlorine that might react with organic matters to produce toxic material, natural plant extracts have been studied for inactivation of pathogen on fresh produce. As a by-produce of wine processing and juice industry, Grape Seed Extract (GSE) have been extensively studied for many years because of it's a broad spectrum of beneficial health effects, such as cardioprotective, hepatoprotective, anti-cancer, anti-oxidative, anti-inflammatory *(29, 32, 47, 51)*. GSE has also been reported to improve color and flavor stability *(18)*, reduce lipid oxidation *(1)*, exhibit antimicrobial *(3)* and antivirus abilities *(43)*, which gained attention in food applications. The antimicrobial mode of action of GSE was not very clear, but it has been reported to relate with its polyphenolic compounds, containing multiple aromatic rings attached with hydroxyl groups *(31)*. Many factors such as the types of grapes affect the composition and contents of polyphenolics in GSE and thus affect the antimicrobial activities of GSE. This study investigated antimicrobial abilities and characterization of their phenolic compounds of two types of GSE.

Long chain sodium polyphosphate polyP has many functions and approved as generally recognized safe (GRAS) food ingredient by FDA *(11)*. Some studies reported that it posses antimicrobial activities due to its metal ion chelation ability *(17, 21)*. Our group also found it could enhance the antimicrobial activities of plant essential oil *(46)* and polephonolics rich peanut skin extract *(40)*. We hypothesized that the addition of long chain sodium polyphosphate could enhance the antimicrobial ability of GSE, in this study specifically for *Listeria monocytogenes*. As our knowledge, this is the first report of the enhancement of polyP on the antilisterial ability of GSE.

**Materials and methods**

Bacterial cultures and growth condition

Listeria monocytogenes NADC 2045 (Scott A) was obtained from the USDA National Animal Disease Center (NADC, Ames, IA). Listeria innocua ATCC 33090 was from the American Type Culture Collection (Manassas, VA, USA). Cultures were stored as frozen stocks at -80°C in Microbank$^{TM}$ beads (Pro-Lab Diagnostics, Round Rock, TX). Working cultures were maintained on Tryptic Soy Agar (TSA, BD Diagnostics Systems, Sparks, MD) plates at 4°C. Prior to the experiments, strains were grown in TSB for 18 h at 35 °C, centrifuged at 8000g for 3 min and suspended in phosphate buffered saline (PBS) water. The optical densities of the resulting bacterial cultures were measured spectrophotometrically (model DU720, Beckman Coulter) and diluted to the target inoculum level ($10^5$ colony forming unit (CFU) /ml for Bioscreen testing and $10^8$ CFU/ml for time course plating), as determined by plating on TSA plates.

Antimicrobials

Two types of GSE were purchased from Kikkoman (Gravinol-S, Kikkoman Corporation, Tokyo, Japan) and OptiPure (Gravinol Super$^{TM}$, Grape seed pit extract, Los Angeles, CA). Gravinol Super$^{TM}$ from OptiPure was used in the most tests. Gravinol-S was used to confirm the antilisterial effects of GSE as well as its synergistic effect with polyP. GSE stock solutions of 25 mg/ml were dissolved in 10% (w/v) ethanol and filtered through 0.45 μm filter before use in the experiments. Food grade long chain sodium polyphosphate BEKAPLUS FS was obtained from ICL performance Product (St. Louis, Missouri). A 10% stock polyP solution was prepared in distilled water and filtered through 0.22 μm filter.

## Growth curved based antimicrobial studies by Bioscreen

A unique system Bioscreen C microbiology reader (Growth Curves, Piscataway, NJ) that simultaneously and automatically measures optical density was used to study the antimicrobial abilities of GSE and/or polyP. According to the CLSI methodology for broth microdilution susceptibility tests *(8)*, different antimicrobial combinations of polyP (0.5% and 1%) and GSE (ranged from 0.004% to 0.5%) were prepared and added in each well of Bioscreen honeycomb plate, inoculated with $10^5$ CFU/ml overnight *Listeria monocytongenes* culture at 37°C. TSB media were used in this experiment because the common used antimicrobial media Mueller-Hinton (MH) recommended by CLSI did not well support the growth of *L. monocytogenes*. Each well had a final volume of 200 μL and duplicate wells were used for all treatments. Optical densities (OD) were measured at wideband setting (420 to 580nm) to minimize OD interference by the inherent coloration of GSE *(3)* and data were collected every 30 min with shaking before each reading. After 24 hours incubation in Bioscreen C, a series of growth curves of *Listeria* were obtained through Bioscreen EZExperiment software. Antimicrobial abilities of different treatment can be determined by the growth curve of *Listeria* compared with that *Listeria* control without any treatment. At the endpoint of incubation, in an effort to determine whether the treatment was bacteriostatically or bacteriacidally effective, samples from each well of Bioscreen honeycomb plate were taken and placed on TSAYE media using a custom-made multi-pin inoculation and replication tool. The plate was incubated at 37°C for 24h before observation.

## Cell membrane permeability study

Flow cytometry experiments were conducted in an effort to determine the antilisterial activities of GSE and its combination with polyP. Considering that live *L. monocytogenes*

cells might produce an aerosol hazard, the physiologically similar *L. innocua* ATCC 33090 *(14)* was used in these experiments instead. As previously described, *L. innocua* cells was washed and resuspended in 0.85% saline to make $10^9$ CFU/mL of cells suspension (diluted if needed). Aliquots of 100 μL cell suspension were placed in different microcentrifuge tubes. After pelleting by centrifugation at 6000 ×g for 3min, the supernatant was discarded and cells were then resuspended in 100 μL of 0.5% GSE, polyP (0.5%, 1%) and their combinations. After 15min treatment at room temprature, all tubes were centrifuged at 6000 ×g for 1min to remove antimicrobial supernatant (GSE and/ or polyP). A 50 μL of sample was taken and stained with 50 μL working solution of fluorescence dye propidium iodide (PI, component B from the L13152 Live/Dead BacLight kit, Invitrogen Corporation, Carlsbad, CA) in the dark for 15min. PI is one of cell membrane integrity probes that bind to nucleic acids. It is membrane impermeant, generally excluded from live cells and thus only has ability to stain permeabilized or dead cells to red color. Two times of PI working solution was prepared by dissolving the contents of one applicator in 5 ml of molecular biology grade distilled water (Sigma-aldrich, St. Louis, MO).

After staining with PI, two hundred fifty microliters of 0.85% saline water was added into the solution and submitted to cytometric analysis using an Accuri flow cytometer (BD Biosciences, San Jose, CA). For each sample, data on cell scatter and PI fluorescence (488 nm excitation and 670 nm long-pass emission) were collected for 20,000 events at a flow rate of 10 ml/min. Controls included live cells with or without PI, cells treated with GSE for 15 min without PI staining *(3)*, 75% isopropanol-killed cells (both stained and unstained), and a separate cell control treated with 0.5% ethanol.

## Metal ion chelation of polyP

Long chain polyphosphates were reported to cause cell lysis and exert antibacterial ability as metal ion chelators to bind important metal ions in cell wall of bacteria *(21, 24)*. To determine the chelation of polyP, a rapid testing method of SideroTec Assay Kit (Emergen Bio, Ireland) was used to detect iron chelation molecules. It is a modified Chrome Azurol S (CAS) method *(39)* that uses the ternary complex chrome azurol S/iron(lII)/hexadecyltrimethylammonium bromide with a maximum absorbance at 630nm as an indicator. A strong iron chelator such as desferoxamine or polyphosphate would remove the iron from the dye complex and change the color from blue to pink. A series of polyP solutions (1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03% and 0.015%) were tested for the chelation ability with iron. Additionally, twenty microliter of a series of $MgCl_2$ (Fisher Scientific) or $CaCl_2$ (Fisher Scientific) solutions (0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2M) was added to 0.03% BekaPlus solution (obviously showed chelation ability) mixed with iron/dye complex to determine the competition of $Mg^{2+}$ or $Ca^{2+}$ with iron to bind BekaPlus. All the samples were incubated for 15min at room temperature after adding SideroTec reagents. Absorbance at 630nm for all the samples was measured by Synergy H4 Multi-Mode Reader (BioTek, Winooski, VT).

## Time course plating testing method

Time course plating assay was used to determine antilisterial activity of GSE at different concentrations over time as well as the effects of polyP on this activity. Overnight culture of *L. monocytogenes* NADC- 2045 was washed and resuspended in 0.85% saline to obtain approximately $10^9$ CFU $ml^{-1}$ cell suspension (Bledar Bisha, 2010). Aliquots of 100 µL of the cell suspension were placed to a sterile separate microcentrifuge tube, pelleted by

centrifugation at 7000 x g for 3 min and then resuspended with GSE (0.5%), polyP (0.5% or 1%) or their combination. All the tubes were incubated at room temperature statically. At 1h, 2h, 4h and 18h, the samples were pelleted by centrifugation at 7000 x g for 3 min and resuspended in 0.85% saline. Following this, a serial dilution were made in 0.1% Peptone Water and plated on TSAYE (TSA and 0.6% Yeast Extract) media and incubated at 37°C for 24h. The experiment was conducted three times independently to obtain statistical data.

Significant differences between treatments for all treatment time points (p<0.05) were determined by the GLIMMIX procedure with Tukey adjustment using SAS 9.3 program. All the results were expressed as mean and standard deviations of three independent experiments.

LC-MS analysis of GSE polyphenols

Four GSE samples were prepared for LC-MS analyses. GSE of 25 mg/ml (2.5%) from two companies Kikkoman and OptiPure was dissolved in 10% ethanol respectively and filtered through a 0.45-µm filter. In order to study the effects of heat treatment on polyphenolic compounds, two additional GSE samples at same concentration (Kikkoman, OptiPure) were heated at 80°C for 14 hours. LC-MS analyses GSE samples were performed using a system consisting of an Agilent 1100 HPLC instrument (Santa Clara, CA) and an API 6310 ion trap mass spectrometer (Santa Clara, CA). Chromatography was carried out on a Phenomenex Synergi 4µ MAX-RP 80A HPLC column (150 × 4.6mm) (Waters, Milford, MA). The 10 µL column effluent was introduced into the mass spectrometer using electrospray ionization (ESI) in the negative ion mode. Mobile phases for negative mode were (A) 0.1% formic acid (v/v) in LC-MS grade water (B) 0.1% formic acid (v/v) in methanol. Mobile phases for positive mode were (A) 25% acetonitrile in LC-MS grade water (B) 0.1% formic

acid in acetonitrile. After sample injection, a linear gradient from 10% B to 100% B over first 10 min and 10% B was held for 2min. The flow rate was kept at a 1.0 mL/min and the column temperature was 35 °C. The ESI $N_2$ gas pressure was 25 psig, gas temperature was set to 350 °C and drying gas flow rate was 10.0 L/ min.

**Results and discussion**

Growth curved based antilisterial ability

The inhibitory effects of GSE at a series of concentrations, 1% polyP and their combination were determined by growth curved Bioscreen testing method. With a low concentration of 0.008% GSE, 1% polyP and their combination all exhibited strong inhibition against *L. monocytogenes*, among which the combination showed the best effectiveness (Fig. 1) (all the OD values were standardized by subtracting background OD caused by media or GSE). However, the plating tests results showed that *L. monocytogenes* survived at endpoint of incubation, which explained that 0.008% GSE, 1% polyP and their combination had bacteriostatic rather than bactericidal effects against *L. monocytogenes*, which was consistent with the findings of Zaika and Kim*(49)* when they used a higher level of GSE at 2.0%. They also suggested that these compounds would be useful in food to inhibit proliferation of *L. moncytogenes* especially at low temperature and in combination of sodium chloride. Another gram positive *Staphylococcus aureus* was also very sensitive to polyP with MIC of 0.1% *(19)*. In order to further study of the damage effects on *L. monocytogenes*, higher concentrations of GSE were suggested for use in the following two tests: flow cytometry and time course plating.

Cell membrane Permeability

Flow cytometry (FCM) is a very useful tool used for many antimicrobial studies *(3, 5, 25)* to analyze the cell population characteristics of light scatter and fluorescence intensity and provide valuable information of cell membrane integrity and the heterogeneity of individual cells. FCM can determine the physiological changes of cells such as cell lysis and clumping after treated with antimicrobials. With fluorescence probe such as PI, FCM can be utilized to compare the antimicrobial abilities among different compounds based on the cell scatter related with the cell size and heterogeneity as well as fluorescence intensity related with damaged level of cells. In our study FCM was used to further compare the antilisterial effects of GSE, polyP and their combination. Considering the possibility of live cell aerosols that might be produced during flow cytometry studies, *L. innouca* ATCC 33090 was used as a surrogate of *L. monocytogenes* because of their similar physiology *(23)* and their similarity to *L. monocytogenes* in susceptibility to GSE *(35)*. Due to bacteriostatic effects of low levels of GSE, polyP and their combination, higher level of concentration of those compounds (0.5%) were used in FCM tests to determine how many percent of cells could be permeabilized. It was also hypothesized that the combination of GSE and polyP would have ability to permeablize more *L. innocua* cells than each alone. Fig. 2 showed that minimal *L. innocua* cells (0.2%) were permeabilized after exposure to 0.5% polyP for 15min, while 0.5% OptiPure GSE and its combination with 0.5% polyP permeabilized 9.5% and 36.6% respectively. In terms of cell membrane permeability, GSE from OptiPure in our study was much less effective than the reports of Bisha et al.*(3)*, which showed that 0.01% Kikkoman GSE was able to permeabilize 69.8% *L. innouca* cells after 2min exposure. This could be due to the variation from GSE produced from different varieties of grapes grown in different

climatic condition, harvested from different production sites, or in different maturity degree
*(42)*. The same FCM experiments using Kikkoman GSE were repeated in an effort to
determine if the source of GSE was a factor affecting the result. At very low level of 0.016%
Kikkoman GSE had ability to permeabilize 14% *L. innocua* (Fig 3), showing its effectiveness
was between OpitPure GSE and the result of Bisha and others *(3)*. However, despite the
antilisterial variation from OptiPure GSE, in this study 0.5% polyP also enhanced the
permeability of Kikkoman GSE against *L. innouca*, increasing the percentage of
permeabilized cells from 14.0 % to 61.4%. Therefore, combination of GSE (either Kikkoman
or OptiPure) and polyP clearly showed better antilisterial effects than each alone by
permebilization of *Listeria* cell membrane, which was consistent with growth curve based
Bioscreen antimicrobial study (Fig 1).

Time course inactivation

The results of both growth curve based bioscreen test and flow cytometry test using
two sources of GSE (OptiPure and Kikoman) indicated that polyP enabled to enhance the
antilisterial effects. Both culture-independent approaches showed that the combination of
GSE and polyP were very effective. In addition, more commonly used culture-based plating
study would provide us confirmatory information of this enhancement over time. The time
course plating method modified by Bisha et al. *(3)* was used to prevent overestimation of
antimicrobial activity of residual GSE. No significant difference were found among three
treatment groups of 1% polyP, 5% polyP and 0.5% GSE, showing no obvious effectiveness
to kill *L. monocytogenes* after exposure for 18h (Table 1). However, the combination of 0.5%
GSE and polyP (1% or 5%) was significantly effective than the other treatments, reducing 2
$\log_{10}$ CFU/mL at 4h and 4 $\log_{10}$ CFU/mL of *L. monocytogenes* at 18h. This highlighted the

enhancement of polyP on the antilistieral effectiveness. No significant difference between two combinations indicated that polyP exhibited its enhancement at a certain level and higher concentration of polyP would have buffering agent that could decrease antimicrobial activity of antimicrobial system *(50)*. OptiPure GSE used in this study was shown less effective than Kikkoman GSE in Bisha's study *(3)*. This might also due to the variable active polyphenolic compounds from different sources of GSE *(22, 42)*.

To confirm the different effectiveness of different sources of GSE, Kikkoman GSE was also tested using time course plating method. Similar to Bisha's study *(3)*, the results showed Kikkoman GSE was more effective to *L. monocytogenes* than OptiPure GSE. However, colonies on the plate were not ten times between dilutions when a serial of ten-fold cell dilutions was made and plated after treated with even a very low level of Kikkoman GSE (0.008%). The enhancement of polyP on the activity of Kikkoman GSE was not as obvious as shown using OptiPure GSE. This could be explained by two important observations in our studies. Firstly, relative to Fig 3A (0.016% Kikkoman GSE), Fig 3C showed a higher concentration of Kikkoman GSE (0.25%) caused increased side scatter of *L. innocua* cells due to cell clumping, which was consistent with findings of Bisha et al. *(3)* in both fluorescent microscopy and flow cytometry permeability test. These clumped cells could not be dispersed homogeneously by vortexing. Secondly, long chain polyP has been known to serve as an emulsifying agent for use in food. Mizuno and Lucey *(27)* found one of long chain polyP sodium hexametaphosphate enabled to bind casein-bound Ca and therefore disperse casein micelles. In our study fluorescence miscroscopy also showed that the addition of polyP was able to disperse GSE stained with PI very well (data not shown). Therefore, the possible explanation of unrepeated plating data of enhancement of polyP using Kikkoman

GSE would be that relatively effective Kikkoman GSE caused cell clumping or aggregation and polyP might enable to disperse the clumped cells and then get more survived colonies.

The results also indicated the big difference of polyphenolics might exist between Kikkoman GSE and OptiPure GSE and highlighted the importance to analyze the polyphenolics composition and contents in each kind of GSE.

<u>Metal Ion Chelation of polyP</u>

All the tested concentrations of polyP (0.015%-1%) was able to change the dye from blue to pink, among which the lowest tested polyP solution of 0.015% made color become rose and the rest of them all turned dye reagents to pink, explaining that polyP at as low as 0.015% level had ability to react with iron. This suggested that polyP might enable to remove structurally essential ions such as $Mg^{2+}$ and $Ca^{2+}$ in the cell wall of yeast and bacteria by chelating with them.

The graph below showed in the presence of 0.03% polyP, when the concentration of $Mg^{2+}$ or $Ca^{2+}$ added was increased, the absorbance at 630nm of dye ferrocomplex increased (becoming more blue), indicating less and less iron was being removed by polyP from dye ferrocomplex due to the saturation of polyP by $Mg^{2+}$ or $Ca^{2+}$. This result was consistent with previous studies *(20, 49)*, confirming that long chain sodium polyphosphate poyP had a strong chelation with these two metal divalent ions $Mg^{2+}$ or $Ca^{2+}$. Combined with flow cytometry permeability data, this observation also indicated that cell permeability caused by polyP is likely due to its chelation with important metal ions in cell walls of yeast and bacteria.

LC-MS analysis of GSE polyphenolics

Electrospray Ionisation mass spectrometry analysis of GSE in the negative ion mode (Fig 5. and Fig 6.) showed that GSE (Kikkoman, OptiPure) was a complex mixture containing methyl catechin (epicatechin) (m/z 304.9), epicatchin or catechin dimer (m/z 576.9), trimer (m/z 865.0) and gallate of diametric proanthocyanidins (m/z 728.9) and trimeric proanthocyanidins (m/z 1017.0) and some unknown compounds (m/z 351.9, m/z 639.9). Those catechin (epicatechin) derivatives were also found in the other GSE HPLC-mass spectrometry studies *(33, 48)*. Different from some studies *(33, 37, 42)*, monomeric catechin or epicatechin (m/z, 289) was not found in either OptiPure GSE or Kikkoman GSE. Tetramer or polyphenolics with higher molecular weight were not able to be identified by LC-MS in our study and Matrix-assisted laser desorption ionization (MALDI) mass spectrum would be suggested to analyze low levels of polyphenols with high molecular weight *(10)*.

Among all the phenolic compounds, catechin (epicatechin) dimer (m/z 576.9) was the most dominant one that was consistent with the other study *(12)*, followed by the two major unknown compounds (m/z, 351.9; 639.9). It was noticed that the difference of molecular weight between those two unknown compounds is one catechin monomer (m/z, 288), which means they are also important metabolites of catechin (epicatechin). To further identify these two major compounds, LC-MS/MS analysis in the multiple reaction monitoring mode would be recommended to perform, which uses fragmentation of the ions between the stages and provides more information about the structure and composition of the molecule. The data revealed the similarity of polyphenolics composition and the difference of their contents between Kikkoman GSE and OptiPure GSE. Two sources of GSE both contain methyl catechin(epicatechin), dimer, trimer and dimer gallate ester and two major unknown

compounds. However, the contents were different in each kind of GSE. Each of these compounds in Kikkoman GSE was higher than that in OptiPure GSE, especially for the unknown compound (m/z 351.9), the concentration of which in Kikkoman was two times that in OptiPure. This concentration difference of this compound might be the important reason to explain why Kikkoman GSE was more effective than OpitPure GSE, indicating the importance to identify the unknown compounds by more high-resolution Mass spectrometer.

During this study, we noticed that the old batch of Kikkoman stored for 6 years at room temperature had less effective antimicrobial activity than new batch of Kikkoman, indicating the storage condition (time, temperature, light) might affect the antimicrobial activity of GSE. An accelerated shelf life study of treating GSE at 80°C for 14h *(44)* was performed in an effort to determine the change of polyphenolic composition and content in GSE. With comparison with MS polyphenolics spectrum of unheated Kikoman GSE (Fig. 5A), the unknown compound (m/z 351.9) was substantially deceased and the catechin dimer and another unknown compound (m/z 639.8) was slightly increased in the heated Kikkoman GSE (Fig. 6A). This could be explained that the heating treatment might cause the reaction among the polyphenolics compounds. However, compared with the OptiPure GSE (Fig. 5B), almost all the major polyphoenolic compounds decreased in the heated OptiPure GSE (Fig. 6B), which revealed the reactions caused by heat among polyphenolics in OpitPure GSE were different from those in Kikkoman GSE. These results further confirmed the big difference between OptiPure GSE and Kikkoman GSE.

## Factors affecting GSE polyphenolics

There are many factors reported affecting the content and composition of polyphenolics in GSE (shown in Table) such as verities of grapes, climatic condition,

production site, maturity/ripening degree, extraction method and storage condition. All those

factors could lead to the variation of product-to-product or batch-to-batch in the content and

composition of grape seed polyphenolics *(28)*. The big difference of Kikkoman GSE and

OptiPure GSE in polyphenolic contents and the antilisterial activities could be due to some of

those affecting factors.

Factors affecting polyphenolic content in grapes or grape seed extract

| Affecting factor | Findings and significance |
|---|---|
| Variety of grapes | Liang et al. found *(22)* there was significant variation among and within species for the total polyphenolic content of grape *Vitis Germplasm*. |
| | Great differences in contents of polyphenolic compounds depended on cultivar and vintage. *(15)* |
| | Fuleki et al. *(12)* found that the seeds of Gamay, Pinot noir, Baco noir and Vincent had relatively high content of polyphenolics. |
| | Different GSEs had different polyphenol content and proportion leading to different antipica effects in cisplatin-treated rats *(45)*. |
| Climatic condition | Revilla et al. *(34)* found the year of production had effects on the content of catechins and procyanidins in the seeds of cv. Muscat of Hambourg in El Encin, Spain even though they had similar maturity. |
| Site of production | The catechins and procyanidins of grapes were variable, depending on the geographic origin of grapes *(34)*. |
| Degree of maturity and ripening | Romeyer et al. *(36)* found during maturation flavan-3-ols decreased and more dimeric procyanidins accumulated, suggesting more dimeric procyanidins were synthesized from monomeric forms during maturation |
| | A few varieties of grapes had the highest catechins and procyanidins in the early stages of development but decreased dramatically during September *(34)*. |
| | Kennedy et al. *(16)* concluded that polyphenols in grape seeds decreased substantially with a 60% decrease in procyanidins and 90% decrease in flavan-3-ol monomer forms. |
| | Boido et al. *(4)* found the content of flavan-3-ols did not change much but dimer and oligomer forms increased during ripening. |
| Extraction | The extraction temperature and extracting phase composition played an |

| method and condition | important role in extraction yield of polyphenols (30). |
|---|---|
| | Shi et al.(41) found ethanol concentration and extraction temperature had the most important effect on the yield of total phenolics. |
| Storage condition | The catechins, epicatechin and procyanidins from grape seeds of Romanian cultivar "Merlot Recas" were not stable when stored in water at 4°C (7). |

To use grape seed extract as dietary supplements and food preservatives, quality control and standardization especially for its phenolic compounds is an important issue for consumers, researchers and manufacturers (15, 28, 45). Similar to the standardization of herbal medicine (13), quality control of GSE can be achieved by planting genetically uniform monoculture of the grape in standardized farmers, applying the same process and extraction method and same appropriate storage condition. Those fully standardized conditions need to be further studied. Some big food ingredient companies have developed their own standardization method to control GSE in a good and uniform quality.

**Conclusions**

The results of multiple antimicrobial methods (growth curve based Bioscreen method, time course plating and flow cytometry analysis) showed that GSE and polyP had a promising cooperative antilisterial effects. The mechanism of synergistic antimicrobial effects of polyP and GSE could be: polyP chelated the cell membrane structurally essential metal ions such as $Mg^{2+}$ and $Ca^{2+}$, which assisted GSE to further permeabilize the bacterial cell membrane. Product-product and batch-batch variations in GSE polyphenolic compounds and antilisterial abilities are due to many affecting factors that need further study to be exploited. The combination of GSE and polyP is a promising natural intervention for *L. moncoytotogenes* on fresh produce if standardization of GSE quality especially its

polyphenolic compounds can be achieved.

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**Table 1**. Time course inactivation of *L. monocytogenes* in 0.85% saline

| Treatment time (h) | 0.5% GSE [A] | 0.5% polyP [A] | 0.5% GSE+ 0.5% polyP [B] | 1% polyP [A] | 0.5% GSE+ 1% polyP [B] | Control |
|---|---|---|---|---|---|---|
| 1 | 7.90±0.06 | 8.00±0.05 | 7.49±0.10 | 7.99±0.09 | 7.57±0.23 | |
| 2 | 7.74±0.13 | 7.98±0.09 | 6.72±0.79 | 8.06±0.08 | 6.82±0.50 | |
| 4 | 7.65±0.32 | 7.92±0.03 | 5.06±0.90 | 7.99±0.09 | 5.69±0.82 | 8.10±0.02 |
| 18 | 7.48±0.19 | 7.93±0.06 | 3.21±0.38 | 8.03±0.14 | 3.20±0.45 | 8.10±0.04 |

The same letter marked for each treatment group meant no significant difference, while different letter meant significant difference existed.
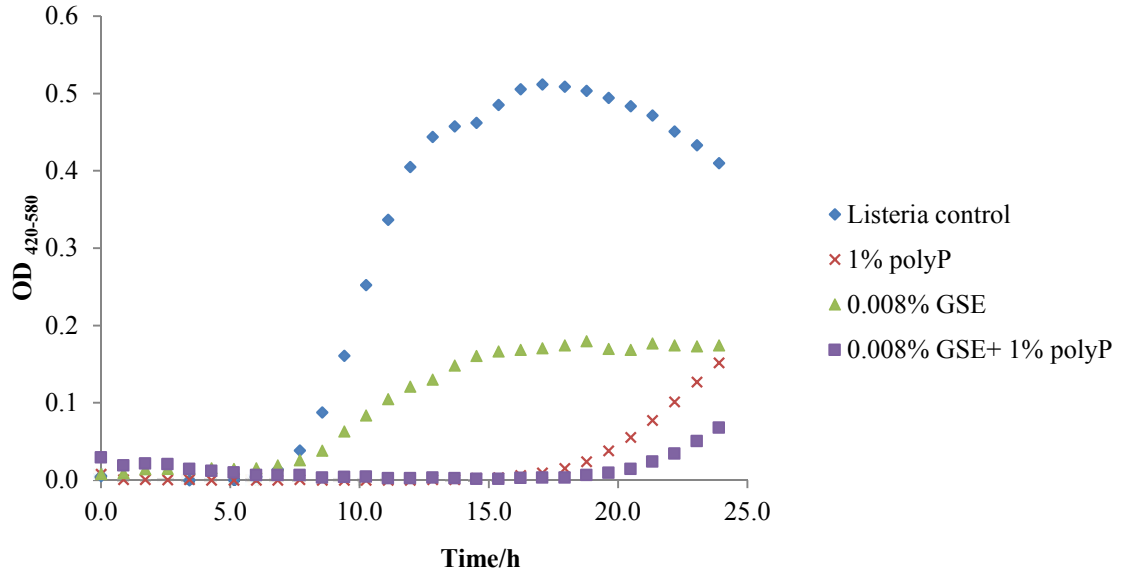
**Fig 1**. Growth curve based inhibitory effects of GSE and or polyP against *L. monocytogenes*
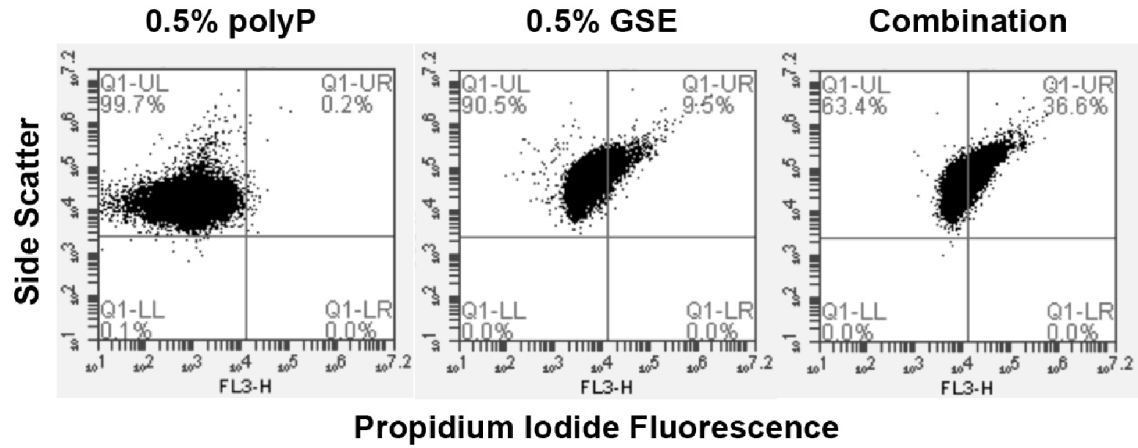
**Fig. 2**. Flow cytometry permeability of *L. innocua* by OptiPure GSE, polyP and/or combination
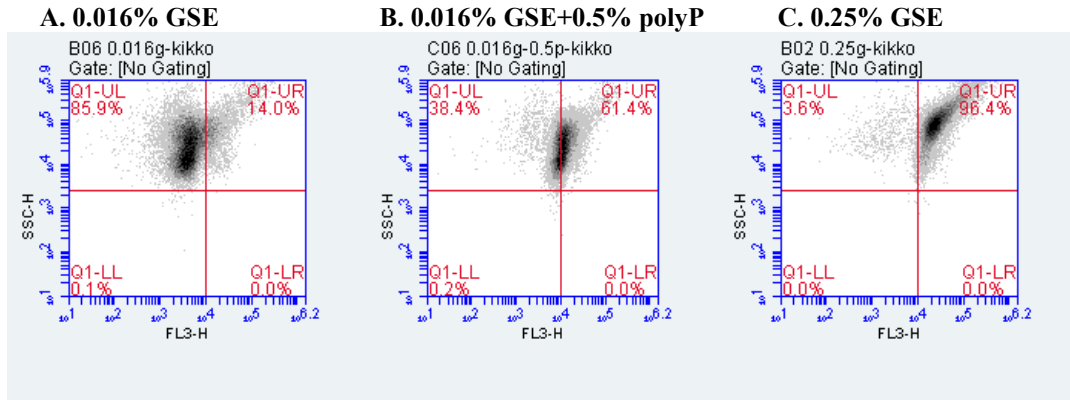
**Fig 3**. Flow cytometry permeability of *L. innouca* by Kikkoman GSE and its combination with polyP
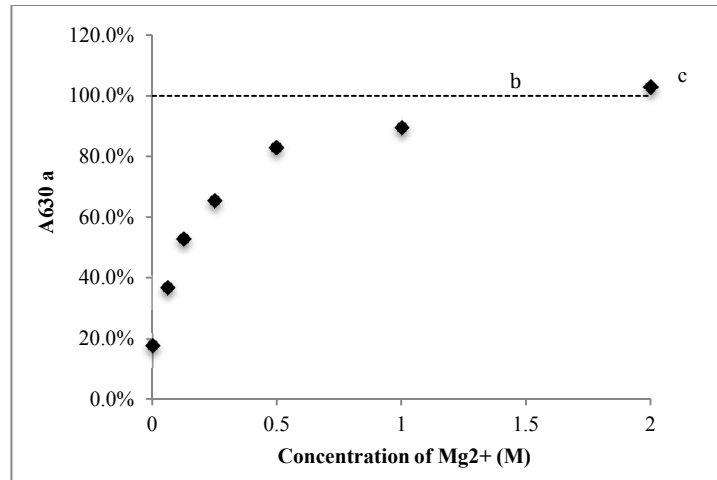
**Figure 4**. Competition of $Mg^{2+}$ with $Fe^{3+}$ to chelate polyP

The tested $Mg^{2+}$ concentrations were 0.0312M, 0.0625M, 0.125M, 0.25M, 0.5M, 1M, 2M.

a.  Y-axis was shown as the percentage of OD of dye ferrocomplex in the presence of polyP after adding $Mg^{2+}$ divided by OD of intact dye ferrocomplex without $Mg^{2+}$ or polyP added.
b.  The dashed line showed the absorbance value of the intact dye ferrocomplex.
c.  When $Mg^{2+}$ was increased to 2M, the absorbance was a little higher than the value of the intact dye ferrocomplex, this can be caused by experimental errors. Or it can be explained by the assumption that when polyP was saturated by $Mg^{2+}$, the rest of $Mg^{2+}$ might also bind dey ferrocomplex and interfere absorbance value.

A. Kikkoman GSE
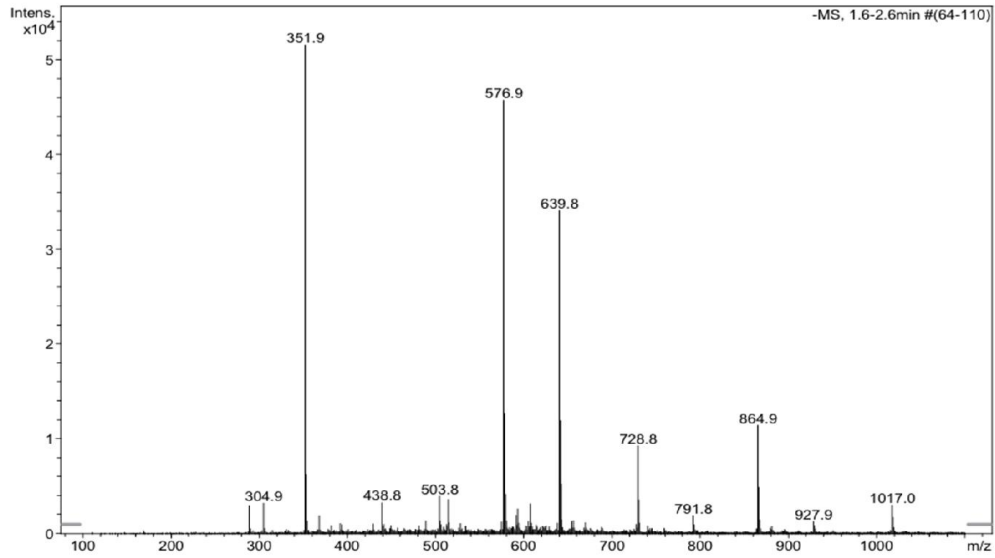


B. OptiPure GSE
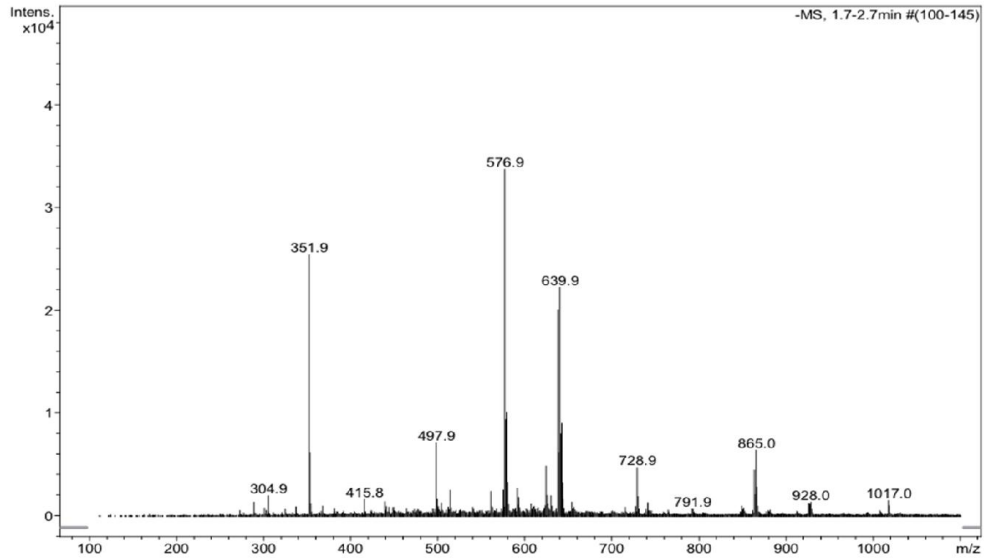


**Fig 5**. ESI-MS polyphenolics spectrum of the GSE solutions

A. Kikkoman GSE-heated
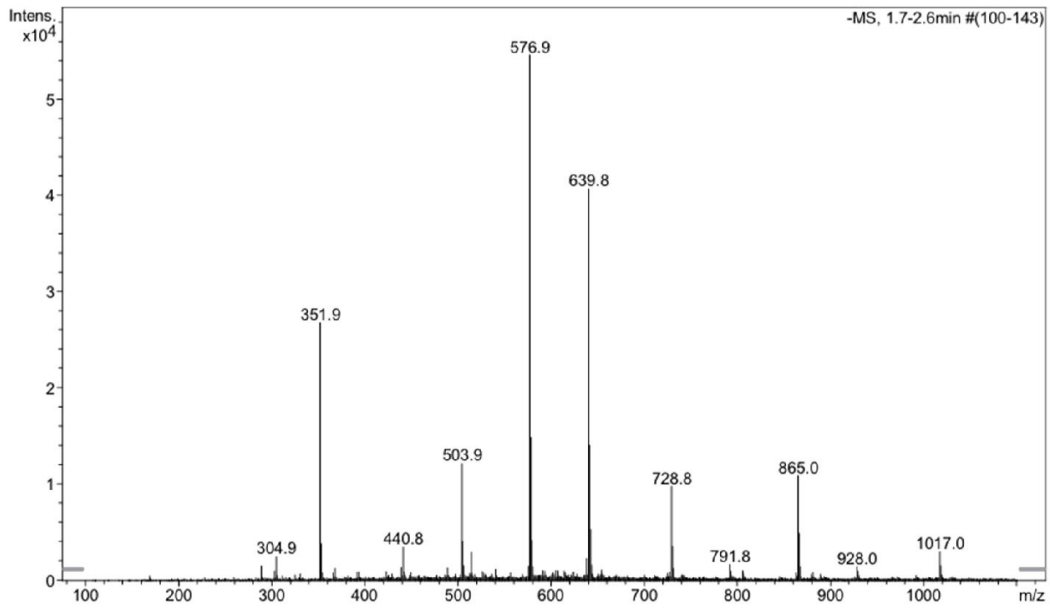


B. OptiPure Heated
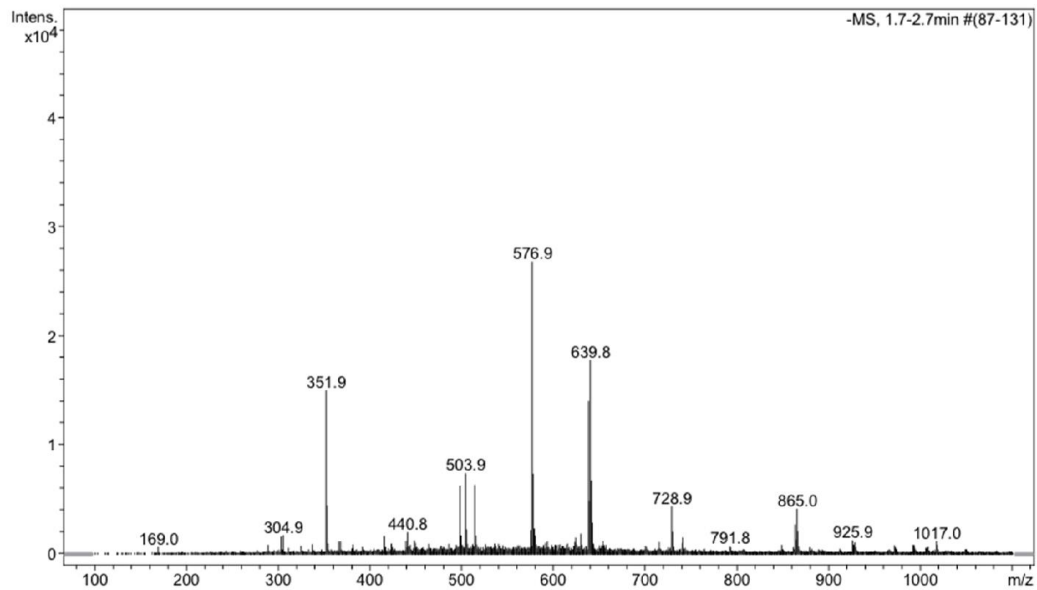


**Fig 6**. ESI-MS polyphenolics spectrum of the heated GSE solutions

## CHAPTER 8. GENERAL CONCLUSIONS

This dissertation demonstrates natural and value-added agricultural approaches for pathogen control in fresh produce, food operations as well as medical materials. Chapter 3, 4 and 5 of this dissertation discuss the synthesis and characterization of environmentally-friendly polyurethane dispersion and films produced from soybean oil or castor oil based polyols. Determined by multiple conventional and novel antimicrobial testing methods, those polyurethane coatings exhibited good antimicrobial activities against human foodborne and nosocomial pathogens. Their antimicrobial mode of action against multiple bacterial and yeast pathogens has been determined such as morphology change, cell membrane permeability, and intracellular leakage measurement and metal ion chelation. Castor oil-based polyurethane coating was found to suppress the hyphal growth of *C. albicans* and enhance antibiotics effectiveness against methicillin-resistant *S. aureus* and *C. albicans*. The vegetable oil-based polyurethane dispersions can find many applications in food industries such as food non-contact shipping pallets in food storage or shipping operations, as well as in medical devices and clinical materials such as bandage dressings.  These inexpensive antimicrobial coatings will continue to be developed for a wide range of applications.

Chapter 6 and 7 focus on the development of natural antimicrobial systems for fresh produce and their antimicrobial activities against three major foodborne pathogens. The enhancement of sodium polyphosphate on antimicrobial effects on Grape Seed Extract and organic acids demonstrated the promise for multicomponent antimicrobial systems with potential applications in fresh produce, due to their natural source, GRAS status and their

promising synergistic antimicrobial ability. The findings suggest that the different compounds can effectively and cooperatively target and permeabilize the outer or cytoplasmic membrane, thereby allow the active compounds entry into the cell. More research will need to be conducted to explore the mechanisms of action for natural antimicrobial system and their effects on produce quality and sensory characteristics in order to examine the potential of this approach in fresh produce industry. Other plant extract rich in polyphenols such as green tea extract or rosemary extract might be also potential antimicrobial agents that would posses synergistic inhibitory effects with organic acids and sodium polyphosphate against foodborne pathogens associated with fresh produce outbreaks.

Due to the concern of chemical materials that might pose risk to human health, demand of natural and biorenewable green technology is increased especially for food, food packaging or medical devices and materials, this research is very important to demonstrate natural and inexpensive value-added approaches in this wide range of applications.

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This thesis is dedicated to the loving memory of my mother, whose role in my life was and always remains immense.