Evaluation of Peanut Skin Extract, Grape Seed Extract, and Grape Seed Extract Fractions to Reduce Populations of Select Foodborne Pathogens

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ABSTRACT

Grape seed extract (GSE) and peanut skin extract (PSE) are waste products in the wine and peanut industries. Both extracts have high concentrations of polyphenols, known to possess antioxidant and antimicrobial properties. A subcategory of polyphenol is procyanidin, which can be divided into two types, type A and type B. Type A (PSE), contains two single bonds connecting the phenolic groups while type B (GSE), contains one single bond connecting the phenolic groups. The minimum inhibitory concentration (MIC) of the two extracts was evaluated for their antimicrobial effect on *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium using the pour plate method. GSE was found to have a significantly lower MIC ($p \leq 0.05$) than PSE for *L. monocytogenes* (GSE=60.60ppm, PSE=not found), *S. aureus* (GSE=38.63ppm, PSE=51.36ppm), and *S.* Typhimurium (GSE=45.73ppm, PSE=60.60ppm). There was no significant difference in inhibition of *E. coli* O157:H7 (GSE=47.44ppm, PSE=51.13ppm). Since GSE, contributed to greater pathogen inhibition, its extract was fractionated into monomer and oligomers components. Growth curves of all four pathogens inoculated in the monomer and oligomer fractions were compared using the BioScreen method. Oligomers inhibited growth of *L. monocytogenes*, *S. aureus*, and *E. coli* O157:H7 while monomers inhibited growth of *S.* Typhimurium. These results indicate that an extract with type B procyanidins that are high in oligomers may be more effective as antimicrobials. Type B procyanidins have also been shown to

prevent bacterial adhesion, as is the case with urinary tract infections, and may aid in the prevention of biofilms.

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Dedication

I dedicate this work to my family: Mom, Dad, Dave, and Kathleen. Thank you for always being so loving, supportive, and encouraging me to pursue my dreams. I love you.

ATTRIBUTION

Several colleagues aided in the writing and research behind one of my chapters presented as part of this thesis. Other colleagues aided with data collection for Chapter 3 and the Appendix. A brief description of their contributions is included here.

Renee R. Boyer – Ph.D. (Assistant Professor, Department of Food Science and Technology, Virginia Tech) is the primary advisor and chair of the author's thesis committee. She provided assistance with the selection of test compounds and aided in the design of this research project. She also provided funds to support the work presented in this thesis.

Chapter 3: Evaluation of Peanut Skin Extract, Grape Seed Extract, and Grape Seed Extract Fractions to Reduce Populations of Select Foodborne Pathogens

Andrew P. Neilson – Ph D. (Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. He provided the grape seed extract and was very knowledgeable of polyphenolic compounds.

Sean F. O'Keefe – Ph.D. (Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. He provided assistance with the isolation of peanut skin extract and aided in the design of this research project.

Robert C. Williams – Ph.D. (Associate Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. His knowledge and expertise in antimicrobial interactions greatly contributed to this work.

Melanie R. Dorenkott – M.S. (Department of Food Science and Technology, Virginia Tech) aided the author with extraction and fraction protocols.

Katheryn M. Goodrich – Ph. D. Candidate (Department of Food Science and Technology, Virginia Tech) assisted the author with UPLC and chemistry knowledge.

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CHAPTER 1

Introduction

The Centers for Disease Control and Prevention (CDC) estimate that each year 1 in 6 Americans, or 48 million people, become ill from foodborne diseases. Among those, 128,000 are hospitalized and 3,000 die (Osorio, Flores et al. 2010, Scallan, Griffin et al. 2011, Scallan, Hoekstra et al. 2011). Both fresh and processed foods can become contaminated with foodborne pathogens during the entire food production/processing continuum. The use of preservatives in food production is a critical step to eliminate pathogens or prevent their growth within the food matrix, increase shelf life, and ultimately prevent outbreaks (De Roever 1998).

Traditionally, foods are thermally processed or preserved with chemical compounds such as sodium benzoate, nitrites, sodium meta-bisulfate, and others that are generally recognized as safe (GRAS) (Davidson and Harrison 2002). Recently, consumers have pushed for the use of nature–based compounds to replace synthetic chemicals as preservatives in food commodities (Jayaraman, Sakharkar et al. 2010). This drive for perceived healthier, safer food ingredients has led to the exploration and discovery of antimicrobial compounds from natural sources (Aoki, Shen et al. 2010).

There are many natural compounds that have health benefits, such as antioxidant, anti-inflammatory, anti-carcinogenic, and anti-aging effects (Haslam 1996, Gil, Tomas-Barberan et al. 2000, Nassiri-Asl and Hosseinzadeh 2009). Some products derived from plants and microorganisms (traditionally herbal medicines rich in polyphenols) contain compounds that may also possess antimicrobial properties (Haslam 1998). Antimicrobials

are substances that inhibit growth of microorganisms including bacteria, viruses, and fungi (Davidson, Sofos et al. 2005).

One group of compounds that have been studied extensively is the polyphenol group. Polyphenols are secondary metabolites produced by plants, which play a role in plant physiology and have potential bioactive components of interest in human nutrition and medicine, mainly as antioxidants, anti-allergic, anti-inflammatory, anticancer, and antimicrobial. Certain classes of polyphenols have shown antimicrobial characteristics and these compounds have promise in the development of new food preservatives (Rodríguez Vaquero, Aredes Fernández et al. 2010).

There are many subclasses of polyphenols; however, the subclasses of flavan-3-ols, flavonols, and tannins have received the most attention due to their higher antimicrobial activity when compared to other polyphenols and the ability to suppress a number of microbial virulence factors (Daglia 2012). These three subclasses have been shown to suppress a number of microbial virulence factors, such as inhibition of biofilm formation and reduction of host ligand adhesion, as well as show synergism with antibiotics (Daglia 2012). The group of interest in this research is the flavanol subclass, which can be further broken down into two basic structure types, procyanidin A and procyanidin B, characterized by the occurrence of a double or a single linkage connecting two flavanols units respectively (Slade, Ferreira et al. 2005).

Grape seeds and peanuts skins are two sources of procyanidins that need more exploration in their use as antimicrobials. Extracts produced from grape seeds (GSE) have been shown to possibly possess antimicrobial properties (Ahn, Grün et al. 2007); and have Generally Recognized as Safe (GRAS) status to be used in food products

according to the Food and Drug Administration (Ahn, Grün et al. 2007). Peanut skins have also been shown to possess antioxidant activity (Lou, Yuan et al. 2004) and possesses much higher *in vitro* antioxidant activity than vitamin C and trolox (Yu, Ahmedna et al. 2010), which suggests that PSE may have the potential to be a potent antimicrobial (O'Keefe and Wang 2006). Type A procyanidins are found in cranberries, which have been shown to aid in the prevention and treatment of urinary tract infections.

One of the benefits to examining these two products is that they are considered waste products by the industries that produce their parent products, grape juice/wine and peanuts (Shi, Yu et al. 2003). Peanut skins are low value ($12-20/ton) byproducts of peanut blanching operations and see limited use in cattle rations (Sobolev and Cole 2004). Yu *et al.* reported that three classes of phenolics were found in peanut skins, including phenolic acids, flavonoids, and stilbene. Natural phenolic compounds can be extracted from peanut skins and hulls, low value byproducts of peanut roasting and blanching (Seo and Morr 1985). Identifying value in these products will result in less waste, complete utilization of raw products, and provide an all-natural alternative to synthetic preservatives.

This research focused on evaluating the antimicrobial properties of grape seed extract, a type B procyanidin, and peanut skin extract, a type A procyanidin, against four pathogenic bacteria (*L. monocytogenes*, *S. aureus*, *S. enterica* serovar Typhimurium, and *E. coli* O157:H7). GSE was fractionated into its varying degrees of polymerization (DP, which is the number of monomeric molecules that make up the whole polymer) of monomers and oligomers. The different degrees of polymerization were then tested for MIC values to discover the most effective component of the extract.

Objectives

1) Determine which crude extract, grape seed or peanut skin, demonstrates the most inhibition against four common foodborne pathogens (*Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Escherichia coli* O157:H7)
2) Determine which fraction (separated by degree of polymerization) of the extract with the greatest antimicrobial ability, contributes the most to the inhibition of four common foodborne pathogens (*L. monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *E. coli* O157:H7)

CHAPTER 2

Review of Literature

Natural Antimicrobials

Inactivation, growth delay, or growth prevention of spoilage and pathogenic microorganisms are the main objectives of food preservation. Antimicrobial agents are chemical compounds added to, or present in, foods that retard microbial growth or cause microbial death (Davidson, Sofos et al. 2005). The World Health Organization (WHO) defines "antimicrobial" as the term for any compound with a direct action on microorganisms used for treatment or prevention of infections. Antimicrobials include anti-bacterials, anti-virals, anti-fungals and anti-protozoals (WHO, 2000). There has been an increasing demand for more "natural" foods with minimal to no artificial, or chemically changed in a way not found in nature, additives which has prompted the search for alternative natural antimicrobial agents or combinations that can be used by the food industry (López-Malo, Alzamora et al. 2000). This has prompted the study of a wide range of natural systems including animals, plants, and microorganisms, for naturally formed antimicrobials that may be effective in food systems (Nychas 1995, López-Malo, Alzamora et al. 2000, Montville and Matthews 2008). Naturally occurring antimicrobial compounds are present in plants, animals, or produced by microorganisms that are considered within this classification (Davidson, Sofos et al. 2005). These naturally occurring antimicrobial compounds may be abundant in the environment.

The antimicrobial compounds in plant materials are commonly contained in the essential oil fraction in leaves, flowers and flower buds, bulbs, rhizomes, fruit, or other parts of the plant (Shelef 1984, Nychas 1995). These plant derivatives and isolated

compounds contain a large number of substances that are known to inhibit various metabolic activities of bacteria, yeast, and molds (Davidson, Sofos et al. 2005). More than 1,340 plants are known to be potential sources of active compounds, and that list is growing (Wilkins and Board 1989). The major contributor to antimicrobial activity in plants, herbs, and spices are phenolic compounds (Wojdyło, Oszmiański et al. 2007).

There are a wide variety of organisms that affect food products which include viruses, yeasts and molds, and bacteria (Montville and Matthews 2008). Bacteria are a major concern due to their ability to grow and multiply in a food. There is a wide variety of bacterial species that affect food; some cause spoilage and ruin the quality of foods while others cause major illnesses and, in extreme cases, death. This study focused on four major pathogens: *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium.

Polyphenols

Polyphenols are defined as a compound containing more than one phenolic group and their derivatives (Figure 1). Shelef (1984) mentioned that simple and complex derivatives of phenol are the main antimicrobial compounds isolated from spices. Derivatives of phenol, called phenolics, contain a phenol molecule with one or more ligands, which may increase its antimicrobial activity (Davidson, Sofos et al. 2005). In 1867, phenolic compounds extracted from coal tar were used as antimicrobial agents in the form of a sanitizer (Davidson 1993). In addition to being antimicrobials, phenolic compounds are studied because they also act as antioxidants, which contribute to overall health. Today, several common flavoring agents, such as garlic, turmeric, black pepper, clove, ginger, cumin, rosemary, are currently being used as therapeutic and antitoxigenic

agents to reduce inflammation, prevent cancer, and treat acute respiratory diseases (Arora and Kaur 1999, Low Dog 2006). Their effectiveness as an antimicrobial agent depends on the chemical structure of their components and on their concentration (Shelef 1984).

Polyphenolic compounds, as food antimicrobials, can be classified as those currently approved (such as parabens), those approved for other uses (such as antioxidants), and those found in nature (such as polyphenolics and phenols) (Davidson 1993). Many phenolic antioxidants are already incorporated in food products. For example, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) are used to prevent rancidity in fats, oils, and lipid foods (Shahidi, Janitha et al. 1992). BHT, BHA, and TBHQ also possess antimicrobial activity against bacteria, fungi, and viruses (Branen, Davidson et al. 1980).

Polyphenols are generally divided into flavonoids and nonflavonoids (Daglia 2012). Flavonoids share a common carbon skeleton of diphenyl propanes (Figure 2), two benzene rings joined by a linear three-carbon chain. More than 4000 flavonoids have been identified in fruits, vegetables, and plant derived beverages, such as tea and wine, and the list is constantly growing (Gruenwald, Brendler et al. 2004, Daglia 2012). These flavonoids can be further divided into many subclasses, which includes flavanols, flavones, flavanones, flavonols, tannins, and isoflavones (Figure 3) (Packer, Rimbach et al. 1999). Among polyphenols, flavan-3-ols, flavonols, and tannins have received the most attention due to their higher antimicrobial activity in comparison with other polyphenols, and because most are able to suppress a number of microbial virulence factors and show synergism with antibiotics (Daglia 2012). Flavanols are sub-classified

into proanthocyanidins (oligomers); and catechins and epicatechins (monomers) (Thompson, Jacques et al. 1972, Davidson, Sofos et al. 2005). The subclass of proanthocyanidins is the focal point of the study due to being the major subclass making up oligomeric polyphenols.

Proanthocyanidins

In addition to the monomeric flavanol groups, catechins and epicatechins, the major group of interest for this project is a subgroup proanthocyanidin group, procyanidins. Procyanidins can be further broken down into two basic structure types, procyanidin A and procyanidin B (Figure 4), characterized by the occurrence of a double or a single linkage respectively, connecting two flavanols units respectively (Slade, Ferreira et al. 2005). Procyanidins are found in fruits, bark, leaves, and seeds of many plants (Daglia 2012). Procyanidin type A and type B are found in peanut skin extracts (PSE) and grape seed extracts (GSE) respectively (Lee and Jaworski 1987, Monagas, Garrido et al. 2009). The biological properties of procyanidins in vivo are greatly dependent on their bioavailability, which at the same time is influenced by their chemical structure features, which can include the degrees of polymerization (DP) and overall structural composition (Manach, Scalbert et al. 2004). Discovering which type (A or B) of procyanidin possesses greater antimicrobial activity may aid both the food safety industry and further research of natural antimicrobials by revealing where future efforts should be made.

NATURAL EXTRACTS

Grape Seed Extract

Vitis, or grapes, are considered the world's largest fruit crop, with an approximate annual production of 58 million metric tons (FAO 1997). *Vitis* is native to southern Europe and Western Asia and contains several active components including flavonoids, polyphenols, anthocyanins, and procyanidins (Nassiri-Asl and Hosseinzadeh 2009). In the wine industry, the grape seeds are a waste product and disposed of either immediately in the case of white wine or after several days of contact with the grape juice in the case of red wine. The contents of grape seed extract (GSE) is high in monomers, dimers, trimers, tetramers, and pentamers, or oligopolymers (Bozan, Tosun et al. 2008, Terpéniques 2011). Ninety-seven percent of GSE is soluble in water at 37°C (Terpéniques 2008). The GSE used in this project was extracted from French white, sauvignon blanc, grapes from the southwest region of France (Terpéniques 2011). One of the ways GSE is created is when grape seeds are powdered and the fatty material is extracted in a Soxhlet extractor with petroleum ether (60-80°C) for 6 hours. The defatted brownish powder is then extracted with acetone:water:acetic acid (90:9.5:0.5) for 8 hours followed by being concentrated under vacuum to obtain crude extracts (Jayaprakasha, Selvi et al. 2003). The procyanidin composition of grape seeds is type B, which means there is a single bond linkage connecting the phenol base groups instead of two single bonds linkage (Lee and Jaworski 1987).

There have been studies that have examined the antimicrobial properties of GSE. Jayaprakasha *et al.* (2003) found GSE was more effective against Gram-positive bacteria than Gram negative. In his study, using the pour plate method and calculating the inhibition using the formula % Inhibition = (1-T/C)x100, where T=cfu/ml of test sample and C=cfu/ml of control, found the MIC for *S. aureus* to be 1000 ppm and *E. coli*

O157:H7 to be 1250 ppm (Jayaprakasha, Selvi et al. 2003). Baydar *et al.* (2004) found similar results with Gram-positive bacteria being more inhibited than Gram-negative bacteria at concentrations of 4% w/v powdered grape seeds using disc diffusion method. Sivarooban, Hettiarachchy et al. (2008) found *L. monocytogenes* to be more sensitive and was reduced one log CFU/ml after 1 h incubation at 25°C. *E. coli* O157:H7 and *S.* Typhimurium only showed 0.1 and 0.2 log CFU/ml reductions, respectively (Sivarooban, Hettiarachchy et al. 2008). Ahn, Grun et al. (2004) demonstrated GSE reduced the *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium by 1-logCFU/g in raw ground beef after 9 days of incubation at refrigerated incubation. However, the prolonged incubation time at refrigerated temperatures may have been major contributors to the increased reduction. GSE has also been seen to slow the growth of tumors by inducing insulin-like growth factor binding proteins (Singh, Tyagi et al. 2004, Akhtar, Meeran et al. 2009).

Peanut Skin Extract

Peanut skins are traditionally consumed as part of peanuts in many areas of the world without adverse effects and should qualify as a GRAS product if evaluated as a food additive (Yu, Ahmedna et al. 2010). Nuts are proven to be a source of antioxidants, polyphenols, and other phytochemicals such as phytosterols and carotenoids (Chen and Blumberg 2008, King, Blumberg et al. 2008). In nuts, the total polyphenols content accounts for 34-2052 mg/100g (Kornsteiner, Wagner et al. 2006). Several procyanidins have been identified in peanut skins and shown to have antioxidant activity (Lou, Yuan et al. 2004). Phenolic compounds in nuts are mainly located in the skin or testa, which is usually removed by blanching or roasting for the use of the kernel in the food industry

(Senter, Horvat et al. 1983, Milbury, Chen et al. 2006, Monagas, Garrido et al. 2007). Research has indicated that natural phenolic compounds can be extracted from peanut skins and hulls, which are low value byproducts of peanut roasting and blanching (Seo and Morr 1985). The procyanidin composition of peanut skins is type A, which means there are two single bonds connecting each monomer residue (Lou, Yamazaki et al. 1999, Yu, Ahmedna et al. 2005).

There is very limited work identifying that peanut skins possess antimicrobial properties. Vaughn (1995) found antifungal effects of 5,7-dihydroxychromone from peanut shells, not peanut skins. Yu *et al.* (2010) found PSE exhibited significant inhibitory effects on *E. coli* O157:H7, *S.* Typhimurium, and *S. aureus* at 0.1% w/w with MIC values determined to be 0.3% (w/w) in raw ground beef. Sarnoski *et al.* (2012) found PSE inhibited yeasts at low concentrations, reporting a minimum inhibitory concentration around 10mg/ml. Though these results may have led to an overestimation of antimicrobial susceptibility because the Bioscreen method does not account for injured cells since it relies on turbidity and not necessarily growth (Lambert, Johnston et al. 1998, Sarnoski, Boyer et al. 2012), this is a promising sign of the overall capabilities of PSE.

Polyphenolic Mode of Action

Polyphenolic compounds may have a common mode of action since they are similar in structure and share the ability to inhibit microorganisms. There are a variety of factors that influence how effective an antimicrobial may be on a particular microorganism, including the specific strain tested and the conditions the tests are run (Paster, Juven et al. 1990). Polyphenolics may exert antimicrobial activity by injuring

lipid membranes, which leads to leakage of intracellular contents (Davidson, Sofos et al. 2005). Polyphenols have been proven effective against a variety of bacteria, however Gram-positive bacteria tend to be more sensitive (Shelef 1984, Davidson, Sofos et al. 2005). Branen, Davidson et al. (1980) reported inhibitory effects of phenolic antioxidants against several bacteria, including *S.* Typhimurium, *S. aureus*, *E. coli* O157:H7, and *Clostridium perfringens*. The phenolic compound mode of action is likely concentration dependent, meaning that at lower concentrations, polyphenols may affect enzyme activity, especially if those enzymes are associated with energy production. Conversely, it is suggested that at greater concentrations polyphenols may cause protein denaturation (Viuda-Martos, Ruiz-Navajas et al. 2007). Polyphenols could also interact with membrane proteins, causing a deformation in its structure and functionality (Fung, Taylor et al. 1977). The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds of altering microbial cell permeability, leading to the loss of macromolecules from the interior (Davidson, Sofos et al. 2005).

Limitations

Many of the publications regarding the application of GSE as antimicrobials have been performed in model and laboratory systems with fewer done in a real food matrix (Board and Gould 1991). Generally, testing performed in food matrices has shown that antimicrobial efficacy is much higher in microbial media (Shelef 1984). This reduction in the effectiveness observed *in vivo* is a very important limitation in to the use of phenolic antioxidants as antimicrobial agents in food (Juven, Kanner et al. 1994). The interactions among phenolic groups and proteins, lipids, and aldehydes, could explain the reduction of

the antimicrobial effect of the extracts (Davidson, Sofos et al. 2005). Additionally, the antimicrobial potential of essential oils also depends on pH, temperature and level of microbial contamination.

Degrees of Polymerization

According to the Encyclopedia of Polymer Science and Technology, the degree of polymerization (DP) is defined as the average number of base units per molecule if the molecules are composed of regularly repeating units, or as the average number of monomeric units (mers) per molecule. The varying degrees of polymerization categories are defined as DP = 1 as monomers, DP = 2-10 as oligomers, and DP >10 as polymers (Gu, Kelm et al. 2002). These varying DPs can be separated by fractionation using normal-phase high performance liquid chromatography (HPLC) (Hammerstone, Lazarus et al. 1999). There has not been much research on GSE or PSE on the antimicrobial effects of varying DPs. However, chitosan, a polymer which is found in shrimp and crab shell chitin, research has shown a DP of less than four seems not to be biologically important; while a DP greater than six appears to be most active (Rabea, Badawy et al. 2003). The antibacterial effect has been shown dependent on the DP, with higher DP possessing the greater antimicrobial effect (Jeon, Park et al. 2001).

PATHOGENS OF CONCERN

***Escherichia coli* O157:H7**

Escherichia coli are Gram negative, rod shaped bacteria that are part of the Enterobacteriaceae family. *E. coli* O157:H7 was first recognized as a foodborne pathogen during an outbreak of hemorrhagic colitis associated with the consumption of raw hamburgers in 1982 (Riley, Remis et al. 1983). *E. coli* O157:H7 is a member of a group

of *E. coli* species called Shiga toxin producing *E. coli* (STEC). *E. coli* O157:H7 is the most commonly found shiga toxin-producing bacteria in North America and can cause complications such as hemolytic uremic syndrome (HUS) (Montville and Matthews 2008). Shiga toxin, the toxin produced by *E. coli* O157:H7 adds to the virulence of the pathogen. *E. coli* O157:H7 causes an estimated 63,000 foodborne infections and 20 deaths per year in the United States (Scallan, Hoekstra et al. 2011). In most cases, illness associated with this pathogen is relatively self-limiting (lasting between five and seven days) and includes acute symptoms such as abdominal cramping, fever, nausea, diarrhea and hemorrhagic colitis (bloody diarrhea) (Pai, Ahmed et al. 1988, Montville and Matthews 2008). *E. coli* O157:H7 is spread through the fecal-oral route and typically causes severe diarrhea and cramping. Outbreaks associated with the bacterium have been linked to contamination of meat products, juices, ciders, water supplies, and even cheeses (Montville and Matthews 2008).

***Salmonella enterica* serovar Typhimurium**

Salmonella enterica is a Gram negative, rod shaped bacterium that belongs to the non-spore forming portion of the Enterobacteriaceae family (Montville and Matthews 2008). It is responsible for approximately 42,000 reported cases and an estimated over 1 million cases of foodborne Salmonellosis and approximately 378 deaths in the United States every year (Scallan, Hoekstra et al. 2011). Ingestion of low levels of *Salmonella enterica* serovar Typhimurium (as few as 17 cells) typically produce physiological symptoms within 12-72 hours of consumption (Blaser and Newman 1982, Services 2012) The symptoms of Salmonellosis include abdominal cramps, diarrhea, and fever as soon as

eight hours after consumption (Montville and Matthews 2008). Outbreaks of *S. enterica* have been linked to peanut butter, fresh produce, meats, and juices (CDC, 2013).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive rod bacterium that is facultatively anaerobic. *L. monocytogenes* is responsible for nearly 1,600 foodborne infections and is responsible for an estimated 255 deaths per year in the United States (Scallan, Hoekstra et al. 2011). The organism typically does not produce symptoms in healthy individuals, however, listeriosis, which is the disease cause by *L. monocytogenes*, can cause serious illness to at risk populations, including newborn infants, pregnant women, the elderly, and the immunocompromised (Montville and Matthews 2008). Patients who have contracted Listeriosis, present with symptoms of high fever and muscle aching. In extreme cases, the pathogen may cross the blood-brain barrier, infect the meninges, and cause meningitis encephalitis (Gaillard, Berche et al. 1991). The organism is also capable of crossing the placental barrier of pregnant women, leading to prenatal infections and abortion (during the second and third trimester) (Services 2012). *L. monocytogenes* is a psychrotroph and is capable of growth between temperatures of 0 to 45°C but grows slower at colder temperatures. Outbreaks have been linked to contamination of ready-to-eat-foods, meats, dairy products, vegetables, juices, and fruit (Montville and Matthews 2008).

Staphylococcus aureus

Staphylococcus aureus is Gram-positive cocci shaped bacterium that produces a very heat stable enterotoxin. Onset of symptoms can be as quick as 30 minutes after consumption of the preformed toxin. Symptoms of the food poisoning include vomiting,

nausea, cramps, diarrhea, and headaches (Montville and Matthews 2008). Remission of these symptoms usually occurs after 24 hours. In extreme cases, toxic shock syndrome and sepsis have been known to occur (Le Loir, Baron et al. 2003). Staphylococcal food poisoning is responsible for approximately 241,000 foodborne illnesses and six deaths in the United States each year (Scallan, Hoekstra et al. 2011). Outbreaks have been like to contamination of meats, poultry, delicatessen salads, milk and dairy products, and bakery products. The toxin is very resilient since it is stable at 100°C for over one hour at sea level, which makes it very difficult to destroy during thermal processing (Montville and Matthews 2008).

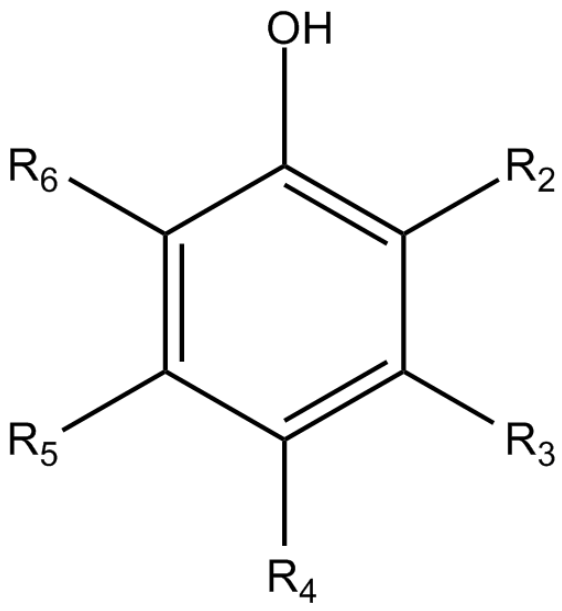


Figure 1. Phenolic base derivative

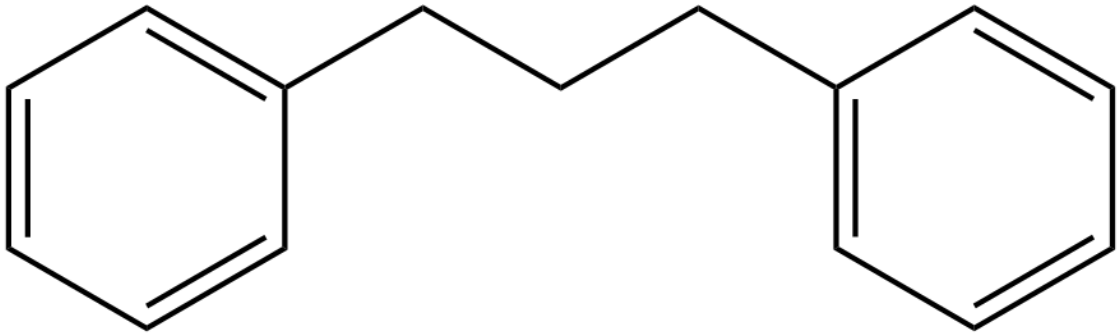


Figure 2. Diphenylpropane backbone

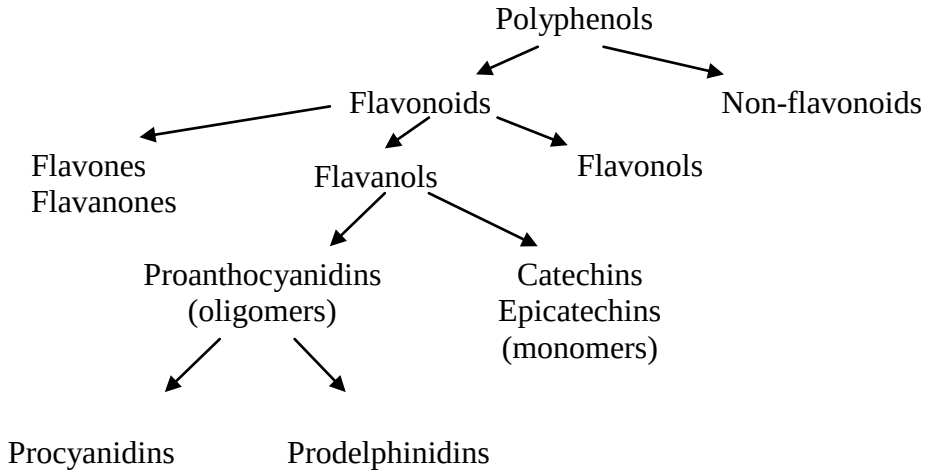
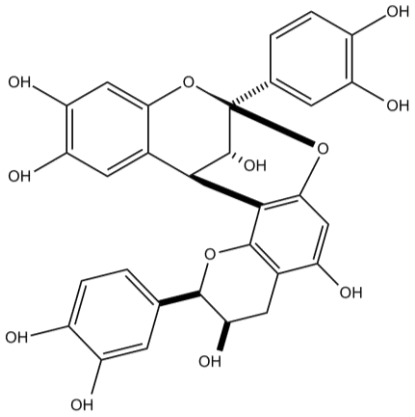
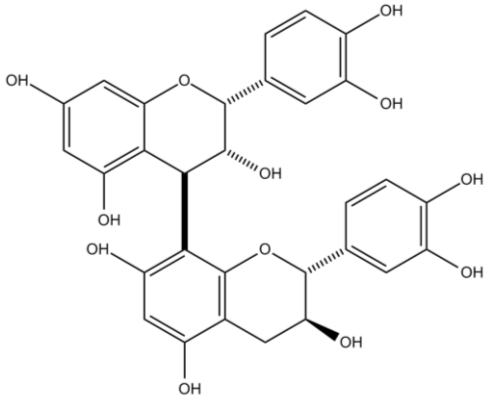


Figure 3. Polyphenol compound hierarchy



Type A



Type B

Figure 4. Example of type A and type B procyanidins

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CHAPTER 3

Evaluation of Peanut Skin Extract, Grape Seed Extract, and Grape Seed Extract Fractions to Reduce Populations of Select Foodborne Pathogens

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Key Words: Foodborne pathogens, antimicrobials, Grape Seed Extract, Peanut Skin Extract, standard agar dilution method

ABSTRACT

Grape seed extract (GSE) and peanut skin extract (PSE) are waste products in the wine and peanut industries. Both extracts have high concentrations of polyphenols, known to possess antioxidant and antimicrobial properties. A subcategory of polyphenol is procyanidin, which can be divided into two types, type A and type B. Type A (PSE), contains two single bonds connecting the phenolic groups while type B (GSE), contains one single bond connecting the phenolic groups. The minimum inhibitory concentration (MIC) of the two extracts was evaluated for their antimicrobial effect on *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium using the pour plate method. GSE was found to have a significantly lower MIC ($p \leq 0.05$) than PSE for *L. monocytogenes* (GSE=60.60ppm, PSE=not found), *S. aureus* (GSE=38.63ppm, PSE=51.36ppm), and *S.* Typhimurium (GSE=45.73ppm, PSE=60.60ppm). There was no significant difference in inhibition of *E. coli* O157:H7 (GSE=47.44ppm, PSE=51.13ppm). Since GSE, contributed to greater pathogen inhibition, its extract was fractionated into monomer and oligomers components. GSE monomers consist primarily of catechins, epicatechins, and epicatechin gallates; and oligomers consist of dimers, trimers, tetramers, up to decamers. Growth curves of all four pathogens inoculated in the monomer and oligomer fractions were compared using the BioScreen method. Oligomers inhibited growth of *L. monocytogenes*, *S. aureus*, and *E. coli* O157:H7 while monomers inhibited growth of *S.* Typhimurium. These two extracts were evaluated to see if type influenced antimicrobial activity. These results indicate that an extract with type B procyanidins that are high in oligomers may be more effective as

antimicrobials. Type B procyanidins have also been shown to prevent bacterial adhesion, as is the case with urinary tract infections, and may aid in the prevention of biofilms.

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimate more than 48 million illnesses due to foodborne illnesses annually in the United States (CDC, 2011). Some of these outbreaks were caused by *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium (CDC, 2011). Traditionally, foods are thermally processed and/or preserved with chemical compounds such as sodium benzoate, nitrites, sodium meta-bisulfate, and others that are generally recognized as safe (GRAS) to prevent spoilage organisms and pathogens from growing. There has been a push from consumers to replace these compounds with more naturally–derived compounds for use in food commodities (Jayaraman, Sakharkar et al. 2010). This emphasis on what is perceived as "healthier", and "safer" food ingredients has led to the exploration and discovery of antimicrobial compounds from natural sources (Aoki, Shen et al. 2010).

Two compounds that have received some attention in recent years are Grape Seed Extract (GSE) and Peanut Skin Extract (PSE). One of the benefits to examining these two products is that they are considered waste products by the industries that produce their parent products, grape juice/wine and peanuts (Shi, Yu et al. 2003). Identifying value in these products will result in less waste, complete utilization of raw products, and provide an all-natural alternative to synthetic preservatives. These extracts are high in a compound called polyphenols, which is a major contributor to antioxidant and antimicrobial properties (Daglia 2012).

Grape (*Vitis vinifera*) seeds contain several active components including flavonoids, polyphenols, anthocyanins, and procyanidins (Figure 1) (Nassiri-Asl and

Hosseinzadeh 2009). The procyanidin composition of grape seeds is type B, which means there is a single bond linkage connecting the monomer residues (Lee and Jaworski 1987). The antimicrobial properties of GSE have been preliminarily examined. Generally, GSE is more effective at reducing growth of Gram-positive bacteria than Gram negative (Jayaprakasha et al., 2003). Concentrations of GSE at 1200 and 1000 ppm reduced *L. monocytogenes* by 9-logs over 12 hours incubated at 37°C and 1.5 logs after incubation at 37°C respectively (Theivendran et al., 2006, Sivarooban et al, 2007).

Alternatively, the procyanidin composition of peanut skins is type A, which means there are two single bonds connecting each monomer residue. Peanut skins are traditionally consumed as part of peanuts in many areas of the world without adverse effects and would qualify as a GRAS product (Yu, Ahmedna et al. 2010). Nuts are a source of antioxidants, polyphenols, and other phytochemicals such as phytosterols and carotenoids (Lou, Yamazaki et al. 1999, Yu, Ahmedna et al. 2005, Chen and Blumberg 2008, King, Blumberg et al. 2008). There has been very limited work performed evaluating the efficacy of PSE on pathogenic bacteria. However, Yu *et al.* (2010) found PSE exhibited significant inhibitory effects on *E. coli* O157:H7, *S.* Typhimurium, and *S. aureus* in raw ground beef at 0.1% w/w with MIC values determined to be 0.3% (w/w).

Procyanidins (the focus of this study) are made up of monomers (1 unit), oligomers (2-10 connected monomeric units), and polymers (10+ connected monomeric units). In many cases, the oligomer form is included into the polymer group, with polymers being any compound made up of more than one monomer. Procyanidin monomers consist primarily of catechin and its derivatives, while polymers include dimers, trimers, hexamers, etc. of basic catechin compounds (Manach, Scalbert et al.

2004). The amount of monomers, oligomers, and polymers that are present in a procyanidin are averaged to calculate a degree of polymerization (DP), with a higher DP indicating more polymeric structures versus monomeric structures (Bruice 2004).

The objective of this study was to assess the antimicrobial effects of PSE (type A procyanidin) and GSE (type B procyanidin) against select foodborne pathogens (*Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* O157:H7, and *Salmonella* Typhimurium). The extract with the greatest inhibition against the organisms tested were then fractionated into monomers and oligomers. The fraction with the greatest inhibition of the four pathogens will be determined.

Ultimately, identifying which component of the procyanidin-rich polyphenol is most effective may inform researchers on the factor that contributes the most to antimicrobial properties. This may help with the selection of other natural extracts using their compound profiles to determine which ones will have the best potential.

MATERIALS AND METHODS

Preparation of Inoculum

To activate cultures prior to each experiment, one loopful of each stored culture from the TSA or TSAYE slants was transferred to tubes of 10ml TSB or TSBYE and incubated at 37°C for 24 hours. Cells from each tube were then centrifuged at 1192 g-force for 10 minutes. The liquid media was decanted, and the cells were resuspended in 10ml of sterile 0.1% peptone. The cells were centrifuged once more at 1192 g-force for 10 minutes. The liquid was decanted once more and the cells were re-suspended in peptone water.

Preparation of extracts:

Extracts were prepared prior to each experiment. The GSE used was a commercially-available extract (Vitaflavan®) and purchased from Les Dérivés Résiniques & Terpéniques (Dax, France).

The PSE was prepared from crude peanut skins donated by Seabrook Ingredients, Edenton, NC. Methodology followed that of Adamson, Lazarus et al. (1999) and Robbins, Leonczak et al. (2012) with modification. Briefly, 50g of peanut skins were weighed and placed in a strainer and shaken over a garbage can until the dust was gone. The peanuts were then transferred to a 1000ml beaker and hexane (approximately 750ml) was added to cover all of the peanut skins. The hexane and peanut skins were mixed thoroughly with a spatula for two minutes and sonicated (FS20H, Fisher Scientific, Hampton, NH) for ten minutes. The hexane containing the fat was decanted and discarded. This hexane washing process was completed two more times. After the final

sonication, the remaining hexane was allowed to evaporate off the peanut skins under the hood for 24 hours.

The weight of the peanut skin sample was recorded and a mixture of 70:28:2 (v/v/v) of acetone:water:acetic acid was added to the peanuts in a 1000ml beaker, enough to cover the peanuts (approximately 400ml). The peanut mixture was sonicated for 10 minutes and the supernatant was placed in a filtered stomacher bag (to aid in the separation of the solids and liquids). The supernatant was then transferred to centrifuge tubes. The peanut skins were added to the stomacher bag with 40ml of the 70:28:2 mixture and was stomached for two minutes. The liquid was transferred to enough 50ml centrifuge tubes to accommodate all the supernatant while the peanut skins remained in the stomacher bag. The process of adding of acetone:water:acetic acid, peanut skins, and stomaching was repeated three more times. After the supernatant was collected, it was centrifuged at 4000 rpm for 20 minutes. The supernatant was then transferred to 1000ml Rotovapor flasks (approximately 500ml into each flask). The contents were placed in the Rotovapor (RV 10, IKA, Wilmington, NC) at 37-40°C, 100 rpm with a vacuum of 20 psi for 3-4 hours (until boiling stopped, indicating the removal of the acetone fraction). The remaining liquid (predominately water) was placed into plastic cups and frozen for 12 hours. The frozen extract was then placed into a 1000ml beaker. The contents were freeze dried for 2-3 days on a FreeZone 1L freeze drier (Labconco, Kansas City, MO).

Determining Minimum Inhibitory Concentration of GSE and PSE

The methods used in this study were similar to those described in Jayaprakasha *et al* (2003). To assess the antimicrobial effect of each concentration of extract the agar dilution method was used.

One loopful of *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium was separately added to 10 ml tubes of TSB and incubated at 35°C for 24 hours. Additionally, one loopful of *L. monocytogenes* was added to a 10 ml tube of TSBYE and incubated at 35°C for 24 hours. After 24 hours, each pathogen was serially diluted using 9 ml 0.1% peptone water blanks to reach a final concentration of 10^5 cfu/ml.

Quantities of GSE and PSE were dissolved in 100 ml of sterile DI water to reach final concentrations of 11.36, 22.72, 34.09, 38.64, 40.91, 45.45, 56.82, and 68.81ppm (Table 1). One ml of each concentration of GSE or PSE was dispensed onto sterile petri dishes and 20 ml of molten TSA was added (40-42°C). 100 µl of each pathogen solution was dispensed onto duplicate sterile petri dish plates (final concentration of 10^4 cfu/ml) for all concentrations of GSE and swirled for 20 seconds to mix thoroughly. These plates were incubated at 35°C for 24 hours. Likewise, *L. monocytogenes* was incubated at 35° C for 48 hours. A negative control sample was prepared by pour plating 20 ml of molten TSA with the pathogens. A positive control of 22.72 ppm trans-cinnamaldehyde was used (Mahmoud 1994). After the allotted time, the plates were observed for the presence or absence of growth. The MIC was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium tested. The experiment was performed in triplicate.

Fractionation of GSE

GSE was fractionated according to methods described by Dorenkott, Griffin et al. (2014) with modification. Briefly, Waters tC18 and C18 Sep-pak columns (20cc, 5g Milford, MA) were connected by Waters adapters to form 10 total 2-stage columns. Each column was preconditioned by running 10ml methanol through the columns followed by

10ml of DI water. The eluents were discarded after each conditioning step. 3g of GSE were dissolved in 15ml of 70:28:2 acetone:DIwater:acetic acid solution by sonicating for 15 minutes. 1.5ml of the GSE solution was loaded onto each of the 10 columns with the use of a vacuum and the elutents were discarded. Approximately 40ml of diethyl ether were pulled through the columns, via vacuum, and the monomer eluents were collected and placed in a RotaVap flask. The step of the addition of diethyl ether was performed two more times and then the eluents were Rotavapped until most nearly all of the liquid was gone. Approximately 40ml of methanol were pulled through the columns, via vacuum, and the oligomer eluents were collected and placed in a RotaVap flask. The step of the addition of methanol was performed two more times and then the eluents were Rotavapped until most nearly all of the liquid was gone. Approximately 25ml of DI water was added to each RotaVap flask in order to thoroughly remove the contents into separate beakers. These mixtures were frozen and placed on the freeze dryer for 2 days.

Determination of which Degrees of Polymerization Contributes to Inhibition

The oligomer and monomer fractions were diluted to 50% the GSE MIC values for each pathogen found in this study. The concentrations used were 650ppm, 425ppm, 500ppm, and 500ppm for *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium, respectively. A portion, 100µl or 150µl, of the fractionated GSE were placed in each well on a honeycomb 100 well plate. Each well was then inoculated with 100µl pathogen (1:1 treatment:pathogen) or 50µl pathogen (1:3 treatment:pathogen), respectively, for a total volume of 200µl/well. The oligomer fraction was placed into wells 1-40 (10 wells for each pathogen) with 20 wells being filled with 100µl of the fraction and the other 20 wells being filled with 50µl of the fraction. The monomer

fraction was used for wells 41-80 (10 for each pathogen) with 20 wells being filled with 100µl of the fraction and the other 20 wells being filled with 50µl of the fraction. Unfractionated GSE was used in wells 81-88 (2 wells of each pathogen while wells trans-cinnamaldehyde was used in wells 91-95. The negative controls were wells 96-100 and filled with TSB and the respective pathogen. See Table 1 in appendix for setup parameters. The plate was read in a BioScreen Growth Curve machine (Growth Curves USA, Piscataway, NJ) for 48 hours, being shaken and read every 15 minutes at 35°C. There were 5 wells per treatment.

Ultraperformance Liquid Chromatography-Mass Spectrometer Analysis

UPLC separations were performed to confirm the monomer and oligomer fractions were enriched for procyanidin monomers and the oligomers, respectively based on the method of (Dorekott *et al*. 2014) fraction consisted of oligomers. UPLC was performed on a Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm particle size). The column temperature was set to 40°C, and the samples were maintained at 10°C. The binary mobile phase system was comprised of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in ACN (phase B). Solvents for UPLC0MS/MS were LC-MS grade (VWR, Radnor, PA). The system flow rate was 0.6 mL/min. Elution was performed based on the following linear gradient: 95% A at 0 min held until 0.5 min, 65% A at 6.5 min, 20% A at 7.5 min held until 8.75 min, 95% A at 8.85 min held until 10.0 min. MS/MS analysis of column effluent was performed by (−)-electrospray ionization (ESI) on a Waters Acquity TQD (triple quadruple) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.25 kV,

and the source and desolvation temperatures were 150°C and 400°C, respectively. The desolvation gas and cone gasses were N_2 at flow rates of 900 L/hr and 75 L/hr, respectively. The MS/MS collision gas was Ar. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points/peak with an average peak width of 6 s. The auto-dwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s for each transition. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest (Table 2). Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μl/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min. Intellistart automatically selected the most abundant daughter ion, optimized the source cone voltage and MS/MS collision energy, and generated a single MRM transitions for each compound.

Quantification

All compound peaks were processed and quantified using the TargetLynx function of MassLynx software. Quantification parameters for native compounds are shown in Table 2. Compounds were quantified based on external standard curves of authentic standards; compounds for which authentic standards were not available were quantified based on external standard curves of similar compounds. Peaks between the lower limits of quantification and detection (LLOQ and LLOD) were quantified and included in the data, but means containing values in this region are indicated in the data table. Peaks below the LLOD were not quantified and were included in the data as concentrations of 0.

Total Phenolics Count

PSE and GSE were diluted to 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 g/L water. This was done by mixing 5mg extract with 50 ml water (PSE 25ml of acetone was added). The standard was compared with gallic acid with the same concentrations. 100 µl aliquots of samples were separately mixed with 2.5 ml 0.2 N Folin-Ciocalteau reagent and vortexed. Then, 2.0 ml saturated sodium carbonate was added and mixed on the vortexer. These solutions were allowed to sit for 2 hours after which they were read by the spectrophotometer at 765 nm. Each concentration was done in duplicate.

Statistical Analysis

The growth of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium in the varying concentrations of GSE and PSE was performed in duplicate on four separate days. MIC values for each respective bacterium were recorded based on presence or absence of growth. Error bars were created using standard deviation. All BioScreen points were recorded and plotted on Microsoft Office Excel 2007 (Microsoft, Redmond, WA). Microsoft Office Excel 2007 was also used to calculate the area under the curve for each compound. Significant differences were analyzed with ANOVA, Tukey-Kramer HSD method, and Chi Squared method on JMP Pro 10 (SAS Institute Inc, Cary, NC) with p-values < 0.05.

All BioScreen points were recorded and plotted on Microsoft Office Excel 2007 (Microsoft, Redmond, WA). Microsoft Office Excel 2007 was also used to calculate the area under the curve for each compound. Significant differences between the areas under the curve were analyzed with ANOVA and Tukey-Kramer HSD method on JMP Pro 10 (SAS Institute Inc, Cary, NC).

Regression was conducted in Microsoft Excel between absorbance vs. concentrations of standard solutions. The results from the each concentration from each sample were averaged and compared to the standard solution and a percentage was calculated.

RESULTS

Confirmation of Bacterial Cultures

Each of the bacterial cultures was confirmed on solid media. Confirmation of *E. coli* O157:H7 on Sorbitol MacConkey agar plates was positive as indicated by growth of colorless colonies. Growth on the XLD-4 agar plates was positive (black colonies indicating H_2S production) for the presence of *S. enterica*.. Growth on the Modified Oxford agar plates was positive (media became black with colony growth) for the presence of *L. monocytogenes*. Growth on the Baird Parker agar plates was positive (black colonies with zone of clearing) for the presence of *S. aureus*.

Minimum Inhibitory Concentration of GSE and PSE

Throughout the experiment, the negative control plates (inoculated TSA with no extract) had a lawn of growth. The positive control plates (inoculated TSA with 500 ppm trans-cinnamaldehyde) had no apparent bacterial growth. GSE and PSE both solublized easily in water.

MIC values can be seen in Figure 1. There were no significant differences ($p = 0.26$) when comparing the inhibition of *E. coli* O157:H7 between PSE (MIC = 51.13) and GSE (MIC = 47.44). There was significantly more inhibition of *L. monocytogenes* ($p=0.0005$), *St. aureus* ($p=0.01$), and *Sa*. Typhimurium ($p=0.01$) with GSE than PSE. The pH of GSE was 6.6 and 5.6 for concentrations of 11.36ppm and 68.18ppm respectively. The pH of PSE was 6.5 and 5.9 for concentrations of 11.36ppm and 68.18ppm respectively. Since GSE was the more inhibitory of the two extracts evaluated, all further experiments were completed with GSE. Chi Square analysis was able to reject GSE and

PSE being linked (*Sa.* Typhimurium<0.02, *L. monocytogenes*<0.01, *St. aureus*<0.04) except for *E. coli* O157:H7 (probability>0.40).

Compound Profile of Grape Seed Extract Fractions

The UPLC compound profiles of the GSE found high amounts of catechin (0.06811 mg/mg fraction), epicatechin (0.06265 mg/mg fraction), and epicatechin gallate (0.01870 mg/mg fraction) present in the monomer fraction. The GSE oligomer fraction contained high amounts of dimer B1 (0.02260 mg/mg fraction), dimer B2 (0.01530 mg/mg fraction), and dimer B2-gallate (0.01332 mg/mg fraction). Complete results can be found in Table 3.

Grape Seed Extract Monomeric and Oligomeric Effects on Bacterial Growth

The BioScreen provided an optical density reading every 15 minutes, however, the results are expressed in five-hour increments since such minute changes were seen over 15 minute increments. The areas under the curve were calculated for each curve produced from each fraction/compound combination (Table 4). The smaller the area, the more inhibition from the compound. The largest area under the curve was always the negative control, except for *E. coli* O157:H7, where the largest area under the curve was oligomer 1:3 for each pathogen. For every pathogen, oligomer 1:1 was significantly better at slowing growth than the negative standard, except for *L. monocytogenes*. Likewise, oligomer 1:1 was statistically better than oligomer 1:3 fractions for every pathogen. The monomer 1:1 fraction for *S.* Typhimurium had a very small area of growth, significantly smaller than any other treatment. Similarly, this phenomena also happened for the oligomer 1:1 fraction of *E. coli* O157:H7, where it was drastically lower than the other treatments.

Total Phenolics Count

Grape seed extract was found to contain an average of 96% total phenolics compared to peanut skin extract, which contained 76% total phenolics (Figure 2).

DISCUSSION

Effect of GSE on Four Foodborne Pathogens

S. aureus had the largest definitive difference between MIC values for GSE and PSE. Studies have looked at the efficacy of GSE for pathogenic inhibition and for pathogenic death. Theivendran, Hettiarachchy et al. (2006) looked at the lethality of GSE and found at a concentration of 1200 ppm had a 9-log reduction over twelve hours against *L. monocytogenes*. Though this study looked at inhibition, this study found GSE to be effective in preventing growth at a similar concentration (MIC=60.60 ppm). Another study looking at bacterial death found that *L. monocytogenes* was reduced by approximately 1.5-logs after 24 hrs of incubation at 37°C with a concentration of 1000 ppm GSE (Sivarooban, Hettiarachchy et al. 2007). This, again, is a similar concentration to invoke activity in this study. Ahn, Grün et al. (2007) found at a concentration of 1000 ppm GSE, *L. monocytogenes* increased after 3 days of refrigerated storage, which could be due to the organism's psychrotrophic nature. This study only looked at GSE at 1000 ppm, which is far lower than the concentrations believed to be effective.

Complete inhibition of *S. aureus* with GSE at 38.63 ppm has been reported, which is the same result found in the current study (Jayaprakasha, Selvi et al. 2003). The structural activity of correlation assays revealed that the three hydroxyl groups of the polyphenolic compounds were effective for antibacterial activity and all the substituents of the benzene rings were effective against *S. aureus*. The biggest differences in MIC values were found to be the Gram-positive bacteria, which suggests they are more susceptible to the treatments.

Previously reported MIC values for *E. coli* O157:H7 have also been 47.44 ppm which correlates well with this study (MIC=1000 ppm) (Jayaprakasha, Selvi et al. 2003). Another study, looking at bacterial death, found a more than 1-log reduction in *E. coli* O157:H7 after 3 days of refrigerated storage with 1000 ppm GSE (Ahn, Grün et al. 2007). This study saw activity at the same concentration. The trend of GSE having a lower MIC (GSE=45.73 ppm) value than PSE remains true for *S.* Typhimurium. Again, Ahn, Grün et al. (2007) found bacterial death of more than 1-log reduction in *S.* Typhimurium after 3 days of refrigerated storage with 1000 ppm GSE.

Effect of PSE on Four Foodborne Pathogens

In the current study, PSE exhibited no inhibition against *L. monocytogenes* at the highest concentration of 68.18 ppm. To date, no other studies have looked at the effects of PSE on *L. monocytogenes*. PSE was found to be effective against *S. aureus* with complete inhibition at a concentration of 0.3% w/w (or 3000 ppm) which contradicts the results of this study (MIC=51.36 ppm) (Yu, Ahmedna et al. 2010). The use of the microplate assay method may be the reason for the differences in the MIC values since they looked at levels 1000ppm, 2000ppm, 3000ppm and 4000ppm (Yu, Ahmedna et al. 2010).

PSE has been reported to be active against *E. coli* O157:H7 and *Sa*. Typhimurium, resulting in complete inhibition, at a concentration of 0.3% w/w (or 3000ppm) which is a concentration much higher than evaluated in this study. Much lower MIC for *E. coli* O157:H7 and *Sa*. Typhimurium were found in this study (51.13 and 60.60 respectively) (Yu, Ahmedna et al. 2010). The use of the microplate assay method may be the reason for the differences in the MIC values since they looked at

levels 1000ppm, 2000ppm, 3000ppm and 4000ppm (Yu, Ahmedna et al. 2010). Further studies must be performed to see if there is a significant difference between these two extracts.

For each pathogen, the type B procyanidin, GSE, performed better than the type A procyanidin, PSE. Type B procyanidins only contain one single bond between each monomer residue whereas type A procyanidins have two single bonds between each monomer residue. The availability of the chains in the type B procyanidin may allow for more hydroxyl groups that may interfere with the cellular membrane and metabolic processes. The openness may also allow for less intercellular charges that may lead to more interactions with the environment. Performing the exact same methodologies simultaneously leads to very little variation in conditions and an accurate comparison of the two extracts. A possible scenario where an extract high in type A procyanidins may perform better than an extract high in type B procyanidins would be on biofilms on food contact surfaces within a processing plant. Cranberries, which are high in type B procyanidins, are consumed to help prevent urinary tract infections since they prevent the attachment of the bacteria to the lining of the urinary tract. The analysis of total phenolic count revealed that GSE contained almost 25% more total phenolics than PSE did per weight. This could be another factor that was not incorporated when calculating the concentrations of each extract.

Composition of GSE Fractions:

The UPLC results indicate that the monomer-rich fraction consisted of catechin, epicatechin, and epicatechin gallate. There were negligible amounts of oligomer compounds in the monomer-rich fraction. The oligomer-rich fraction consisted of mostly

dimer B1, dimer B2, and dimer B2-gallate, as well as trace amounts of catechin, epicatechin, and epicatechin gallate. These results confirm the technique to separate the GSE monomers and GSE oligomers was successful. The profile of GSE provided by Les Dérivés Résiniques & Terepéniques indicates that it contains approximately 25% monomers and approximately 45% dimers and trimers for a total of approximately 70% of lower degrees of polymerization (Terepéniques 2009). Since GSE has a negligible amount of polymers, the extract for this study was only fractionated into monomers and oligomers. The UPLC results were consistent with the information provided by Les Dérivés Résiniques & Terpéniques.

Oligomer fractions appeared to inhibit pathogenic growth better than monomer fractions for the four pathogens. This may be because there are more branches for one compound to disrupt the bacterial cell membrane. Studies have found the main mode of action for oregano essential oil to be damage in membrane integrity (Lambert, Skandamis et al. 2001). Overall, the 1:1 ratio for both oligomers and monomers performed better; statistically better for *S. aureus* and *S.* Typhimurium. This difference is most likely due to the 1:1 ratio being closer to the original concentration. However, the positive standard was never significantly better than the negative standard. Cinnamaldehyde, the positive standard, is a much darker compound than the plain broth, which may have led to an inaccurate reading in the BioScreen. The BioScreen measures the amount of light can pass through the well over time. This can skew the results since the BioScreen cannot account for bacterial cell that dies since their cells do not get broken down. Prior to each reading, the wells were shaken lightly to make spread the cells throughout the well;

however, the cells tend to stick to one another at the bottom of each well. This is another limitation of the BioScreen method that may contribute to discrepancies in results.

This shows that having a higher oligomer concentration present in an extract will lead to better inhibition of bacterial growth. The size and number of branch derivatives oligomers possess may be the biggest factor contributing to the capabilities of GSE as an antimicrobial. Osorio, Flores et al. (2010) found the monomer and oligomer fractions from pecan nut shells, pomegranate husks, and creosote bush leaves to be very effective against plant pathogenic fungi. Though this does directly correlate with the research conducted in this study, it shows that individual fractions can thwart growth of pathogens.

FUTURE DIRECTIONS

For future research, the GSE fractions, which were evaluated via BioScreen, need to be evaluated using the same technique as the crude extract, standard agar dilution method. Other techniques, such as spiral gradient endpoint, could be used to compare the results against the standard agar dilution method conducted in this study. These tests will allow for a more accurate comparison and will help remove the biases inherent in comparing different tests. More pathogens should be tested against such as *Campylobacter* and *Clostridium* species. Testing should also be done for viruses like Hepatitis A and Norovirus. For this experiment, the compounds were concentrated by weight and not by per mole basis. How many total monomer compounds were present versus oligomer compounds? Now that there is a better idea of the percentage of compounds presents in the fractions, there needs to be research looked at on a mole-to-mole basis. More type B extracts that are high in oligomers need to be tested of its efficacy against pathogens. There needs to be more studies done testing GSE in food matrices, perhaps sprays for produce or in juices since it is water soluble. Along with this, sensory testing should be done to see the effects the extracts will have on the foods. There has been, and will continue to be, research conducted in search of natural antimicrobials to stand alone and aid in hurdle technology. There are a few antibiotic resistant strains of *S. aureus*, where GSE and PSE should be tested to see how effective they are as antibiotics.

Table 1. Concentration measurements for GSE and PSE in sterile DI water

Concentration (PPM)	W/W (g/100g DDi water)
11.36	0.025
22.72	0.050
34.09	0.075
38.64	0.085
40.91	0.090
45.45	0.100
56.81	0.125
68.18	0.150

Table 2. Mass Spectrometer Settings for Detection of Native Monomers and Procyanidins

Compounds	t_R[a] (min)	MW (g mol^{-1})	$[M - H]^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
procyanidin dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[c]	2.92	578.136	577.136	425.102	36	16
(+)-catechin	2.99	290.028	289.028	245.057	36	14
procyanidin trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[c]	3.29	578.136	577.136	425.102	36	16
procyanidin dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
procyanidin trimer C_1	3.82	866.218	865.218	287.085	46	32
cinamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
procyanidin dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
procyanidin octamers	4.04	2307.17	1152.58	125.17	48	68
unknown dimer 3[c]	4.07	578.136	577.136	425.102	36	16
procyanidin pentamers	4.10	1442.820	720.410	125.022	26	44
procyanidin hexamers	4.23	1731.038	864.519	125.020	32	56
procyanidin nonamers	4.33	2586.36	864.12	125.17	28	46
procyanidin heptamers	4.41	2018.80	1008.40	125.17	36	56
(−)-epicatechin gallate	4.60	442.076	441.076	168.968	38	18
procyanidin decamers	4.60	2883.55	960.18	125.17	30	52
procyanidin dimer B_5	4.64	578.136	577.136	289.107	30	26

[a]Retention time

[b]m/z values represent monoisotopic masses detected by Intellistart; all MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M - 2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M - 3H]^{3-}$)

[c]Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

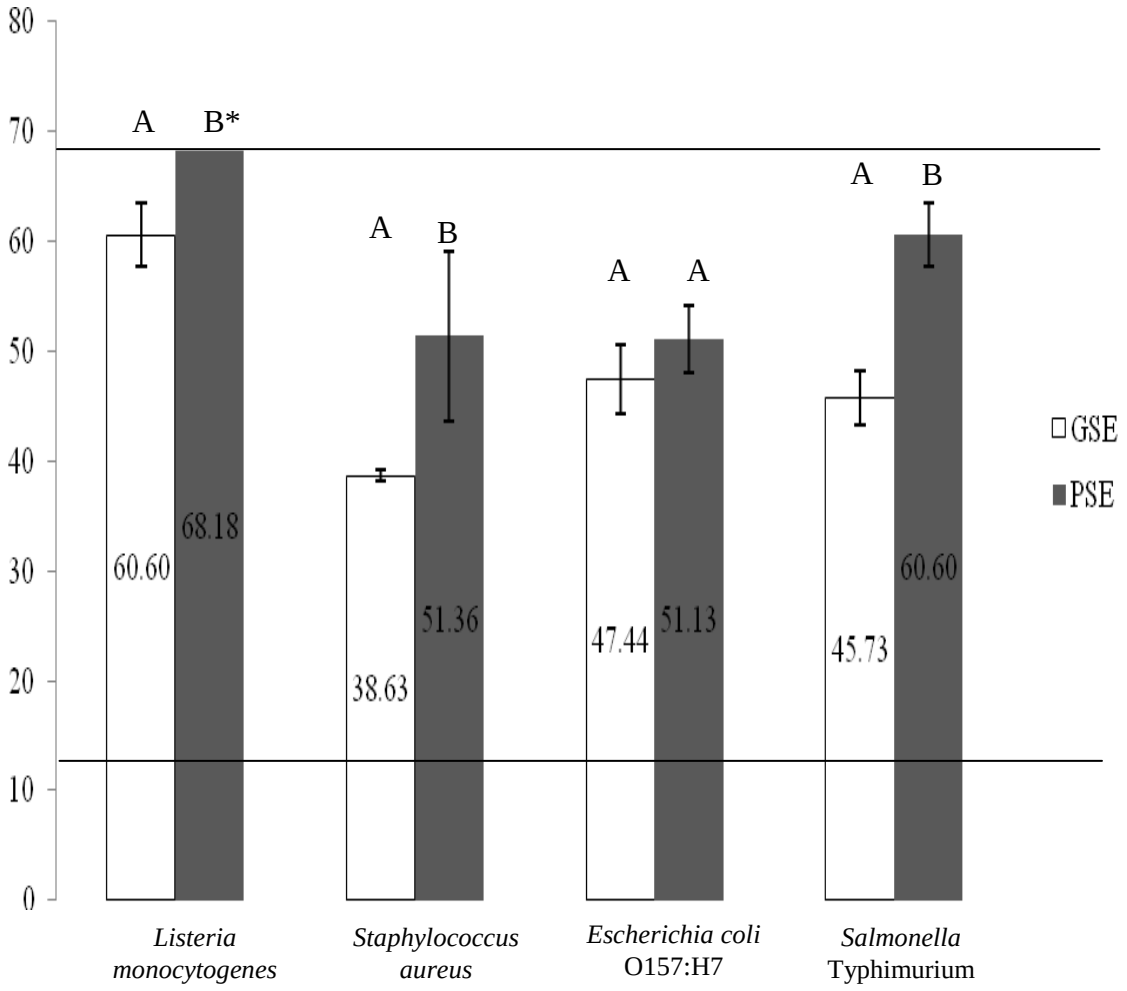

Figure 1. MIC values for comparison GSE and PSE for *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium at 35°C. Error bars represent standard deviation.

Lines indicate the highest and lowest concentrations tested
*Indicates there was no inhibition at highest concentration tested.

Table 3. Polyphenol profile for the monomer and oligomer fractions (µg/mg fraction)

Compound	GSE Monomer Fraction	GSE Oligomer Fraction	Monomer:Oligomer Ratio
Catechin	68.11	2.42	28.14
Epicatechin	62.65	3.81	16.44
Epicatechin Gallate	18.70	0.28	66.79
Epigallocatechin Gallate	0.20	0.02	10.00
Dimer B1	0.75	22.60	0.03
Dimer B2	0.79	15.30	0.05
Dimer B5	1.92	6.77	0.28
Dimer B2-gallate	1.62	13.32	0.12
Unknown Dimer 1[B]	0.36	6.38	0.06
Unknown Dimer 2[B]	0.32	4.54	0.07
Unknown Dimer 3[B]	1.62	2.23	0.73
Tetramer A2	6.97	11.46	0.61
Trimer C1	nd*	2.55	undefined
Trimer T2	4.16	0.13	32.00
Pentamer	nd*	0.39	undefined
Hexamer	nd*	2.69	undefined

*Not detected

[A] Denotes the bond location where the monomers are connected

[B] Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Table 4. Area under the curve from BioScreen for *L. monocytogenes, S. aureus, S.* Typhimurium, and *E. coli* O157:H7.

Compound	LM	SA	ST	EC
Olig 1:1	31.59±1.84^{B}	22.00±3.17^{C}	30.01±0.85^{C}	12.83±3.26^{C}
Olig 1:3	36.40±1.37^{A}	30.43±2.79^{B}	39.24±1.03^{A}	27.32±2.45^{A}
Mono 1:1	32.91±0.83^{B}	36.12±1.14^{A}	5.90±3.37^{D}	20.10±1.10^{B}
Mono 1:3	34.50±0.75^{B}	28.30±3.19^{B}	39.61±0.58^{A}	19.57±2.22^{B}
GSE	37.64±0.56^{A}	32.48±0.92^{AB}	32.30±0.67^{BC}	20.28±0.38^{B}
Standard[1]	35.28^{AB}	35.24±^{AB}	39.32^{AB}	20.39^{AB}
Cinn*	30.56^{B}	34.45^{AB}	34.59^{ABC}	20.80^{AB}

*trans-Cinnamaldehyde

Columns with different superscript letters are significantly different

[1]Standards were growth of organism in 0.1% peptone water

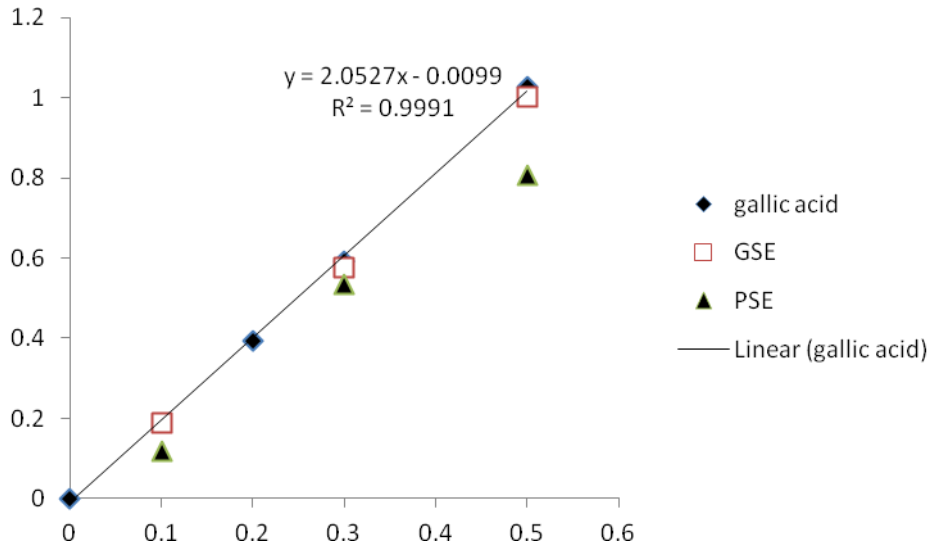

Figure 2. Total phenolic contents of GSE and PSE plotted with the standard (gallic acid)

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APPENDIX

Well	Concentration	Organism	Fraction	PPM
1-5	1:1	LM	Oligomer	30.30
6-10	1:3	LM	Oligomer	15.15
11-15	1:1	SA	Oligomer	19.32
16-20	1:3	SA	Oligomer	9.66
21-25	1:1	ST	Oligomer	22.87
26-30	1:3	ST	Oligomer	11.43
31-35	1:1	EC	Oligomer	23.72
36-40	1:3	EC	Oligomer	11.86
41-45	1:1	LM	Monomer	30.30
46-50	1:3	LM	Monomer	15.15
51-55	1:1	SA	Monomer	19.32
56-60	1:3	SA	Monomer	9.66
61-65	1:1	ST	Monomer	22.87
66-70	1:3	ST	Monomer	11.43
71-75	1:1	EC	Monomer	23.72
76-80	1:3	EC	Monomer	11.86
81-82	1:1	LM	GSE	30.30
83-84	1:1	SA	GSE	19.32
85-86	1:1	ST	GSE	22.87
87-88	1:1	EC	GSE	23.72
89-90	Blank	Blank	Blank	n/a
91-94	1:1	LM,SA,ST,EC	Cinn	11.36
95	1:1	Blank	Cinn	11.36
96-99	1:1	LM,SA,ST,EC	Broth	n/a
100	1:1	Blank	Broth	n/a

Table 1. Parameters for BioScreen well setup

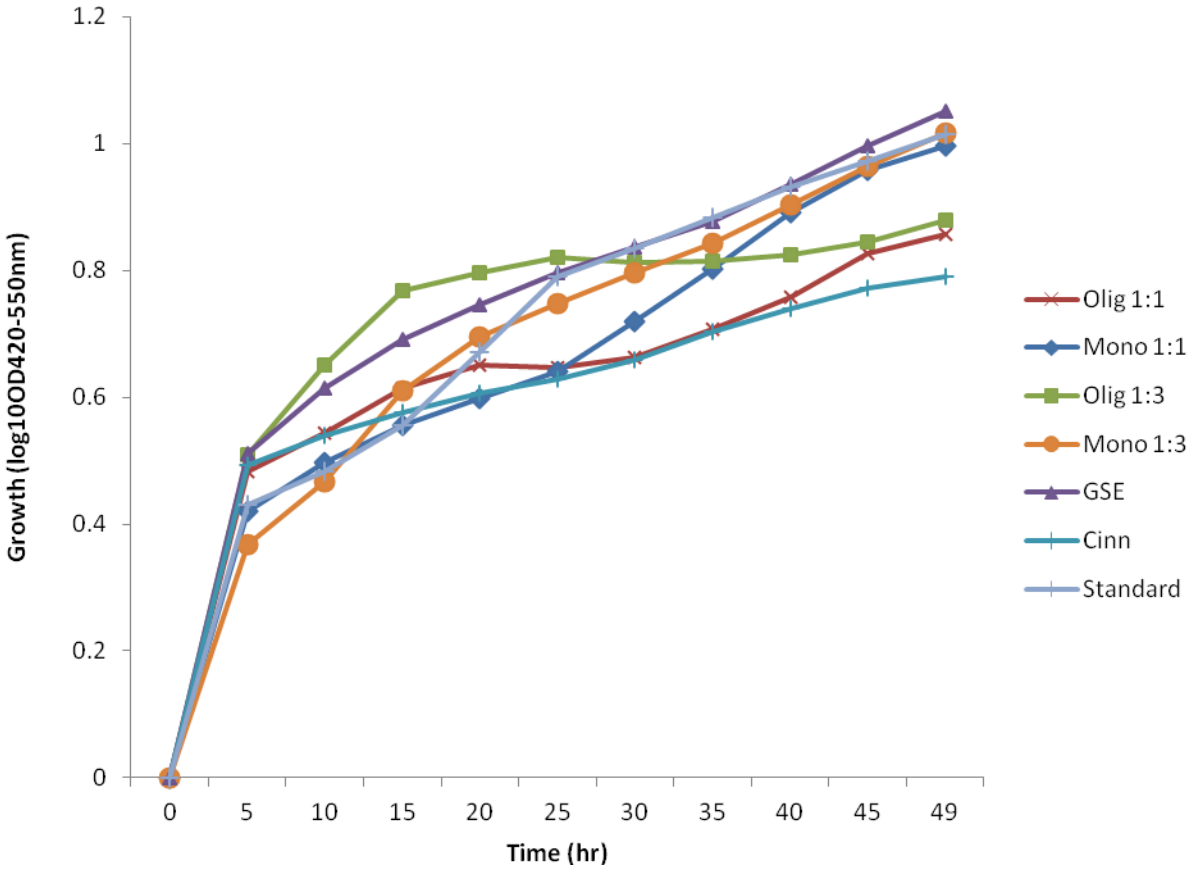

Figure 1. Growth curves for *L. monocytogenes* in the presence of oligomer fractions, monomer fractions, negative control, and cinnamaldehyde (positive control).

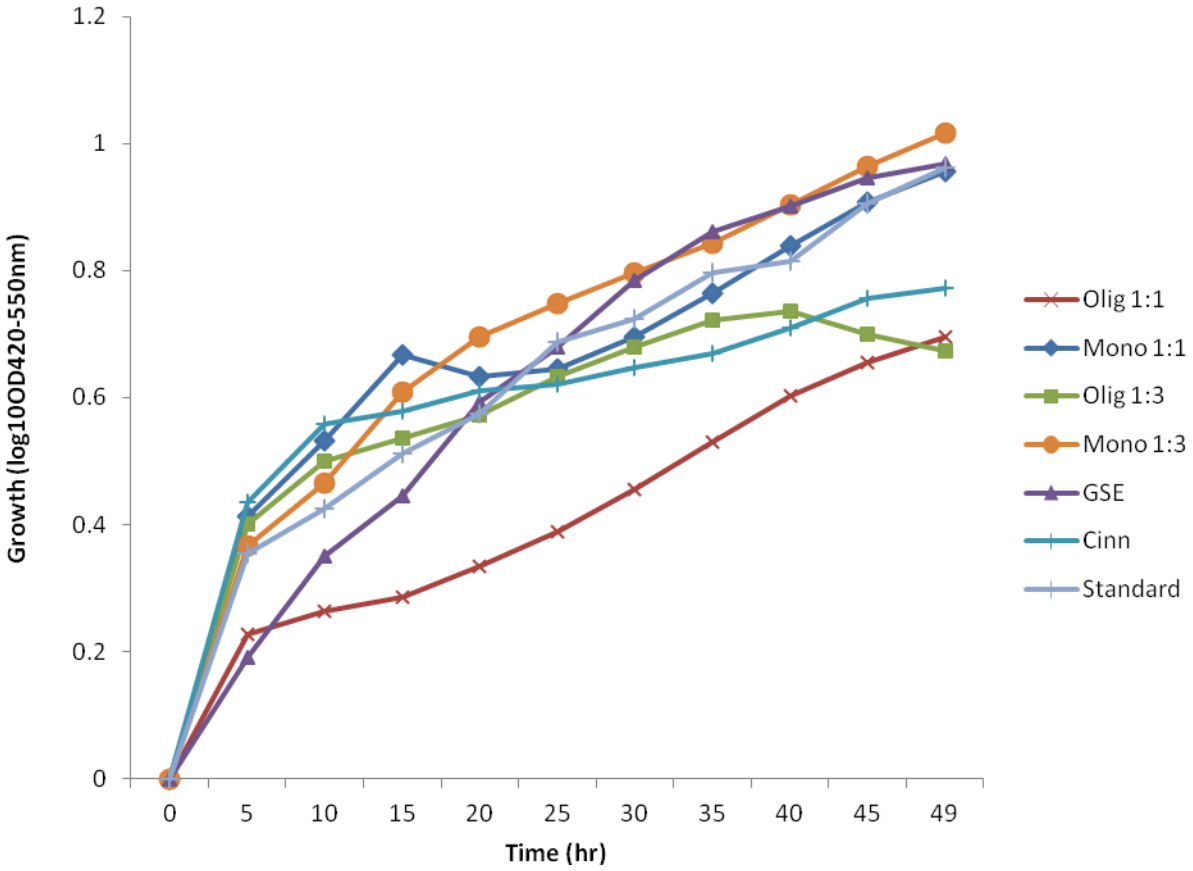

Figure 2. Growth curves for *S. aureus* in the presence of oligomer fractions, monomer fractions, negative control, and cinnamaldehyde (positive control).

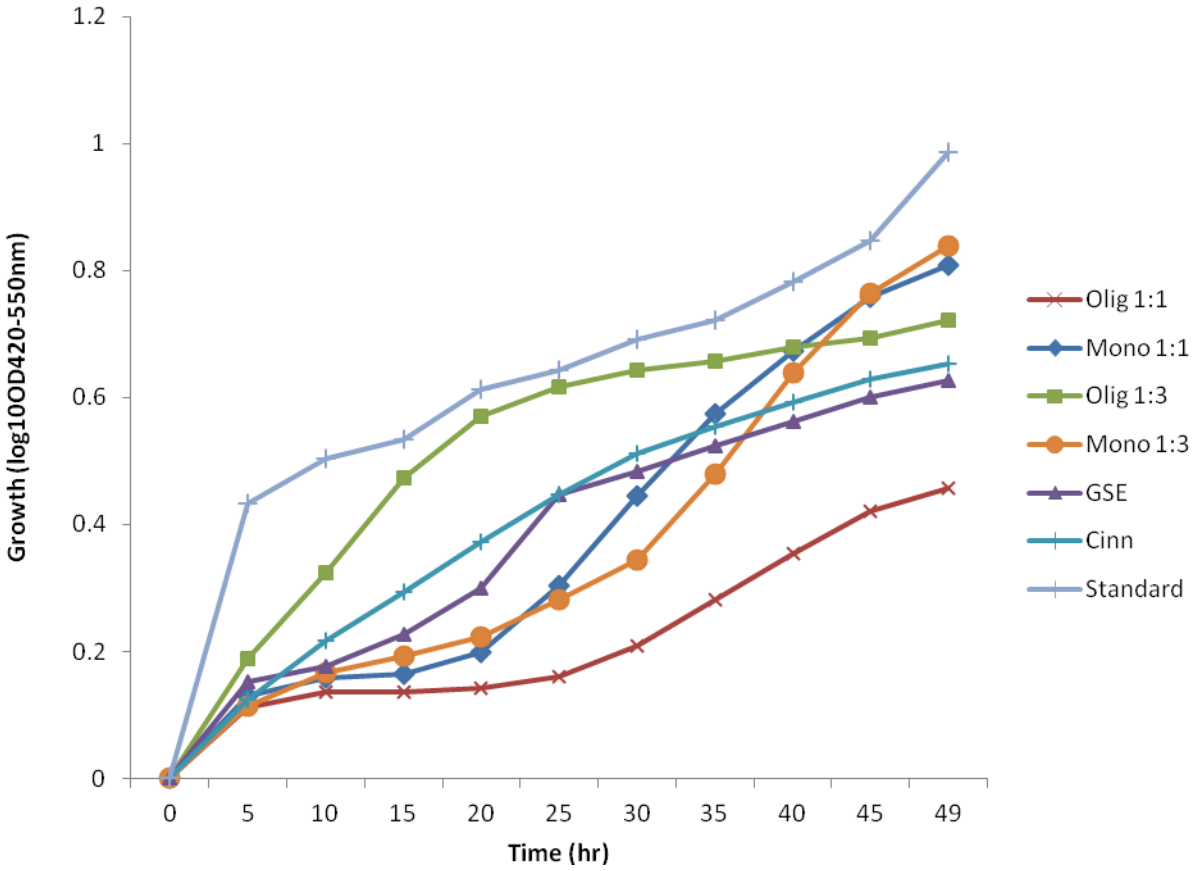

Figure 3. Growth curves for *E. coli* O157:H7 in the presence of oligomer fractions, monomer fractions, negative control, and cinnamaldehyde (positive control).

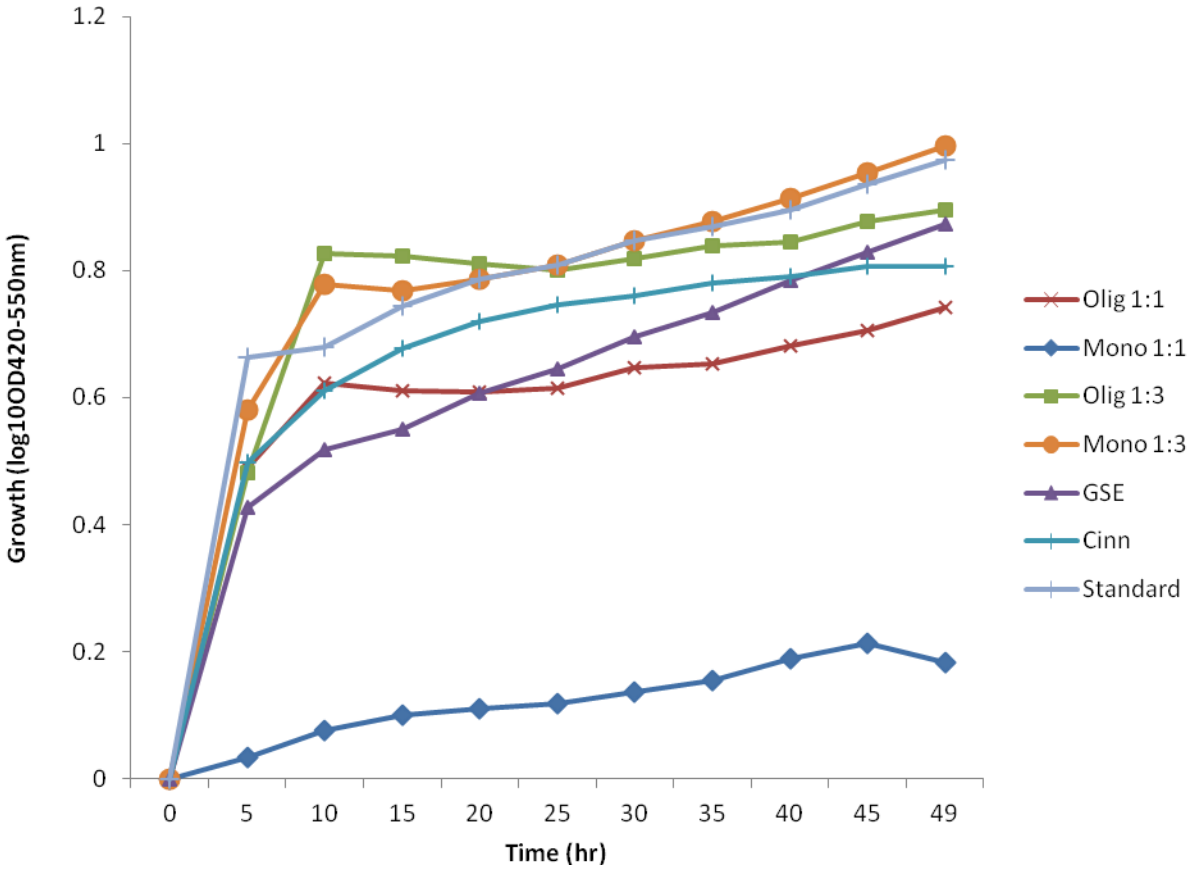

Figure 4. Growth curves for *S.* Typhimurium in the presence of oligomer fractions, monomer fractions, negative control, and cinnamaldehyde (positive control).