Sodium polyphosphate enhances the antimicrobial activities of whole and fractionated peanut skin extract against food spoilage yeasts in a model juice system

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Sodium polyphosphate enhances the antimicrobial activities of whole and fractionated peanut skin extract against food spoilage yeasts in a model juice system

by

Merike Nicole Seaman

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

From January 2011-September 2011, there were approximately 174 new fruit/vegetable juices and nectars (a beverage diluted with water and other additives besides fruit juice) introduced into the U.S. market (Canada 2011). According to Doyle (2007), processed fruits and vegetables accounted for 8.6% of the food lost in the U.S. in 1995. Recalls from beverages contaminated with yeasts and molds are rare and not highly publicized. However, in 2000 there were two different recalls of apple juice due to yeast and mold contamination, eight states and nationwide, respectively (Vasavada 2003). This is of considerable importance, as the average U.S. consumer drank approximately 30.0 liters of fruit or vegetable juice in 2010, spending approximately $52.50 (Canada 2011). In addition to antimicrobial processing steps such as concentration and freezing, methods aimed at limiting spoilage of fruit juices and juice-containing beverages include pasteurization and chemical preservation. This is problematic, as the yeasts responsible for spoilage of processed fruit juices, including Zygosaccharomyces bailii and Z. bisporus, are often resistant to traditional widely used preservatives (Deak 2008). This property, along with their intrinsic tolerance to acidic and low water activity environments makes these yeasts ideal juice spoilage organisms. Complicating these issues are trends in consumer demand to move away from the use of chemical preservatives, forcing food processors to explore alternative, and ideally, natural preservative approaches (Zink 1997). Therefore, research is required to investigate natural approaches for effective control of yeast spoilage in fruit juices.
Literature Review

JUICE SPOILAGE YEASTS

Three yeasts of major concern as spoilage organisms are *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus*. Salient characteristics of these yeasts and their physiology are described in greater detail below.

*Zygosaccharomyces bailii*

There are eight documented species of *Zygosaccharomyces*, three of which pose a serious spoilage threat to processed juice manufacturers (Erickson and Mckenna 1999). *Zygosaccharomyces bailii* exhibits several characteristics that cause it to be problematic in various fruit drinks, including growth under conditions of high osmolarity, vigorous glucose fermentation and broad resistance to various classes of food preservatives. These properties are described further below. Cells of *Z. bailii* exhibit similar heat resistance to *S. cerevisiae*, however the ascospores were found to be significantly less heat tolerant than those of *S. cerevisiae* (Thomas and Davenport 1985).

*Z. bailii* is indistinguishable from many other yeasts and other *Zygosaccharomyces* spp. Using macroscopic or microscopic observation techniques, appearing as elongated ovoid cells with multilateral budding (Erickson and Mckenna 1999). *Z. bailii* is a vigorous glucose fermenter and can be distinguished from other food and beverage spoilage yeasts by variable slow growth in sucrose, variable growth at 37°C and acetic acid resistance (Erickson and Mckenna 1999).
A notable characteristic of *Z. bailii* is its ability to survive and reproduce in extremely stressful environments – it can survive and grow at pH as low as 2.0 and water activity as low as 0.85 (Erickson and Mckenna 1999). For example, it is able to colonize environments with high osmotic conditions, such as dried or fermented fruit and juices. Normally, *Z. bailii* does not present a spoilage problem in unprocessed foods; it seems to only be a problem in processed foods where there are intrinsic factors that reduce the competition from other organisms, such as low pH, low water activity (a\textsubscript{w}), and preservatives (Erickson and Mckenna 1999).

*Z. bailii* is notoriously known for its resistance to preservatives, and is resistant to most weak acids, such as acetic, lactic, benzoic, and sorbic acids, which are traditionally used in juice drinks. Several strains of *Z. bailii* are tolerant to concentrations of 600 μg/mL or higher of benzoic or sorbic acid (Patrignani et al. 2010). A 10-20% sugar concentration produces the maximum resistance response, an energy-dependent process that is facilitated by glucose levels (Erickson and Mckenna 1999). In fruit drinks and salad dressings containing >1000ppm of potassium sorbate, it is not unusual to observe *Z. bailii* spoilage (Erickson and Mckenna 1999). One strain has even been reported to require benzoic acid for growth (Stratford 2006).

The preservative resistance mechanism of *Z. bailii* has been thoroughly studied and is well understood. *Z. bailii* uses an active transport system to pump undissociated preservative molecules from the cell, preventing acidification of the cytoplasm. Because of this pump, *Z. bailii* is the most notorious for its resistance to widely used weak acid preservatives (Erickson and Mckenna 1999).
Zygosaccharomyces spp. Ferment sugar in a unique way, where fructose is more rapidly fermented than glucose (Erickson and Mckenna 1999). Sucrose, a disaccharide composed of fructose and glucose, is hydrolyzed in foods held at low pH. During long storage periods, sucrose in acidic foods such as juices or sodas is broken down, resulting in an accumulation of both glucose and fructose. Spoilage of such foods by Z. bailii may be delayed until the accumulation of these substrates reaches levels able to support fungal reproduction and proliferation (Erickson and Mckenna 1999).

Spoilage by Z. bailii is an issue of broad concern in the food industry as this yeast can survive in a diverse range of foods. Characteristics of foods at risk for spoilage by Z. bailii include low pH, low water activity, presence of a suitable nitrogen source and addition of preservatives (Thomas and Davenport 1985). Examples of frequently spoiled products include acetic acid preserved food such as pickles, mayonnaise, ketchup, syrups, fruit juice concentrates, and various fruit juices (Thomas and Davenport 1985). Levels of gas produced during fermentation are sufficient to cause bottles to explode, cans to split and tetrapaks to burst – events that can occur on store shelves or in consumers’ homes (Thomas and Davenport 1985). Spoilage may also be accompanied by color changes, haze formation, carbonation, off flavors and textural changes. Spoilage-related changes in food composition may also promote colonization by additional microorganisms (Thomas and Davenport 1985).

Zygosaccharomyces bisporus

Z. bisporus shares many of the same characteristics as Z. bailii. However, unlike Z. bailii it cannot ferment sucrose and does not grow at 37°C. However, Z. bisporus can grow in foods with water activities below 0.80 – lower than Z. bailii (Erickson and Mckenna 1999). Z
*G. bisporus* ascospores can survive at 60°C for 10 min, but not 20 min (Pitt and Hocking 2009).

As with *Z. bailii*, high sugar/low aw foods are those most likely to be spoiled by *Z. bisporus*, although the fermentation is typically slower, and its growth can be completely inhibited by sorbic and benzoic acids at levels ≥ 500 ppm (Erickson and Mckenna 1999). Although it is an important spoilage organism, there is relatively little published research on *Z. bisporus*.

SPOILAGE OF FOODS BY YEASTS

The yeasts that cause food spoilage are almost entirely non-pathogenic; although there have been anecdotal reports of minor gastrointestinal disorders following consumption of yeast-spoiled beverages, none have been confirmed (Stratford 2006). While yeasts do not produce toxins harmful to human health, a number of yeasts, including *Candida* spp., are considered as emerging pathogens (James and Stratford 2003). Colonization and infection with these yeasts is particularly problematic in immunocompromised patients (James and Stratford 2003). The potential health hazards posed by food spoilage yeasts is a topic of ongoing research, as a number of these yeasts may also be components of the microbiota responsible for wild fermentations used in cocoa production or the production of foods such as artisanal breads and meats (Bisha et al. 2011).

Fruit juices are often contaminated with yeasts and molds, stemming mainly from wound damage to raw fruit, such as from insects (Wareing and Davenport 2004). Yeasts and molds can also contaminate fruit juices via air, soil, and processing equipment. Most yeasts grow slowly in concentrated juices, but inocula as low as one cell per bottle is enough to cause spoilage over time (Wareing and Davenport 2004). Of the more than 800 yeasts
described, only about 10 are identified in close association with food spoilage (Wareing and Davenport 2004).

There are several overt signs of yeast spoilage, the most visible being the production of gas that leads to swollen containers, “blown” cans or exploding bottles (Stratford 2006). This gas production is the result of fermentation of sugars. The amount of gas produced varies between yeast species, with Zygosaccharomyces bailii being one of the species capable of forming sufficient gas pressure to explode bottles (Stratford 2006). The second most obvious sign is macroscopic appearance of yeast growth, which is manifested as haze, surface films or colonies and sediment in undisturbed bottles (Stratford 2006). In normally clear beverages such as apple juice, yeast haze is visible when cells reach a concentration of $10^5$ CFU/mL (Stratford 2006).

Another sign is the presence of off odors and/or flavors, which, to untrained consumers, are the least obvious indicators of yeast spoilage. There are two theorized ways that yeasts can cause off flavors and odors: 1) removal of flavorsome food components or 2) production of new compounds or byproducts having distinct flavor or aroma profiles (Stratford 2006). Fermentation of sugars by yeasts yields both ethanol and carbon dioxide. Ethanol is easily detected in fruit juices due to its distinct mildly sweet flavor and the sweet flat note it imparts to the juice. Depending on the food, alcoholic aromas may or may not be detectable (Stratford 2006). Finally, spoilage-mediated carbonation may also signal the spoilage of a food. This may be masked in a beverage such as soda, but may be a clear sign of spoilage if present in non-carbonated foods such as fruit juices or condiments.
The economic impacts of yeast spoilage are not easily quantified. It has been estimated that the lost retail value of affected production lots ranges from $57,000 to $7,136,000, with an average loss of $2,262,000 (Lawlor et al. 2010). However, companies have no incentives or regulatory requirements for reporting spoilage data, so such documentation is rare, with most losses unreported (Loureiro and Querol 2000).

PROCESSING TECHNOLOGY OF FRUIT JUICES

Briefly, fruit to be used for juices is harvested, separated, washed, and exposed to mechanical processing. Usually, fruit presses can be used for more than one fruit but certain foods require specially designed equipment. Apples, for example, are subjected to both mechanical treatment and biochemical processing, resulting in almost complete liquefaction of the fruit (Lea 1998).

In a generalized scheme, picked fruit is delivered to the receiving dock of a processing plant, where it undergoes a washing and screening process. From here, it goes through the processing cycle where it is crushed, heated, mixed with enzyme if applicable, then held at a constant high temperature for a specific period of time (Lea 1998). Separation of solids is performed via centrifugation or fruit presses and the resulting juice is either concentrated, processed for aseptic storage and/or subjected to freezing or refrigeration (Lea 1998).

Although many of these newly received fruits are contaminated with a large number of yeasts, many of these are removed during the early processing steps, such as washing or peeling (Worobo and Splittstoesser 2005). However, there are numerous points for recontamination, including conveyor belts, presses, dicing equipment, and filling machine ports (Worobo and Splittstoesser 2005).
Unless specifically labeled as ‘100% juice’, retail juice drinks contain added ingredients, including water, high fructose corn syrup and natural and reconstituted flavors, and some may contain combinations of several juice types. In general, three routes for mixing or formulation are used, each of which can be used individually or combination to obtain the final product. These routes are: “batch”, “flip-flop”, and “continuous” (Lea 1998). In batch blending, all ingredients are combined together in a single tank, which can be heated or cooled as needed (Lea 1998). Flip-flop blending can be thought of as a serial batch process, where one batch is prepared in one mix tank, then pumped directly into a processing or fill line. A second batch is simultaneously prepared in a separate mix tank and also delivered to the line. These mixing and delivery processes are coordinated in a reciprocating (flip-flop) fashion between the two tanks so that a continuous flow of liquid is created in the line (Lea 1998). In continuous blending, separate ingredient streams are delivered on a continuous basis and combined via in-line mixing, with each stream carefully controlled by weight to prevent disruptions in the lines (Lea 1998).

Juice ‘not from concentrate’ is usually screened and heat processed immediately after pressing. There are three main objectives for this: 1) to control growth of spoilage organisms that live on surfaces 2) to destroy pectolytic enzymes that naturally occur in fruit to maintain the opacity or cloudiness of the juice 3) safety – kill pathogens (Lea 1998). Where a clear juice is desired, additional enzymes, such as pectinase, may be added to speed up the clearing process either during the mash phase or in the holding tank (Ribeiro et al. 2010).

Juice ‘from concentrate’ is usually screened to remove large cellular debris. The juice enters a multi-step evaporation process to remove most excess water (Lea 1998). Evaporators are highly efficient processing units and also recover volatile aromatic substances that help
give fruit juice their sensory characteristics (Lea 1998). Concentrated juices are stored until reconstitution, some requiring refrigeration or freezing. Alternatively, juices may be processed aseptically and held or shipped in drum containers or even in “juice tanker” ships equipped internally with large rubber bladders to contain the juice concentrate (Foundation 2013). Transportation of concentrated juices in this manner reduces shipping expenses, making long-distance transportation of juices economically feasible. As these concentrates are processed aseptically, temperature constraints normally required for microbial stability do not apply. Other routes for deterioration, such as non-enzymatic browning may still apply, if temperatures become too high. The activity of endogenous enzymes, such as polyphenol oxidase (PPO), may also contribute to non-microbial degradation under such circumstances (Lea 1998).

There are four main thermal processing methods used for fruit juices: in-pack pasteurization, hot filling, flash pasteurization and aseptic filling (Lea 1998). The method used depends on the initial level of microbiological contamination and the potential of the final product to support or suppress the growth of microorganisms (Lea 1998). These processes are discussed further below.

Flash Pasteurization

Briefly, pasteurization consists of heating the product to a specific temperature and holding at that temperature for a period time so that the organism of concern is killed (Lea 1998). Pasteurization may be used for both food safety and quality purposes, targeting pathogens and spoilage organisms, respectively.
Apple juice can be flash-pasteurized at 0.75s at 121°C (Brandt 2009). However, any combination equivalent would be sufficient (Tandon et al. 2003). Raw juice is heated by hot water in a plate or tubular heat exchanger to the desired temperature and held for a specific time in a holding tube before cooling to filling temperature using chilled water (Lea 1998). Most flash-pasteurizers have an energy saving feature where the incoming raw product is heated by the hot product returning from the holding coil, which is then cooled (Lea 1998). The equipment must be sterilizable using steam or hot water and be resistant to spores attachment (Lea 1998). The FDA mandated in January 2001 that all juice or juice used as an ingredient in a beverage must be pasteurized or processed to a 5-log reduction in microbial contamination (FDA 2001). This is to ensure a longer shelf life as well as kill any pathogens that may be harmful to consumers.

The process must be timed precisely so that treatment time is accurate. Precise timing is achieved through control of product flow rate within a known holding tube volume. The flow rate and ending temperature are critical and must be monitored (Lea 1998). The heat from flash pasteurization can damage several of the quality and nutrient attributes of the juice (Choi and Nielsen 2005). Yeast recontamination is possible through cross-contamination of raw and finished product, inadequate heating and cooling times, and improperly cleaned filling ports.

Hot Filling

In this process, the fruit juice is heated, and filled into containers while hot. The closed containers are then held at or above the pasteurization temperature for a set period of
time, then cooled (Lea 1998). This process results in heat treatment of both the product and the container.

In a typical system, the product is heated to 87°C and sent to the filler bowl (Lea 1998). Product temperature is monitored and the filling process is stopped at temperatures below 85°C, followed by emptying of the filling bowl and refilling with hot product (Lea, 1998). The juice is filled into either plastic or glass containers and kept at an equilibrium temperature of 83-84°C (Lea 1998). The containers are then held at temperatures about 80°C for a specific period of time, with the time-temperature combination serving as a microbial control step. The juice is then cooled below 30°C.

As with flash pasteurization, there is the potential for post-heat treatment recontamination. Since the product is filled hot, the main source of recontamination is inadequate heating. If the process does not reach 87°C, there is the potential for yeast to survive the heating process and grow during the storage period.

In-Pack Pasteurization

This is severest and most microbiologically safe form of heat treatment. The filled sealed package is passed through a tunnel pasteurizer. This pasteurization occurs with water spray at numerous controlled temperatures (Lea 1998). Different zones make up the pasteurizer unit. The first zones are the heating and superheating zones which raise temperature of juice and container (Lea 1998). The next zone is where the product is held at pasteurization time and temperature. In the last zone, the product is cooled to 30°C. To ensure that this process is microbiologically safe, a pasteurization probe is regularly sent through the machine (Lea 1998). In-pack pasteurization is normally referred to as a fool-
proof, however whether or not the product spoils ultimately lies with the package seal (Ashurst 2008). If seal integrity is disrupted, there is potential for yeast spoilage.

Aseptic Filling

Aseptic filling is a method of producing commercially sterile beverages. For this process to be successful, clean containers, products and closures must be brought together in an environment that is free of contamination. This environment must have sterile air, which is achieved by either heat or filtration with 0.2μm filter (Lea 1998). Prior to filing with the UHT-processed beverage, laminate boxes are sterilized with hydrogen peroxide vapor, which is subsequently removed with sterile, heated air. While laminate “Tetra Pak” boxes are commonly used, other container types include bottles, some of which can be injection-molded in a sterile molding instrument, then filled with product without additional chemical treatment (Lea 1998). The primary concern for aseptic filling is for heat resistant spores, which should be used for process determination (McLellan and Padilla-zakour 2005). Continuous aseptic processing is less energy intensive than other thermal processing techniques, however there are more potential for malfunction because of packaging than in other processing equipment such as bottle or glass filling (Reuter 1993). As with other thermal processing systems, there is potential for recontamination of a juice product if the packaging is not properly sterilized before use, potential leaking in sterile environments, non-hermetic seals, and cross-contamination with raw product.

CHEMICAL PRESERVATIVES
Sorbates

Sorbates, a group including sorbic acid and its water-soluble salts, are used extensively throughout the world as preservatives, including in animal feed, pharmaceuticals and cosmetics, and other applications (Stopforth et al. 2005). Sorbates have a wide spectrum of activity and are active against various microorganisms including yeasts, molds, and bacteria. It has been proposed that sorbates may act through alteration of the cell membrane and inhibition of transport functions and metabolic activity (Stopforth et al. 2005). Some organisms, such as molds, can metabolize sorbates through β-oxidation, using these preservatives as a carbon source for growth (Stopforth et al. 2005). The effectiveness of sorbates can be influenced by numerous intrinsic and extrinsic factors, including pH, aw, temperature, preservative concentration, and product formulation.

Benzoates

Benzoates, salts of benzoic acid, have been used as preservatives in foods, as well as drugs and cosmetics, for numerous years, making it one of the oldest chemical preservatives still in use. It is one of the most widely used preservatives because of the low cost, low toxicity, and lack of color. Benzoates are active against yeast, mold, and bacteria. It is thought that the mechanisms for benzoic acid are that it depletes ATP and interferes with cell membrane permeability, resulting in an uncoupling of both substrate transport and oxidative phosphorylation for the electron transport system (Chipley 2005). Benzoic acid naturally occurs in several foods, such as cranberries, tomatoes, and blackberries (Chipley 2005). Commercial food grade benzoates and benzoic acid is synthetically produced. Benzoic acid is
produced almost entirely from toluene by liquid-phase oxidation, while the neutralization of benzoic acid with sodium hydroxide produces sodium benzoate (Wibbertmann et al. 2005).

NATURAL ANTIMICROBIALS

Consumer demands for more “natural” alternatives to synthetic chemical food preservatives have driven academic and industrial research into development of new, “greener” preservative approaches. A variety of compounds occurring in the natural world are known to have antimicrobial activity. These are present in plants, animals or other microorganisms, and include phytochemicals, enzymes or peptides such as plant essential oils, lysozyme from eggs, lactoferricin from milk or bacteriocins from bacteria. These antimicrobials typically play a role within their host systems to ward off infection, prevent microbial utilization of vital biological materials such as eggs or milk or provide bacteria with a competitive edge against closely related organisms occupying the same ecological niche. The fact that the host tissues or environments that these antimicrobials have evolved to operate in are also used by humans as foods suggests their potential purification and addition to foods as naturally-sourced preservatives or preservative systems to produce microbiologically safe and stable foods that are perceived to more “natural” and less “processed”. Phytoalexins, organic acids, phenolic compounds, and essential oils from plants and bacteriocins produced by lactic acid bacteria are described briefly below (Smid and Gorris 2007).

Organic Acids

Organic acids are used extensively in the food industry with citric, malic, and tartaric among some of the most popular. Many of these organic acids are found naturally in fruits
and vegetables (Theron and Lues 2007a). Other organic acids can be naturally occurring in food processing, such as food fermentation. These acids act upon cell walls, cell membranes, metabolic enzymes, genetic material, protein synthesis, and many more (Smid and Gorris 2007). Critic acid, for example, acts as a chelating agent for metal ions (Doores 2005). For these reasons they can be used against a wide range of microorganisms. Several fungi species are resistant to organic acids, either intrinsically resistant or develop resistance over time (Theron and Lues 2007b).

Phytoalexins

The term “phytoalexin” describes a class of low molecular weight phytochemicals having broad-range antimicrobial activities against fungi and bacteria. Phytoalexins are produced in plant tissues in response to external stimuli such as microbial infection or exposure to various “elicitor” molecules (Smid and Gorris 2007). The most important class of phytoalexins are the isoflavonoids. Isoflavonoids are characterized best by the C₆-C₃-C₆ structure, and greater lipophilicity results in increased antifungal activities (Smid and Gorris 2007). Another important class is the proteinaceous phytoalexins, represented by thaumatins (with thaumatin also functionally useful in foods as a proteinaceous sweetener), plant antimicrobial peptide analogs such as the thionins and enzymes such as chitinases, which degrade chitin, the major component of both fungal cell walls and insect exoskeletons (Smid and Gorris 2007). Although use of phytoalexins for food preservation is intriguing, they have not yet been widely applied in this role, as high local concentrations are typically required for efficacy (Smid and Gorris 2007). One exception is the use of trans-resveratrol as a surface application to fruits to prevent fungal spoilage, which was found to be both effective and organoleptically acceptable (Urena et al. 2003). It has also been reported that peanuts and
peanut products contain levels of resveratrol ranging from 0.06 ppm – 5.1 ppm, depending on the processing technique used (Ballard et al. 2009).

Phenolic Compounds

Phenolic compounds aid in plant defenses against a wide variety of microorganisms. Phenolics also contribute to the taste, aroma, and appearance of plants and can contribute to the nutritional qualities of the plants pre- or post-processing. Phenolic compounds can be divided into three groups: simple phenols and phenolic acid, hydroxycinnamic acid derivatives, and flavonoids (Smid and Gorris 2007). Flavonoids are the most important of these groups and contain catechins, proanthocyanins, anthocyanidins, and flavonols (Smid and Gorris 2007). Flavonoids, especially catechins, have been extensively researched. Catechins have shown promise against *Vibrio cholera*, *Steptococcus mutans*, and other microorganisms (Cowan 1999). The antimicrobial activity of phenolic compounds is attributed to enzyme inactivation, protein denaturation, and interaction with cell membranes (Bisha et al. 2010).

Tannins are polyphenolic compounds that can precipitate proteins out of solution (Scalbert 1991). Mechanisms of tannin toxicity could include: enzyme inhibition and substrate deprivation, actions on membranes, and metal ion deprivation (Scalbert 1991). Tannins reducing the availability of metal ions can affect metalloenzymes, and they have been shown to be good inhibitors of fungal metalloenzymes (Scalbert 1991). Tannins will be discussed in more detail below.

Many phenolic compounds have been studied for their antimicrobial activity, such as phenolics from tea, olives, coffee, and grapes. The phenolic compounds naturally present in
grape seed extract have been shown to have the same effect as a chemical preservatives in cooked meats, such as improving shelf life and color (Bisha et al. 2010).

Essential Oils

Essential oils are derived from spices, herbs, and fruits and have long been valued for their properties as flavorant and aroma compounds. Essential oils show a wide variety of interesting biological activities, including anti-inflammatory, insect repellent and antimicrobial properties and have been used traditionally in folk remedies and food preservation. Many essential oils are already approved for use in foods as GRAS compounds, although their use in some foods may be limited by their sparing solubility in water (Smid and Gorris 2007). Methods for extraction of essential oils include physical pressing, steam distillation, use of organic solvents, enfleurage (extraction into odorless animal fat) and use of supercritical carbon dioxide.

Essential oils have been extensively studied on pathogenic bacteria. Smith-Palmer et al identified that bay, cinnamon, clove, and thyme oils were most inhibitory against *Campylobacter jejuni, Escherichia coli, Salmonella enteritidis, Listeria monocytogenes*, *Staphylococcus aureus*, and at levels of 0.075% or less (Smith-Palmer et al. 1998). According to their study, gram-positive bacteria are more sensitive to plant essential oils than gram-negative bacteria (Smith-Palmer et al. 1998). It has also been found that allspice, cinnamon, clove, garlic, onion, oregano, savory, and thyme exhibited inhibitory effects against food spoilage yeasts (Conner and Beuchat 1984). Of these essential oils, garlic was the most potent inhibitor at concentrations as low as 25ppm (Conner and Beuchat 1984). Muriel et al recently conducted a study using oregano essential oil (OEO) vs. citral as an
active compound on ethylene vinyl alcohol (EVOH) coated polypropylene (PP) film (Muriel-Galet et al. 2013). However, consumers preferred the film with citral in sensory studies, while the citral also out-performed the OEO in antimicrobial tests against entrobacteria and yeasts and molds (Muriel-Galet et al. 2013).

Since there are several different classes that make up essential oils it is nearly impossible to pinpoint a specific mechanism for the inhibitory characteristics displayed. As evident by the class name, essential oils contain a high degree of hydrophobicity. This could be the mechanism behind some antimicrobial or antifungal effects. Because of this hydrophobicity, the essential oil may transplant itself in the lipid membrane of the bacterial or fungal cell, increasing its bioavailability and decreasing cell vitality (Smid and Gorris 2007). It should be noted, that the yield of the active compounds from a plant are dependent on genotype and extraction methods and the composition can differ between the same species in different environments and geographical locations (Smid and Gorris 2007).

Applications of Natural Antimicrobials from Plant Sources

Natural antimicrobials from plant sources have been used for years in many different food applications. For example, cinnamaldehyde is used as a surface disinfectant for tomatoes (Smid et al. 1996). Tomatoes treated with a 13mM cinnamaldehyde aqueous solution reduced bacteria and fungi by one order of magnitude within 10 and 30 min (Smid et al. 1996). Another example is the use of clove oil in chicken frankfurters. The presence of 1% or 2% (w/v) clove oil inhibited the growth of seven strains of *Listeria monocytogenes* under storage conditions of 5°C and 15°C (Mytle et al. 2004). Thyme, summer savory, and clove essential oils can inhibit the growth of *Aspergillus flavus* in tomato paste at levels of
350 and 500ppm (Omidbeygi et al. 2007). It was also reported that these essential oils at levels of 500ppm were accepted by taste panels (Omidbeygi et al. 2007).

Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) is used in the production of fermented foods and beverages and is one of the oldest preservation techniques used in the industry. LAB starter cultures produce multiple different antimicrobial compounds during the fermentation process that can help out compete competitors. However, since LAB produce numerous acids that can be detrimental to the sensory characteristics of the a product, they are not used as protective cultures in many foods (Smid and Gorris 2007). LAB that produce bacteriocins without much production of acid are currently a potential option for protective cultures (Smid and Gorris 2007).

LAB produce bacteriocins that help combat the growth of many competitor organisms. These bacteriocins are proteins with bactericidal activity against species similar to the bacterium from which they are produced (Klaenhammer 1988). These bacteriocins vary in molecular weight, targets, and biochemical properties (Klaenhammer 1988). Bacteriocins are divided into three classes: Class I, the lantibiotics; class II, the small heat stable labile non lantibiotics; and class III, the large heat labile bacteriocins (Aly et al. 2006). The most well characterized bacteriocins belong to classes I and II. Nisin, a bactericidal bacteriocin is produced late in the growth cycle by Lactococcus lactis subsp. Lactic ATCC 11454 and belongs to class I (Aly et al. 2006). Nisin is active in acidic food matrixes and is effective in inhibiting the growth of gram-positive food borne pathogens (Smid and Gorris 2007). When
used in conjunction with heating, Nisin counteracted heat-resistant spores (Smid and Gorris 2007).

Applications of Natural Antimicrobials from Microbial Sources

Lactic acid bacteria have been shown to have promise as protective cultures for many food products. In meat, lactic acid bacteria have been shown to reduce *L. monocytogenes* in many products including cured pork, sliced Brühwurst and turkey summer sausage (Smid and Gorris 2007). LAB also reduced the number of *L. monocytogenes* in fish and seafood (Wessels and Huss 1996). In dairy products, LAB have been used to reduce or prevent the growth of numerous pathogen bacteria including *Clostridium sporogenes* and *L. monocytogenes* (Smid and Gorris 2007).

VALUE-ADDED AGRICULTURE

Increasing the value of primary agricultural commodities through manufacturing processes is called value-added agriculture. Value-added agriculture may include products from organic produce or locally grown crops. There are five distinct way to add value: quality, functionality, form, place, time, and ease of possession (Anderson and Hanselka 2009). The form aspect means converting raw material into a finished or semi-finished product thus increasing the usability of the product. Place or location is providing the product at a desired place and providing an assortment of products. Timing is important because providing a product at a desired time and using marketing windows all add to the value of a product. The product needs to meet or exceed consumer expectations for quality and provide the function needed of it all to add value.
Examples of value added products may be from: wheat, woody plants, and cattle. The wheat straw can be harvested and processed into building material (Anderson and Hanselka 2009). Wheat value can also be increased by allowing cattle to graze, which can increase the yield by causing “tillering” of the plant and “beefing” up of the cattle (Anderson and Hanselka 2009). Wheat can also be grown organically and sold at health markets, thus increasing the value. Wood, especially cedar, can be used to make building materials or recap oil wells (Anderson and Hanselka 2009). Other woods, such as mesquite, can be used in jewelry boxes or jewelry, and outdoor furniture, as well as flavors such as liquid smoke (Anderson and Hanselka 2009). In the beef industry, value can be increased by obtaining a feed lot. Development of specialty products, such as sausages, can also increase the value of the beef.

Another example of a value added crop includes peanut products. Peanuts are grown over approximately 42 million acres around the world (Putnam et al. 2013). Behind soybean and cotton, it is the third major oilseed. Over the past 25 years India, China, and the United States have grown over 70% of the world’s crop (Putnam et al. 2013). The United States exports an average of 200,000 and 250,000 metric tons annually (SoyaTech). There are seven states that produce a majority of the peanut crop in the US: Texas, Alabama, North Carolina, Florida, Virginia, and Oklahoma (SoyaTech).

Peanuts can be processed into peanut flour, peanut oil, roasted peanuts, and the most famous, peanut butter. Peanut flour is naturally processed with a low fat content and a strong roasted peanut flavor (Council 2011). Peanut flour can be used in many applications and can be found to extend the shelf life of many confections (Council 2011). In addition, it can contribute a good source of protein and be a strong flavoring agent (Council 2011). Peanut oil
is extracted by hydraulic pressing, expeller pressing, or solvent extraction. It has a strong roasted peanut flavor and aroma and can be used as a flavoring agent in confections, sauces, and baked goods (Council 2011). Roasted peanuts can be found in several different products and in several different flavors. Peanut butter is available in many different forms in the US. In peanut butter, the peanuts are roasted, blanched, and ground to a creamy consistency. Over 1.5 billion pounds of peanut butter and peanut products are consumed by Americans every year (Board 2012).

However, there are potential downfalls with the peanut industry. A major outbreak of Salmonella typhimurium occurred between September 2008 and April 2009. Affecting nearly all 50 states (46 to be exact), 714 cases were identified with 166 hospitalized and 9 deaths (Cavallaro et al. 2011). This was one of the largest recalls in U.S. history and cost approximately $1 billion in loss of peanut sales (Cavallaro et al. 2011). This outbreak illustrated how many products that peanuts and peanut products are in. Another pitfall to peanuts is the allergenicity. Having a peanut allergy is potentially life-threatening and it is estimated that approximately one-third of American children are hypersensitive to peanuts (Scurlock and Burks 2004). However, the peanut proteins that cause allergies can be altered by various processing techniques, including boiling or frying (Scurlock and Burks 2004). The third downfall of the peanut industry is the potential presence of mycotoxins, specifically aflatoxin. Aflatoxin is classified as a human carcinogen and is strictly controlled in the U.S. (Chang et al. 2013). In order to be of edible quality grade, peanuts must contain less than 15 ppb of aflatoxin with the FDA able to pursue legal action if the levels reach 20 ppb or above (Chang et al. 2013). If a crop is contaminated with aflatoxin, various treatments can be
applied that could eliminate such as ozone, irradiation, and high temperature roasting (Chang et al. 2013).

The waste from peanut crops can be turned into peanut by-products such as peanut hay, peanut meal, peanut hulls, and peanut skins. Peanut hay amounts vary by peanut variety and cultivation, however a farmer can obtain two-thirds of a ton per acre of peanut hay (Lynch 1930). Peanut hay is fed to stocker cattle, beef replacement heifers, and wintering cow herds as a winter feed supplement (Hill 2002). In 2001, it was estimated that peanut hay was produced on 25-40% of the peanut acreage (Hill 2002). Peanut hay is highly nutritious for cattle if harvested and stored properly. It has been estimated that peanut hay contains 10% crude protein, 20.9% crude fiber, and 8.6% ash, which is similar to alfalfa (Hill 2002). Peanut meal can also be used as a supplement to cattle feed. It contains approximately 6.3% ash, 52.3% crude protein, and 77.0% total digestible nutrients, which is similar to soybean meal and cottonseed meal (Hill 2002). Peanut meal has been substituted for soybean meal without affecting the performance of growing pigs (Hill 2002). In 2001, approximately 312,000 metric tons of peanut hulls were produced in the United States (Hill 2002). These hulls can be used as roughage in livestock diets, fuel in manufacturing processes, mulch, bedding, and a number of specialty uses.

Peanut skin extracts (PSEs) are value-added agricultural products with promising antioxidant, nutraceutical and/or antimicrobial potential. Typically, peanut skins are removed at the blanching step or after dry roasting (Constanza and White 2012). They are rich in antioxidant procyanidin oligomers and the antioxidant capacity of PSEs has been shown to exceed that of both Trolox (a vitamin E derivative) and vitamin C at the same concentrations (Yu et al. 2006). However, their high procyanidin content limits their incorporation into
animal feeds, as it interferes with protein digestion at levels beyond the 5-8% used in feeds. In humans, however, the antioxidant capacity of procyanidins may have wide-ranging positive impacts on conditions such as cancer, heart disease and inflammation (Constanza and White 2012; Yu et al. 2006).

Apart from direct disposal or value-added incorporation into animal feeds, these waste materials may also be reintroduced into peanut butter for nutritional or functional (antioxidant) fortification (Constanza and White 2012; Yu et al. 2006). Previous studies have also examined the use of PSEs as natural antioxidant preservatives in meats ranging from raw or cooked ground beef to salami (Larrauri et al. 2013; O’Keefe and Wang 2006; Yu et al. 2010). O’Keefe and Wang (2006) found that PSE at a level of 200 ppm significantly reduced oxidative spoilage of meats, extending shelf life in ground beef without negative impacts on color or aroma. Yu et al. (2010) extended this work to demonstrate inhibition of undesirable microbes in ground beef, while confirming earlier findings on product quality (i.e. color retention and prevention of lipid oxidation).

The antioxidant properties of PSEs can be affected by the method used for extraction and parallel observations have been made for whole peanut extracts, where the extraction method used was shown to impact immunogenic capacity (Schmitt et al. 2010; Yu et al. 2006). These observations suggest that, if immunogenicity is determined to be problematic for PSEs, that these negative effects might be minimized through use of carefully selected extraction techniques.

Interestingly, almond skin extracts have also been shown to possess antimicrobial activities against Gram-positive and Gram-negative bacteria, with this activity attributed to phenolic compounds such as protochatechuic acid, naringenin and epicatechin (Mandalari et
al. 2010). These similarities to PSE’s suggest that waste streams from various nut-processing operations might also yield similar value-added compounds that can be used advantageously in the food industry.

Polyphenols

As mentioned above, tannins are found in peanut skins. Tannins are a group of polyphenolic compounds that are found in virtually every plant family. Polyphenolic compounds are the most abundant group of antioxidant in today’s diet. It wasn’t until the mid-1990s that the antioxidant effects of polyphenols began to be studied (Scalbert et al. 2005). Polyphenols have been suggested to contribute to the prevention of cardiovascular diseases, cancers, and osteoporosis (Manach et al. 2004). However, the experiments that generate these suggestions are performed in vitro or in animals at concentrations much higher than would be consumed by humans in the diet (Scalbert et al. 2005). Although, there has been significant progress to establish a connection between polyphenols and reducing cardiovascular risk (Scalbert et al. 2005). However, difficulties arise when studying the health effects of polyphenols. For example, there are numerous different phenolic compounds present in foods that exhibit different biological activities (Scalbert et al. 2005). Many of the compounds that are tested in in vitro studies that are not native to the food, but they are most likely metabolized in the body into active compounds (Scalbert et al. 2005).

Phenolic compounds can be divided into 16 classes based on chemical structure (Bravo 1998). Flavonoids are the most widely distributed group of polyphenols and are found in nearly all plants. One important group of flavonoids are anthocyanins, which are the glycosides of anthocyanidin (Bravo 1998). Anthocyanins are dissolved pigments usually
found in flowers and fruit (Manach et al. 2004). Anthocyanins have the ability to decrease capillary permeability and fragility and have anti-inflammatory and anti-oedema activity (Kong et al. 2003). Studies have been conducted to test the effectiveness of red wine anthocyanins against suppressing tumor growth and cardiovascular disease (Kong et al. 2003).

Tannins, as mentioned above, are another group of plant phenolics. These molecules are responsible for astringency in some plant foods. This astringency is produced because of the insoluble complexes that tannins can form with proteins and carbohydrates (Bravo 1998). There are two major groups of tannins that are consumed by humans: 26ydrolysable and condensed tannins (Cowan 1999). The first group, or 26ydrolysable tannins, can be easily hydrolyzed with acid, alkali and hot water, or enzymatic activity to produce polyphydric acid and phenylcarboxylic acid (Bravo 1998). Gallic acid and its dimeric condensation product make up this group of tannins (Bravo 1998). The second group, or proanthocyanidins, may participate in the prevention of cancers, protect against oxidation, and inhibit platelet aggregation that leads to cardiovascular diseases (Santos- Buelga and Scalbert 2000). The antimicrobial activity of tannins is not well known, but possibly related to the ability to inactivate cell membrane transport proteins among other activity (Cowan 1999).

Several polyphenolic compounds are also used as antimicrobials and come from value-added products, such as olive oil, tea, and grapes. For example, the polyphenolics in virgin olive oil have been found to be stable under simulated gastric juices for several hours (Romero et al. 2007). These polyphenols have shown to exhibit a strong bactericidal effect against 8 strains of Helicobacter pylori, which can cause ulcers (Romero et al. 2007). The catechins and theaflavins present in teas have shown to have antimicrobial activities against Bacillus cereus (Friedman et al. 2006). Two catechins in green tea, (-)-epigallocatechin
gallate and (-)-epicatechin gallate, EGCG and ECG, respectively, exhibited the most potent antimicrobial activity against pathogenic bacteria (Perumalla and Hettiarachchy 2011).

Grapes, specifically grape seed extract (GSE), is rich in (+)-catechin and (-)-epicatechin, which are important for antimicrobial activity (Perumalla and Hettiarachchy 2011). The phenolic compounds in GSE have shown to have inhibitory effects against *S. aureus* and *E. coli* (Perumalla and Hettiarachchy 2011).

The polyphenols previously mentioned come from value added products. They are extracted from the foods themselves, or are extracted from by-products of the processing stages. For example, grape seed extract is produced from the seeds left over from wine production. Before the use of the polyphenols from these grape seeds, the seeds and skins from the grapes, called pomace, was used traditionally to produce pomace brandy and up until recently was used as fodder and fertilizer.

There are several pros and cons of value added products. Pros can include a higher profit, more innovation, less waste, less pesticides and antibiotics. Cons can include more processing steps, allergenicity, more diseases, and less weed control. For grape seed extracts for example, the pros outweigh any of the cons because with the extraction of phenols from the seeds produce less waste from the wine production industry. However, with the value added products from peanuts, the cons may be of concern to several people, mainly those with food allergies. Several of the products produced from peanuts will have traces of the proteins that cause allergies and when added to foods increase the potential for causing reactions in allergy prone people.
POLYPHOSPHATES

Phosphorus, an element available in every food we consume, is a cofactor in various enzymatic systems that are involved in metabolism. Phosphorus is naturally available in every food we consume. Polyphosphates consist of four or more orthophosphates, which are composed of a phosphorus atom surrounded by four oxygen atoms (Prakash 2000). Phosphates are considered superior buffers because of the large pH range of 4.0-12.0 (Prakash 2000). Both long-chain and short-chain polyphosphates are used in foods. Long-chain polyphosphates can interact with proteins, pectins, and starches and act as dispersive and emulsifying agents (Prakash 2000). Both long-chain and short-chain polyphosphates can interact with metal ions, which may be the basis of the antimicrobial activity they possess (Prakash 2000).

There have been numerous mechanisms proposed for the antimicrobial activity of polyphosphates. The leading mechanism is that the polyphosphates chelate the metal ions in cell membranes leading to loss of membrane integrity and inhibition of cell division (Prakash 2000). When polyphosphates are added to a water solution that contains cells, they tend to aggregate at the cell surfaces. Cell membranes naturally contain metal chelators that ferry metal ions out of the cells. It is thought that polyphosphates compete with the natural membrane chelators and remove ions from the cell membrane, causing lysis and inhibited growth (Prakash 2000). It is also known that alone they are relatively weak antimicrobial agents, however through the damage that is caused by this chelation, they can sensitize microbes to promising antimicrobials (Weinkauf 2009).
There have been numerous studies done on the effects of polyphosphates and various food-borne pathogens. Typically, gram-positive bacteria are more sensitive than gram-negative bacteria to polyphosphates (Obritsch et al. 2008). Also the longer the chain of phosphates, the greater the antimicrobial activity (Obritsch et al. 2008). In a study done on *Staphylococcus aureus*, it was determined that 0.5% of polyphosphate was sufficient to produce bactericidal effects (Jen and Shelef 1986). In this study, it was reported that the antimicrobial activities were pH dependent with the highest sensitivity above 7.4 (Jen and Shelef 1986). Another study done with *Listeria monocytogenes* reiterates that longer chain polyphosphates have a greater antimicrobial effect (Zaika and Kim 1993). In this study, the effect was on lag time, which increased as the concentration of polyphosphate increased and temperature decreased (Zaika and Kim 1993). It was also suggested that adding 2.0% NaCl increased the inhibitory effects (Zaika and Kim 1993). Studies conducted on *Clostridium botulinum* demonstrate that polyphosphates can reduce toxin production (Prakash 2000). In 60% moisture cheese spreads, those without polyphosphates tested positive for toxins after 8 weeks, while those with polyphosphates tested positive after 20 weeks (Prakash 2000).

Nearly all food phosphates are classified under the “GRAS” status by the FDA. Phosphates are used in all types of foods from baby foods to healthy foods to meats and seafood and processed cheeses. Approved use by the FDA is found under Title 21 of the Code of Federal Regulations (CFR). Polyphosphates are allowed for use at levels of <5000 ppm in the food industry (Obritsch et al. 2008). Meats are regulated by the USDA and therefore the use of polyphosphates in these products are limited to 0.5% weight of the final product (Prakash 2000). According to the USDA, all meats and poultry products that contain polyphosphates must be labeled appropriately and need to be approved (Prakash 2000). Any
phosphate that is used for the treatment of wine and alcoholic juices is regulated by the Bureau of Alcohol, Tobacco, Firearms, and Explosives and found under 27 CFR 24.246 (Prakash 2000).

Food-grade phosphates are used for various applications in food production. They may be used for chemical reasons such as adjusting pH; buffers; aiding or inhibiting coagulation; modifying proteins; or dispersing ingredients (Lampila and Godber 2001). Other functions of polyphosphates include: antioxidant activity; thickeners in dairy products; emulsification; color protection; and water binding (Lampila and Godber 2001). In North American baking and cereal production, there is a huge demand for food-grade phosphates. These phosphates are used for leavening of bakery products; pH adjustment; dough conditioning; and enrichment, among other functions (Lampila and Godber 2001). By the meat industry, polyphosphates are used for buffering; pH control; color development and stability; inhibition of oxidation; and water binding (Lampila and Godber 2001).

HURDLE TECHNOLOGY

Polyphosphates have a varying ability to effect bacteria and fungi. Great success has been achieved combining different temperature, pH, or other antimicrobials with polyphosphates. These combinations can be classified as multicomponent antimicrobial systems or hurdle techniques. Hurdle technology combines existing and novel preservation techniques to prevent the growth of any microbial contamination (Leistner and Gorris 1995). These hurdle factors, as stated previously, can range from pH and temperature to combining antimicrobials and specialized packaging.
Hurdle technology is used in almost every aspect of the food industry after being re-invented some 20 years ago in the meat industry (Leistner and Gorris 1995). Many fermented foods have intrinsic hurdle technology that is initiated by an extrinsic factor. For example, the preservatives added to salami will inhibit the growth of certain bacteria, while others will multiple, using up the oxygen, dropping the redox potential, and inhibiting aerobic bacteria (Leistner and Gorris 1995). From here the conditions favor lactic acid bacteria which produce acid, dropping the pH and further inhibiting competitive bacteria (Leistner and Gorris 1995). Other examples of products that use hurdle technology are pickled fruits and vegetables, pastas, meat products, and dairy products.

There are four basic aspects that support the implementation of hurdle technology. They are homeostasis, multiple targets, metabolic exhaustion, and stress reactions (Leistner 2000). Homeostasis is the tendency of a cell to maintain equilibrium or stability internally while dealing with external changes. In order for preservation techniques to be successful, they should have an effect on the homeostasis of an organism. If homeostasis is disrupted, then the cell will not multiply (Leistner 2000). Metabolic exhaustion is the phenomenon in which microbes use up energy exhausting every possible repair mechanism to achieve homeostasis (Leistner 2000). Stress reactions are very important in hurdle technology because they allow the microbe to withstand the stressors in the environment. However, if an organism is exposed to multiple stressors at once, the ability to produce responses to each stressor will be exhaust the energy supply of that cell (Leistner 2000). Attacking multiple targets with different antimicrobials could have a synergistic effect that repeatedly disrupts homeostasis.
By using different ‘hurdles’ to induce stress responses in microbes, food manufacturers are helping to ensure that food products are safe and free of viable microbes. The basis of hurdle technology is to disrupt cell homeostasis, induce metabolic exhaustion, and stress responses in order to eliminate the pathogens. These hurdles may be intrinsic or extrinsic to the foods and work best when in a series that attacks many different targets of the cell.

METHODS OF QUALITY MEASUREMENTS

Quality is the most important aspect of fruit juices. The effects of preservatives on the quality of fruit juice drinks must also be taken into account, because even if a treatment can eliminate the microbial load, if it contributes to unacceptable quality, no one will buy or consume the product and therefore it is useless. The effects of treatments on juice drinks can be measured in several ways. Below are listed several ways that the quality portrait of fruit juices can be measured.

Color

Color is an important attribute in juice for consumers. The color of beverages is very influential to flavor perceptions. For example, in one study 40% of participants identified a cherry-flavored beverage as orange when it was discolored as orange (Zampini et al. 2007). Color is typically measured by a Hunter L*a*b* colorimeter. This system measures lightness/darkness (L*), red/green (+/-a*), yellow/blue (+/-b*) characteristics of a color. The color difference, ΔE, between a sample and standard can also be calculated using the differences between L*, a*, and b*. Chrome and hue may also be calculated.
Aroma

Volatile compounds are another important aspect of foods. Consumers often base food choices on aroma, as an indicator of freshness. One way to measure the volatile compounds is to use gas chromatography (GC). One technique with GC is to test headspace, that is, the area in the package where volatile organic compounds have escaped from the food matrix. Static headspace sampling is conducted when a food sample is placed into a headspace vial, sealed, and allowed to reach equilibrium, then headspace is drawn out with a syringe and injected into the GC injection portal (Wampler 2001).

CURRENT RESEARCH

High Pressure Homogenization (HPH)

High pressure homogenization is when “fluid is forced under pressure through a small orifice and leaves the gap in the form of a radial jet that stagnates on an impact ring, thus leading to fluid micronization” (Bevilacqua et al, 2012). HPH has shown good promise in inactivating spoilage microbes and extending the shelf life in liquid foods (Patrignani et al. 2010). This technology causes cells membranes to rupture causing microbial inactivation (Bevilacqua et al. 2012). One study showed that combining homogenization and antimicrobials extended shelf life, with homogenization reducing initial contamination while antimicrobials control growth during storage (Bevilacqua et al. 2012).

High Pressure Processing (HPP)

High Pressure Processing (HPP) inactivates the enzymes responsible for pectin degradation (Nienaber and Shellhammer 2001). HPP also destroys pathogenic as well as
spoilage organisms. HPP allows the product to retain a fresh-like quality because of the minimal damage it causes (Nienaber and Shellhammer 2001). It has been reported that spoilage yeasts were undetectable after treatment at 350 Mpa for 30.0°C for 1 minute (Nienaber and Shellhammer 2001). In orange juice and a model apple juice system, D-values ranged from 8 s to 10.8 min at 500 and 300 Mpa (Zook et al. 1999).

Ozone

Ozone is more effective than chlorine and works against gram-positive and gram-negative bacteria, viruses, fungi, spores, and protozoa (Cullen et al. 2010). Ozone is mainly applied in gaseous form or in ozonated water, depending on the food product (Cullen et al. 2010). Fruit juice is treated by injecting ozone gas into the juice (Cullen et al. 2010). Ozone can be used to detoxify and eliminate some mycotoxins and pesticides (Kim et al. 1999). Researchers found that Candida parapsilosis was reduced by 2 logs in 1.67 min when exposed to 0.23 to 0.26 mg/L ozone (Kim et al. 1999).

UV Treatment

UV treatment for fruit juices has been approved by U.S. FDA as an alternative to thermal pasteurization (Koutchma and Orlowska 2012). UV light can be used to treat air, water, nonfood and food contact surfaces, packaging, and fresh fruit and cut fruit surfaces (Koutchma and Orlowska 2012). Not only does UV treatment inactivate spoilage organisms, it can also inactivate enzymes that would affect color and cloud formation (Koutchma and Orlowska 2012). In one study, researchers determined that UV light at 230 J L⁻¹ resulted in a 3.0 log₁₀ reduction of yeasts and molds (Keyser et al. 2008).
However, vitamin destruction is a concern when it comes to treatment with ultraviolet light. Studies have found that vitamin C and vitamin A were reduced by 50% when treated with UV light (Koutchma and Orlowska 2012).

CONCLUSION

As this literature review has demonstrated, there are many packaging and preservative methods available for processed fruit juices. However, one strong disadvantage to these preservative methods is microbial resistance. Many spoilage yeasts have internal mechanisms that allow for the removal of these dangerous molecules from cytoplasm of the cell. Therefore, the objective of this study is to evaluate the effectiveness of fractionated peanut skin extract, whole peanut skin extracts, and polyphosphate combinations on inoculated populations of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* in modified apple juice medium and to evaluate the quality of the juice as a result of these treatments. The results of this study will be used to develop low dose combinations of natural antimicrobials to prevent the growth of spoilage yeasts on a large scale operation during processing, packaging, and distribution.

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CHAPTER 2: SODIUM POLYPHOSPHATE ENHANCES THE ANTIMICROBIAL ACTIVITIES OF WHOLE AND FRACTIONATED PEANUT SKIN EXTRACT AGAINST FOOD SPOILAGE YEASTS IN A MODEL JUICE SYSTEM

ABSTRACT

The efficacy of various fractions of Peanut Skin Extract (PSE) in combination with sodium polyphosphate (SPP; BekaPlus™ FS, BK Giulini) were tested in apple juice medium (AJM, a model juice system) against the economically important spoilage yeasts Zygosaccharomyces bailii and Zygosaccharomyces bisporus.

Z. bailii (ATCC 60483) or Z. bisporus (Y108, an industrial isolate) were inoculated in AJM or AJM containing SPP, PSE or combinations of SPP and PSE, then incubated at 30°C for up to 120 h (5 d). Both whole PSE and 9 individual HPLC-separated PSE fractions were tested. A Bioscreen C automated turbidimeter determined the impact of PSE on yeast growth. After 120 h, samples were taken from each Bioscreen well, serially diluted and plated onto Yeast Mold agar for enumeration. Initial experiments with Z. bailii were used to determine the minimum inhibitory concentrations (MICs) of SPP and whole PSE. A single sub-MIC concentration of SPP (0.013%) was then evaluated in combination with a concentration of whole PSE that was not inhibitory alone, yet yielded complete inhibition in the presence of 0.013% SPP. This concentration, 2 mg/mL, was chosen as a “benchmark” level for further testing of PSE fractions with and without 0.013% SPP against both Z. bailii and Z. bisporus. This rational testing approach enabled us to minimize variables, focus our screening approach and avoid wastage of valuable PSE samples.

Twenty-four treatments were tested (in duplicate) including: no-antimicrobial control (AJM only), AJM plus 0.013% SPP and the following PSE treatments with and without
0.013% SPP: whole PSE, nine HPLC-separated fractions derived from whole PSE and designated fractions A-I, and a catechin control. Uninoculated AJM served as an uncontaminated control.

This screen was replicated in 3 separate experiments and data were analyzed using SAS software. Three variables were analyzed: treatment, time, and treatment as a function of time (treatment*time). A Tukey pairwise comparison indicated that whole PSE and the fractions were significantly different from the control.

Endpoint data were analyzed to determine whether the PSE and SPP were killing the cells or keeping them static. The CFU/mL was determined after 120 h of treatment. Based on the significant P-values for both yeasts (P-values <0.0001), it was determined that the treatments had an effect on growth.

A “reconstituted” whole PSE comprised of known levels of each HPLC-separated fraction was formulated and compared to “natural” whole PSE, in which the levels of each component remain unknown. The comparison tested the hypothesis that the reconstituted whole PSE, containing normalized levels of each fraction, might be more inhibitory at lower concentrations than the natural whole PSE, whose activity might be dependent on one or more dominant fractions. However, our results indicated that both PSEs had identical activities against both spoilage yeasts with and without SPP.

The physical mode of action of whole PSE and select fractions against Z. bailii, with and without SPP was investigated at various timepoints during exposure in AJM using a dye exclusion assay and flow cytometric analysis, which was conducted in parallel with optical density and cell enumeration measurements, made over a 24 h period. Controls included AJM-only and SPP-only treatments, to evaluate the impact of the cell cycle and SPP alone on
membrane permeability. Although cell membranes became transiently permeable to propidium iodide at early points during the growth curve without PSE or SPP treatment, these treatments resulted in additional permeabilization of the cell membrane, which serves a primary barrier function for the cell.

Our data demonstrated increased inhibitory action of combined PSE and SPP suggests growth suppression results from the combined effects of two agents capable of permeabilizing the cell membrane. Based primarily on studies involving the effects of SPP on Gram-negative bacteria, the observed cell permeabilization is likely related to the chelation of structurally, nutritionally or metabolically essential cations. This chelation could therefore impact cell physiology (cell membrane permeabilization), availability of required metals (siderophore-like activity), or enzyme systems dependent on these metals as cofactors. It is possible that these antimicrobials also act at other points within the cell, but additional assay approaches, including genomic-based methods may be required for such determinations.

Although clear (and significant) effects/differences were seen between PSE-only and combined PSE-SPP treatments, whether these are additive or synergistic requires additional analyses. The Fractional Inhibitory Concentration (FIC) method integrates data from the MICs of two antimicrobials alone and from their combination to derive a point value indicative of one of three outcomes for antimicrobial interactions: antagonistic effects, additive effects or synergistic effects. A FIC value (1.6) was determined for the effect of whole PSE on Z. bailii, indicating an additive effect.

While the combination of various PSE treatments (whole PSE, fractions F, G, H, I) with SPP resulted in full growth suppression, final cell counts after 120 h indicated a maximum 3 log reduction of viable cells as compared to the control culture (whole PSE), with
other treatments yielding ~1 – 1.5 log reductions. These data indicate that while not all combinations are lethal to these spoilage yeasts, this approach is a promising method for suppressing the growth of contaminants, if present. This conclusion is fairly conservative, given that natural contaminants will likely be present at comparatively low levels, compared to the challenge level used here of $10^5$ CFU/300 μL AJM (roughly a contamination level of $10^8$ CFU per 12 oz container of a soda or juice beverage, an extremely unlikely contamination level for a processor following Good Manufacturing Practices).

The effects of the addition of PSE and SPP to full-strength apple juice and to AJM were analyzed using a Hunter L*a*b* system. Statistical analysis indicated that there was a significant difference between the colors within the groups. To evaluate the potential for negative organoleptic impact of the antimicrobial treatments on both apple juice and AJM, aroma was analyzed using gas chromatography upon the addition of SPP and PSE to both apple juice and AJM. No off aromas were detected in either liquid upon the addition of these compounds, suggesting that the organoleptic properties of the beverage or medium would not be affected by the antimicrobial treatments.

Together, our data suggest that the combined use of PSE and SPP is a promising natural approach for preserving juices or potentially other beverages at risk for spoilage by *Zygosaccharomyces* spp.

**INTRODUCTION**

From January 2011-September 2011, there were approximately 174 new fruit/vegetable juices and nectars introduced into the U.S. market (Canada 2011). The yeast spoilage of processed fruit juices requires immediate attention because of most of the spoilage yeasts are resistant to traditional preservatives used in the industry. According to Doyle
processed fruits and vegetables accounted for 8.6% of the food lost in the U.S. in 1995 (Doyle 2007). Recalls from beverages contaminated with yeasts and molds are rare and not highly publicized. However, in 2000 there were two different recalls of apple juice due to yeast and mold contamination, eight states and nationwide, respectively (Vasavada 2003). Because the average U.S. consumer drinks approximately 9 gallons of juice and 6 gallons of fruit beverages each year (Vasavada 2003), recalls are significant. Yeasts have high acid tolerance and are resistant to many preservatives, making them ideal juice spoilage organisms. Traditionally, fruit juice drinks undergoes various processing methods to prevent spoilage, including chemical preservation, canning, pasteurizing, freezing, or concentrating; with chemical preservatives being one of the most common. However, this poses a problem since some spoilage yeasts including *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* are resistant to widely used preservatives (Deak 2008). Consumer trends also highlight the avoidance to these chemical preservatives and are forcing food processors to explore more novel and natural preservative sources (Zink 1997).

Numerous alternative preservatives and preservative techniques are available for fruit juices, however there are disadvantages associated with each one (including cost, development of microbial resistance, detrimental effects on quality, etc), or they are not effective enough to prolong shelf life. Resistance and detrimental effects on quality are major considerations when deciding which preservative technique to use. Therefore, research is required to develop effective, natural preservative methods for prolonging the shelf lives of juices at risk for contamination with recalcitrant spoilage yeasts such as *Zygosaccharomyces* spp.
The focus of this study was to examine the potential for enhancing the antimicrobial efficacy of peanut skin extract (PSE) by combining it with sodium polyphosphate (SPP; BekaPlus™ FS), to formulate a preservative system for juices or similar beverages against spoilage by *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, using apple juice medium (AJM) as a model beverage. Potential advantages to this approach vs. traditional chemical preservatives include a “clean” label (through use of all-natural preservatives) and possibly, a lower likelihood of resistance for antimicrobials targeting a fundamental cellular barrier structure such as the cell membrane.

We hypothesized that either whole PSE or various HPLC-separated fractions could be combined with SPP to facilitate effective antimicrobial control of the spoilage yeasts *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* in a model beverage system. Further, we expected that additive or synergistic interactions between these two antimicrobials might allow their use at organoleptically acceptable levels, providing an effective, natural preservative system having minimal impact on desirable beverage attributes.

**MATERIALS AND METHODS**

**Experimental Design (General Overview)**

Peanut skin extract (PSE) and BekaPlus™ FS (SPP) were tested alone and in combination for their antimicrobial effects on the spoilage yeasts *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* using a modified apple juice medium (AJM). Including controls, 24 treatments were examined. Controls included AJM without antimicrobials or catechin, a polyphenolic compound used as a standard in quantification of similar molecules present as bioactive components of PSE. The combination of catechin and SPP was also
examined as a control. Test treatments included whole PSE or 9 HPLC-separated PSE fractions, with or without the addition of SPP. AJM was treated as described, inoculated with the challenge organisms (10^5 CFU/300 μL well) and incubated statically at 30°C for 120 h (5 d) in a Bioscreen C Microbiological Reader with optical density (OD) readings performed every 30 min. After 5 d, aliquots of each treatment were removed for plating onto Yeast Mold (YM) agar. The physiological impact of certain treatments was also examined using a spectrophotometric method (Virto et al. 2005), and cell permeability studies were done using a BD Accuri™ C6 flow cytometer to probe the mode of action for these treatments. The potential impact of PSE and SPP on quality attributes of AJM and full-strength apple juice were determined (at 0 and 5 d) using colorimetry and cryofocusing gas chromatography.

**Peanut Skin Extract Source and Standards**

Peanut skin extracts (whole and HPLC-fractionated) were prepared using the aqueous extraction method and fractionation approach described by Sarnoski et al. (2012). These extracts were received dried from Virginia Polytechnic Institute and State University (Blacksburg, VA, U.S.A.) and stored dry at 4°C until reconstituted in AJM for use. The (+)-Catechin standard was purchased from Sigma (St. Louis, Mo, U.S.A.). PSE and (+)-catechin were rehydrated in AJM, without the addition of organic solvents as carriers or miscibility agents, in order to eliminate any confounding contributions of these agents to the observed antimicrobial effects. A commercial preparation of SPP, BekaPlus™ FS, described as having a fungistatic effect at typical food usage levels, was obtained from BK Giuliani Corporation (Simi Valley, California, U.S.A.).
Yeast Cultures and Growth Conditions

The spoilage yeasts used in this study were: Z. bailii (ATCC 60483; American Type Culture Collection, Manassas, VA, U.S.A.) and Z. bisporus Y108 (obtained from scholarly source, VA Tech, Blacksburg, VA, U.S.A.). Before using in experiments, working cultures were grown overnight (18 – 24 h) at 30°C in AJM (single passage) and diluted into fresh AJM to the target inoculum level of $10^5$ CFU/mL, as determined by plating onto YM agar.

The modified apple juice medium (AJM) recipe used here was the same described by Sarnoski et al (2012). The apple juice medium recipe consists of the following: 100mL of apple juice (Motts brand 100% preservative free) purchased from Hy-Vee (Ankeny, IA, U.S.A.), with the addition 46.8g glucose, 59.4g fructose and 1.8g sucrose (Difco, Sparks, PA., U.S.A.). All ingredients were combined and brought to a volume of 1 L with deionized water and the pH was adjusted to 3.5 using 1M malic acid (Fisher Scientific) (Sarnoski et al. 2012). The AJM was filter-sterilized using VWR Vacuum filtration system (VWR International, Radnor, PA).

Reconstitution of Dried PSE and Automated Turbidimetry

Dried PSE samples were rehydrated in 1 ml portions of AJM, diluted 1:1,000 in AJM and the absorbance at 280 nm was measured for each preparation of whole PSE or PSE fraction and this absorbance was compared against a (+)-catechin standard curve, also prepared using AJM to provide a measurement of total phenolic content. These values were used to deliver PSE treatments (whole PSE and 9 individual HPLC-separated PSE fractions) to test wells (100-well honeycomb plates, Bioscreen C Microbiology Reader) at a final
concentration of 2 mg/mL. For treatments containing SPP, a final concentration of 0.013% SPP was used.

Growth curves were generated with the Bioscreen by measuring optical density at 600 nm (OD 600 nm) as a function of time. Controls consisted of AJM without antimicrobial additions, AJM containing SPP and AJM containing (+)-catechin with or without added SPP. Bioscreen plates were incubated at 30.0°C for 120 h (5 d) and Ods were measured at 30 min intervals, with shaking performed immediately prior to each read. Data were collected using EZ Experiment software (Growth Curves U.S.A., Piscataway, NJ., U.S.A.) and exported to Microsoft Excel (Microsoft, Seattle, W.A., U.S.A.) to generate growth curves. All treatments within each experiment were examined in duplicate.

**Endpoint Plating**

Growth suppression (fungistatic effects) or lethality (fungicidal effects) were examined for each treatment by plating from individual Bioscreen test wells onto Yeast Mold agar at the 120 h timepoint. Briefly, 100μL from each well was removed and added to 900μL 0.85% saline, vortexed and serially diluted. Ten-microliter portions were plated in duplicate using the track dilution method of Jett (1997) (Jett et al. 1997) and the number of CFU/mL determined after incubation at 30°C for 48 h. At the outset of each experiment the target inoculum level of 10⁵ CFU/mL was verified by plating, also using track dilution.

**Cell Leakage Assay**

The inhibitory mechanisms of PSE and BekaPlus™ SPP were examined using the spectrophotometric cell leakage assay described by Virto et al (2005). Briefly, cells (10⁸
CFUs/mL, washed with 0.85% saline) were exposed for up to 6 h to various PSE treatments with and without SPP, as well as to SPP alone. One-milliliter suspensions of cells exposed to each treatment were incubated 30°C for periods ranging from 0-6 h. At the appropriate time interval, 100μL of each suspension were removed and added to 200μL 0.85% saline and centrifuged for 10 min at 6,000 x g. The supernatant was removed and measured at both 260 nm and 280 nm. Increases in absorbance at 260 nm were interpreted as indicative of leakage of DNA from the cell, whereas those made at 280 nm were ascribed to release of intracellular proteins. Appropriate controls were used to subtract the contribution of PSE or SPP to absorbance at 260 and 280 nm. All measurements were performed in triplicate.

Comparison of “Natural” and “Reconstituted” Whole PSE

The inhibitory actions of two whole PSE treatments were examined. The first, “natural” whole PSE, was comprised of the crude/unfractionated PSE obtained using the aqueous extract process and containing unknown ratios of component compounds. “Reconstituted” whole PSE was comprised of all nine HPLC fractions of the crude starting materials, with each component fraction normalized to a concentration of 2mg/mL. The relative activities of these two preparations were compared via automated turbidimetry with or without the addition of 0.013% SPP.

Cell Permeability Assay

To test how the whole PSE, fractionated PSE, and sodium polyphosphate affect the cell membrane, a cell permeability study was carried out. In this experiment, a no-antimicrobial control, SPP alone and fractions H and I, with or without 0.013% SPP were examined. Fractions H and I were chosen for further study because of the unique growth
curve pattern displayed against Z. bailii, consisting of a steady increase in OD up to ~12 – 20 h, followed by a precipitous drop in OD to basal levels for the remainder of each 120 h observation period. Fractions F and G also demonstrated a similar behavior, but were present in lower yield than fractions H and I, which were chosen for the study. Bioscreen experiments were carried out as described above (10^5 CFU/mL Z. bailii inoculum, incubation at 30°C), with the exception that a 24 h observation period, which encompassed the “spike” and drop in OD observed earlier prior to the 20 h point. At designated time points (0, 6, 12, 18, 24 h) 160μL were removed from one of the three wells for cell permeability tests, while 10μL were removed for track plating. Triplicate wells (350μL total volume) were used for each treatment, with two of these wells used for destructive sampling (aliquot removal) at time points 6, 12 and 18, with the 3rd used for OD measurement up until the 24 h point, when aliquots were removed for testing. For cell permeability tests, cells were washed with 1mL dH2O, spun down at 14,000 x g for 3 min, and resuspended in 200μL 50mM sodium citrate buffer. Two microliters of 20mg/mL Dnase-free Rnase A was added to each sample and were incubated for 1.5 h at 37°C. Two hundred microliters of propidium iodide (PI, 16μL/mL stock solution) were added to each 200μL cell suspension and the mixture transferred to small polystyrene test tubes. Samples were examined using a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, C.A., U.S.A) on the “fast” setting. All time 0 measurements consisted of Z. bailii alone, without the addition of SPP or PSE.

**Calculation of Fractional Inhibitory Concentration (FIC)**

The type of interaction between whole PSE and SPP was determined through the calculation of the fractional inhibitory concentration (FIC). This FIC index can indicate
whether a combination displays a synergistic, additive, or antagonistic effect (Weinkauf 2009). The FIC can be calculated using the following formula:

\[
\text{FIC}_A = \frac{\text{MIC} \text{ (A in the presence of B)}}{\text{MIC of A alone}}
\]

\[
\text{FIC}_B = \frac{\text{MIC (B in the presence of A)}}{\text{MIC of B alone}}
\]

\[
\text{FIC} = \text{FIC}_A + \text{FIC}_B
\]

The FIC coefficient usually ranges from 0.5 to 4 (Weinkauf 2009). A coefficient of ≤0.5 indicates a synergistic effect, < 0.5 to 1 is an additive effect, > 1 to < 2 is an indifferent effect, and ≥ 2 is an antagonistic effect (EUCAST 2000). However, sometimes an indifferent effect is hard to distinguish from an additive effect, especially if one component is more active than the other (EUCAST 2000). The MIC is the minimum inhibitory concentration established through bioscreen and preliminary testing.

**Measurements of pH of test solutions**

Measurements of the pH of AJM, AJM and PSE, and AJM, PSE, and BekaPlus™ were taken before use. This was done by adding 2mL of each solution into a polystyrene test tube and measuring the pH with a calibrated pH meter (Denver Instrument, Bohemia, NY) fitted with a glass electrode.

**Color Measurements**

The AJM color was measured before and after PSE and BekaPlus™ were added. Glass cuvettes holding 300μL of AJM with or without 2mg/mL of whole PSE and 0.013% SPP were measured in Hunter colorimeter model XE L*a*b*. The Hunter L*a*b* were
measured under D65 light with a port size of 0.4” and an area view of 0.2” with D65 light and 10° standard observer.

Samples were read in triplicate and all the data were recorded. After replicates were completed, ΔE values for each sample v. apple juice or apple juice medium were calculated as follows for two colors \((L_1^*, a_1^*, b_1^*)\) and \((L_2^*, a_2^*, b_2^*)\):

\[
\Delta E_{ab} = \sqrt{\left(L_2^* - L_1^*\right) + \left(a_2^* - a_1^*\right) + \left(b_2^* - b_1^*\right)}
\]

Aroma Measurements Using Gas Chromatography

The AJM with and without PSE and SPP samples was tested for off aroma volatiles. The technique used was cryofocusing gas chromatography. In this technique, 300μL of sample was transferred to a headspace vial and allowed to equilibrate to room temperature for 2 h. The column was inserted into a liquid nitrogen filled Styrofoam cup. With the door open, the sample was injected at a rate of 1mL/min with the splitter off. After 30 s, the splitter was turned on. The column was removed from the liquid nitrogen after another 15 s; 15 s later, the GC and integrator were activated simultaneously. The door was shut after the GC reached “PR” stage. Readouts were produced and compared to readouts from traditional Motts apple juice and AJM.

Statistical Analysis

Microbial and color measurements were analyzed using 2-way ANOVA and Tukey-Kramer Honestly Significant Difference (T-K HSD) post-ANOVA testing. PSE and SPP efficacies were compared by T-K HSD at specific time periods. Data were analyzed using SAS 9.3 (SAS Institute, Cary, N.C., U.S.A.) using \(\alpha=0.05\).
RESULTS AND DISCUSSION

Comparison of studies with PSE and SPP to studies with PSE and no SPP

When comparing PSE fractions with and without SPP against Z. bailii, a 2-way ANOVA was performed. OD readings at specific times (18, 24, 68, 72, 98, 102 and 120 h) were averaged over 4 trials (each in duplicate). SAS 9.3 averaged the OD readings over these 7 time points within each treatment. These values were then compared for significant differences.

The results indicate that the variables “time” and “treatment” were significant (P values <0.0001 for both), however the variable “treatment*time” was not significant (P-value = 0.9932). For Z. bisporus, the variable “treatment” and “time” were significant (P-values <0.0001 and 0.0044), whereas the variable “treatment*time” was not significant (P-value=0.4818). These results are expressed in table 1 and 2 (below).

Since the time variable and the treatment variables were significant for Z. bailii this means that for each treatment the time was a factor and for time the treatment was a factor. However, since the interaction for Z. bailii was not significant it indicates that at each time there was not a difference between treatments. The same holds true for the studies down with Z. bisporus.

Table 1. Tukey distribution: Effects of whole and fractionated PSE on Z. bailii 60483 in apple juice medium (AJM) as a function of OD at 600nm.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.67ab</td>
</tr>
</tbody>
</table>

*LS-means with different letters are significantly different, and α=0.05
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + SPP</td>
<td>0.38de</td>
</tr>
<tr>
<td>B</td>
<td>0.77ab</td>
</tr>
<tr>
<td>B + SPP</td>
<td>0.39cde</td>
</tr>
<tr>
<td>C</td>
<td>0.62ab</td>
</tr>
<tr>
<td>C + SPP</td>
<td>0.30ef</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>0.57bcd</td>
</tr>
<tr>
<td>(+)-Catechin + SPP</td>
<td>0.25ef</td>
</tr>
<tr>
<td>Control</td>
<td>0.78a</td>
</tr>
<tr>
<td>D</td>
<td>0.58bc</td>
</tr>
<tr>
<td>D + SPP</td>
<td>0.30ef</td>
</tr>
<tr>
<td>E</td>
<td>0.63ab</td>
</tr>
<tr>
<td>E + SPP</td>
<td>0.23ef</td>
</tr>
<tr>
<td>F</td>
<td>0.16f</td>
</tr>
<tr>
<td>F + SPP</td>
<td>0.13f</td>
</tr>
<tr>
<td>G</td>
<td>0.14f</td>
</tr>
<tr>
<td>G + SPP</td>
<td>0.13f</td>
</tr>
<tr>
<td>H</td>
<td>0.15f</td>
</tr>
<tr>
<td>H + SPP</td>
<td>0.20ef</td>
</tr>
<tr>
<td>I</td>
<td>0.13f</td>
</tr>
<tr>
<td>I + SPP</td>
<td>0.15f</td>
</tr>
<tr>
<td>Whole PSE</td>
<td>0.79a</td>
</tr>
<tr>
<td>Whole PSE + SPP</td>
<td>0.13f</td>
</tr>
</tbody>
</table>

Table 2. Tukey distribution: Effects of whole and fractionated PSE on *Z. bisporus* Y108 in apple juice medium (AJM) as a function of OD at 600nm.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.01ab</td>
</tr>
</tbody>
</table>

*LS-means with different letters are significantly different, and α=0.05.*
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + SPP</td>
<td>1.06a</td>
</tr>
<tr>
<td>B</td>
<td>0.813abcd</td>
</tr>
<tr>
<td>B + SPP</td>
<td>0.793abcd</td>
</tr>
<tr>
<td>C</td>
<td>0.697cd</td>
</tr>
<tr>
<td>C + SPP</td>
<td>0.655d</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>0.939ab</td>
</tr>
<tr>
<td>(+)-Catechin + SPP</td>
<td>0.355ef</td>
</tr>
<tr>
<td>Control</td>
<td>0.926ab</td>
</tr>
<tr>
<td>D</td>
<td>0.631d</td>
</tr>
<tr>
<td>D + SPP</td>
<td>0.209efi</td>
</tr>
<tr>
<td>E</td>
<td>0.743bcd</td>
</tr>
<tr>
<td>E + SPP</td>
<td>0.213efi</td>
</tr>
<tr>
<td>F</td>
<td>0.348efi</td>
</tr>
<tr>
<td>F + SPP</td>
<td>0.185efi</td>
</tr>
<tr>
<td>G</td>
<td>0.370e</td>
</tr>
<tr>
<td>G + SPP</td>
<td>0.163fi</td>
</tr>
<tr>
<td>H</td>
<td>0.185efi</td>
</tr>
<tr>
<td>H + SPP</td>
<td>0.332efi</td>
</tr>
<tr>
<td>I</td>
<td>0.155fi</td>
</tr>
<tr>
<td>I + SPP</td>
<td>0.152i</td>
</tr>
<tr>
<td>Whole PSE</td>
<td>0.759bcd</td>
</tr>
<tr>
<td>Whole PSE + SPP</td>
<td>0.152i</td>
</tr>
</tbody>
</table>

The Tukey analysis of *Z. bailii* data indicated that every fraction, in addition to whole PSE and the (+)-catechin standard, performed differently (lower OD reading) from the control upon the addition of SPP over the 120 h period (Figure 2, below; Appendix, Figure 5). This is in contrast to the studies without SPP where 5 fractions (D, F, G, H, and I) and the (+)-
catechin standard performed differently (lower OD reading) from the control over 120 h (Figure 1, below; Appendix, Figure 5). When the same analysis was carried out with the *Z. bisporus* data, 7 of the 9 fractions, in addition to whole PSE and the (+)-catechin standard, were different (lower OD reading) from the control upon the addition of SPP (Figure 3, below; Appendix Figure 6), whereas in the studies without SPP only 6 of the 9 fractions (C, D, F, G, H, and I) reacted differently (lower OD reading) (Figure 4, below; Appendix, Figure 6). Sarnorski *et al* (2012) demonstrated that whole PSE completely inhibited *S. cerevisiae* at 10 mg/mL over 120 h. In the present study, it was demonstrated that the addition of 0.013% SPP to 2mg/mL whole PSE or select PSE fractions was sufficient to inhibit the growth of both *Z. bailii* and *Z. bisporus*. These data indicate that the addition of sodium polyphosphate allows effective use of lower levels of PSE in a model juice system.

![Figure 1](image-url). Effects of 2mg/mL various PSE treatments without 0.013% SPP on *Zygosaccharomyces bailii* in Modified Apple Juice Medium at 30°C over 120 h

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**Figure 1.** Effects of 2mg/mL various PSE treatments without 0.013% SPP on *Zygosaccharomyces bailii* in modified apple juice medium at 30°C over 120 h
**Figure 2.** Effects of 2mg/mL various PSE treatments with 0.013% SPP on *Zygosaccharomyces bailii* in modified apple juice medium at 30°C over 120 h

**Figure 3.** Effects of 2mg/mL various PSE treatments without 0.013% SPP on *Zygosaccharomyces bisporus* in modified apple juice medium at 30°C over 120 h
Effects of 2mg/mL Various PSE Treatments with 0.013% SPP on Zygosaccharomyces bisporus in Modified Apple Juice Medium at 30°C over 120 h

Figure 4. Effects of 2mg/mL various PSE treatments with 0.013% SPP on Zygosaccharomyces bisporus in modified apple juice medium at 30°C over 120 h

Enumeration (CFU/mL) of *Z. bailii* after exposure to each treatment was determined by plating on YM agar. According to Student’s t-test, the different treatments significantly impacted final cell counts (P <0.0001). According to Tukey’s distribution, 8 of the 9 fractions, plus whole PSE and the (+)-catechin standard, with SPP were significantly different from the *Z. bailii* control after 120 h. In contrast, in the studies without SPP, none of the fractions, whole PSE, or the catechin standard were different from the control (Table 3, below; Appendix, Figure 7).

Table 3. Tukey Distribution: Average effects of treatments on log CFU/mL of *Z. bailii* before and after 120 h incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean log CFUs/mL and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Z. bailii</em>-0 h</td>
<td>6.24cde</td>
</tr>
<tr>
<td><em>Z. bailii</em>-120 h</td>
<td>7.22a</td>
</tr>
<tr>
<td><em>Z. bailii</em> + SPP</td>
<td>5.79ef</td>
</tr>
</tbody>
</table>

*Means with different letters are significantly different, and α=0.05.*
As with *Z. bailii*, enumeration of *Z. bisporus* was performed after 120 h incubation, and again, the Student’s t-test indicated that the different treatments significantly impacted final cell counts (*P* <0.0001). Several of the treatments (5 of the 9 fractions, whole PSE, and catechin standard) were different from the *Z. bisporus* control upon the addition of SPP, while none of the treatments without SPP were significantly different from the control. These data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean log CFUs/mL and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.93ac</td>
</tr>
<tr>
<td>A+SPP</td>
<td>5.66ef</td>
</tr>
<tr>
<td>B</td>
<td>7.11abd</td>
</tr>
<tr>
<td>B+SPP</td>
<td>6.49ace</td>
</tr>
<tr>
<td>C</td>
<td>6.87ac</td>
</tr>
<tr>
<td>C+SPP</td>
<td>6.27cde</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>6.76ac</td>
</tr>
<tr>
<td>(+)-Catechin + SPP</td>
<td>4.51ij</td>
</tr>
<tr>
<td>D</td>
<td>6.95ad</td>
</tr>
<tr>
<td>D+SPP</td>
<td>6.06bce</td>
</tr>
<tr>
<td>E</td>
<td>6.96ac</td>
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<tr>
<td>E+SPP</td>
<td>5.98cf</td>
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<td>6.80ace</td>
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<td>F+SPP</td>
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<td>6.68ace</td>
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<td>G+SPP</td>
<td>5.81ef</td>
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<tr>
<td>H</td>
<td>6.60ace</td>
</tr>
<tr>
<td>H+SPP</td>
<td>6.12cde</td>
</tr>
<tr>
<td>I</td>
<td>6.60ace</td>
</tr>
<tr>
<td>I+SPP</td>
<td>5.18fi</td>
</tr>
<tr>
<td>Whole PSE</td>
<td>6.77ac</td>
</tr>
<tr>
<td>Whole PSE + SPP</td>
<td>4.17j</td>
</tr>
</tbody>
</table>
are summarized in Table 4 (below; Appendix Figure 8), along with the Tukey pairwise comparison results.

Table 4. Tukey Distribution: Average effects of treatments on log CFU/mL of *Z. bisporus* before and after 120 h incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Log CFU/mL and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Z. bisporus</em> – 0 h</td>
<td>6.59efg</td>
</tr>
<tr>
<td><em>Z. bisporus</em> – 120 h</td>
<td>8.29ad</td>
</tr>
<tr>
<td><em>Z. bisporus</em> + SPP</td>
<td>7.43be</td>
</tr>
<tr>
<td>A</td>
<td>8.34abc</td>
</tr>
<tr>
<td>A + SPP</td>
<td>8.22abc</td>
</tr>
<tr>
<td>B</td>
<td>8.96a</td>
</tr>
<tr>
<td>B + SPP</td>
<td>8.34abc</td>
</tr>
<tr>
<td>C</td>
<td>8.31ab</td>
</tr>
<tr>
<td>C+SPP</td>
<td>7.33bdf</td>
</tr>
<tr>
<td>Catechin</td>
<td>8.26ab</td>
</tr>
<tr>
<td>Catechin+SPP</td>
<td>6.03ij</td>
</tr>
<tr>
<td>D</td>
<td>8.02abc</td>
</tr>
<tr>
<td>D+SPP</td>
<td>6.32ab</td>
</tr>
<tr>
<td>E</td>
<td>8.18ab</td>
</tr>
<tr>
<td>E+SPP</td>
<td>6.22fi</td>
</tr>
<tr>
<td>F</td>
<td>7.86abc</td>
</tr>
<tr>
<td>F+SPP</td>
<td>6.10fj</td>
</tr>
<tr>
<td>G</td>
<td>7.90abc</td>
</tr>
<tr>
<td>G+SPP</td>
<td>6.89cefi</td>
</tr>
<tr>
<td>H</td>
<td>7.75abc</td>
</tr>
<tr>
<td>H+SPP</td>
<td>6.63efi</td>
</tr>
<tr>
<td>I</td>
<td>7.48bde</td>
</tr>
<tr>
<td>I+SPP</td>
<td>6.71efi</td>
</tr>
<tr>
<td>Whole PSE</td>
<td>8.17ab</td>
</tr>
<tr>
<td>Whole PSE + SPP</td>
<td>5.16j</td>
</tr>
</tbody>
</table>

*Means with different letters are significantly different, and $\alpha=0.05$. 
These plating results indicate similar trends between results for *Z. bailii* and those for *Z. bisporus*. Based on the Bioscreen data, several of the fractions (F, G, H, I) without SPP showed a steep “spike” in OD values prior to the 20 h timepoint, followed by a rapid decline in absorbance, suggesting cell lysis in the presence of these fractions. Potential explanations for the spike in OD values include increased susceptibility related to cell cycle/growth phase or cumulative exposure leading to lethality prior to the 20 h timepoint. Plating data indicated that after 120 h, the number of viable cells remaining in these treatments was not significantly different from the original inoculum level. It is interesting to note that a basal level of viable cells remained in these treatments. Whether these represent an intrinsically resistant subpopulation or are able to persist beyond 5 d incubation is not known.

When comparing the two yeasts, 8 of the 9 fractions, whole PSE, and catechin standard upon the addition of SPP tested against *Z. bailii* were significantly different from the *Z. bailii* control after 120 h, whereas 5 of the 9 fractions, whole PSE, and catechin standard with the addition of BekaPlus™ against *Z. bisporus* were significantly different from the *Z. bisporus* control after 120 h. These data indicate that *Z. bisporus* is a more challenging target organism compared to *Z. bailii* for this antimicrobial system, at least for the two strains analyzed.

Overall, the addition of 0.013% SPP to 2mg/mL of whole and fractionated PSE samples was more effective than using 2mg/mL PSE alone. Because these fractions (or whole PSE, see below) were effective at inhibiting these yeasts under optimal growth conditions, our data suggest that their combination with 0.013% BekaPlus FS could be a promising alternative to the chemical preservatives currently used in fruit juice drinks.
Comparison of Whole (unfractionated) and Reconstituted PSE

Whole and a reconstituted whole PSE consisting of known levels of each fraction were compared with each other. The rationale for this comparison were two-fold. First, because the levels of each individual fraction within the whole PSE were unknown, it is possible that the effects observed for whole PSE were dominated by the most abundant fractions. Second, it is possible that one or more essential fractions present in the whole PSE were unaccounted for in the series of fractions isolated via HPLC. Our data indicate that potential biases resulting from differing fraction yields were present and that all essential fractions present in the whole PSE were accounted for in the fractionated samples. The results from the comparison of whole PSE and the reconstituted PSE for *Z. bailii* indicate that the “time” and “treatment” variables were significant (P-value <0.0001), and the interaction variable “time*treatment” was significant (P-value <0.0001). This means that at each time the treatments react differently from each other. These results indicate that the whole (unfractionated) PSE and reconstituted PSE do not react differently from one another upon the addition of SPP, but react significantly different from the control (lower OD). These results are illustrated in table 5 (below). The impact of color change on OD values was not examined, but no clouding or precipitate formation were seen in these treatments, suggesting that this effect was negligible.

Table 5. Tukey Distribution: LS-means effects of whole and reconstituted PSE with and without sodium polyphosphate on OD readings of *Z. bailii* 60483 over 120 h period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014mg/mL Reconstituted PSE</td>
<td>0.772ab</td>
</tr>
<tr>
<td>0.00008mg/mL Whole PSE</td>
<td>0.756ab</td>
</tr>
<tr>
<td>0.10mg/mL Whole PSE</td>
<td>0.755abc</td>
</tr>
<tr>
<td>0.0003mg/mL Whole PSE</td>
<td>0.751bc</td>
</tr>
</tbody>
</table>

*Means with same letter are not significantly different, and α=0.05.*
Table 5. (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.016mg/mL Whole PSE</td>
<td>0.741bc</td>
</tr>
<tr>
<td>3mg/mL Reconstituted PSE</td>
<td>0.729bc</td>
</tr>
<tr>
<td>Z. bailii</td>
<td>0.726bc</td>
</tr>
<tr>
<td>0.002mg/mL Reconstituted PSE</td>
<td>0.725c</td>
</tr>
<tr>
<td>0.00005mg/mL Reconstituted PSE</td>
<td>0.723c</td>
</tr>
<tr>
<td>0.58mg/mL Whole PSE</td>
<td>0.607d</td>
</tr>
<tr>
<td>20.8mg/mL Whole PSE</td>
<td>0.155e</td>
</tr>
<tr>
<td>3.4mg/mL Whole PSE</td>
<td>0.149fe</td>
</tr>
<tr>
<td>20.8mg/mL Whole PSE + SPP</td>
<td>0.135fei</td>
</tr>
<tr>
<td>3.4mg/mL Whole PSE + SPP</td>
<td>0.113fei</td>
</tr>
<tr>
<td>0.58mg/mL Whole PSE + SPP</td>
<td>0.104fi</td>
</tr>
<tr>
<td>0.00005mg/mL Reconstituted PSE</td>
<td>0.104fi</td>
</tr>
<tr>
<td>18mg/mL Reconstituted PSE + SPP</td>
<td>0.102i</td>
</tr>
<tr>
<td>0.10mg/mL Whole PSE + SPP</td>
<td>0.100i</td>
</tr>
<tr>
<td>0.00008mg/mL Whole PSE + SPP</td>
<td>0.100i</td>
</tr>
<tr>
<td>0.003mg/mL Whole PSE + SPP</td>
<td>0.099i</td>
</tr>
<tr>
<td>0.016 mg/mL Whole PSE + SPP</td>
<td>0.098i</td>
</tr>
<tr>
<td>0.014mg/mL Reconstituted PSE + SPP</td>
<td>0.098i</td>
</tr>
<tr>
<td>0.5mg/mL Reconstituted PSE + SPP</td>
<td>0.097i</td>
</tr>
<tr>
<td>0.002mg/mL Reconstituted PSE + SPP</td>
<td>0.093i</td>
</tr>
<tr>
<td>0.00005mg/mL Reconstituted PSE + SPP</td>
<td>0.093i</td>
</tr>
<tr>
<td>18mg/mL Reconstituted PSE</td>
<td>0.092i</td>
</tr>
<tr>
<td>0.0003mg/mL Reconstituted PSE + SPP</td>
<td>0.092i</td>
</tr>
</tbody>
</table>

Results from the study with *Z. bisporus* were the same as with *Z. bailii*. The “treatment” and “time” variables were significant (P-value <0.0001 for both), and the interaction variable was significant (P-value <0.0001). This indicates that at each time the treatments had a different reaction (higher or lower OD reading) from each other. Without the addition of the SPP, several of the reconstituted PSE samples were not significantly different from the control; however several concentrations of whole PSE without SPP did
react differently from the control (lower OD). Again, although several of the concentrations of the reconstituted reacted differently from the control upon the addition of SPP, they did not react differently from the whole PSE upon the addition of SPP. The results from the study with Z. bisporus are displayed in table 6 (below).

Table 6. Tukey Distribution: LS-means effects of whole and reconstituted PSE with and without sodium polyphosphate on OD readings of Z. bisporus Y108 over the 120 h period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS-Means and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5mg/mL Reconstituted PSE</td>
<td>0.894a</td>
</tr>
<tr>
<td>Z. bisporus</td>
<td>0.8941a</td>
</tr>
<tr>
<td>0.00005mg/mL Reconstituted PSE</td>
<td>0.881a</td>
</tr>
<tr>
<td>0.0003mg/mL Reconstituted PSE</td>
<td>0.876a</td>
</tr>
<tr>
<td>0.014mg/mL Reconstituted PSE</td>
<td>0.874a</td>
</tr>
<tr>
<td>0.002mg/mL Reconstituted PSE</td>
<td>0.872a</td>
</tr>
<tr>
<td>0.0005mg/mL Whole PSE</td>
<td>0.844a</td>
</tr>
<tr>
<td>0.00008mg/mL Whole PSE</td>
<td>0.758ab</td>
</tr>
<tr>
<td>3mg/mL Reconstituted PSE</td>
<td>0.674b</td>
</tr>
<tr>
<td>0.016mg/mL Whole PSE</td>
<td>0.374c</td>
</tr>
<tr>
<td>0.10mg/mL Whole PSE</td>
<td>0.374cd</td>
</tr>
<tr>
<td>3.4mg/mL Whole PSE</td>
<td>0.322cde</td>
</tr>
<tr>
<td>20.8mg/mL Whole PSE</td>
<td>0.247cdef</td>
</tr>
<tr>
<td>0.58mg/mL Whole PSE</td>
<td>0.207def</td>
</tr>
<tr>
<td>3mg/mL Reconstituted PSE + SPP</td>
<td>0.176ef</td>
</tr>
<tr>
<td>20.8mg/mg Whole PSE + SPP</td>
<td>0.139f</td>
</tr>
<tr>
<td>0.58mg/mL Whole PSE + SPP</td>
<td>0.122f</td>
</tr>
<tr>
<td>0.016mg/mL Whole PSE + SPP</td>
<td>0.115i</td>
</tr>
<tr>
<td>3.4mg/mL Whole PSE + SPP</td>
<td>0.114f</td>
</tr>
<tr>
<td>0.10mg/mL Whole PSE + SPP</td>
<td>0.114f</td>
</tr>
<tr>
<td>0.00008mg/mL Whole PSE + SPP</td>
<td>0.113f</td>
</tr>
</tbody>
</table>

*Means with same letter are not significantly different, and α=0.05.
Although evaluation of individual HPLC fractions allowed us to observe unique phenomena contributed by individual fractions (i.e. the “spike” phenomena seen in fraction F, G, H and I), the use of reconstituted PSE offers no apparent practical benefits over whole PSE.

**Cell Leakage Assay**

The results from the cell leakage assay performed on the *Z. bailii* were inconclusive. Although leakage of cellular components could not be demonstrated, we sought to perform additional tests to probe for changes in cell membrane permeability, as these could have substantial physiological impact on cells.

**Cell Permeability Results**

It was hypothesized that treatment of *Z. bailii* cells with PSE and/or SPP might modulate cell permeability, as similar polyphenolic extracts (i.e. grape seed extract) have been shown to permeabilize bacteria such as *Listeria monocytogenes* (Bisha et al. 2010), and SPP
has been shown to permeabilize other yeasts, such as *Candida albicans* to exogenous compounds, such as essential oils (Weinkauf 2009). To test this hypothesis, cell permeability (propidium iodide [PI] dye exclusion assay) was measured over the course of 24 h.

Surprisingly, the control culture of *Z. bailii* (no antimicrobials) showed intrinsic permeability to PI at both the 6 and 12 h timepoints, with an intact membrane (effective exclusion of PI) observed at all subsequent timepoints (18 and 24 h). This observation could have resulted from carry-over of dead or moribund cells present in the inoculum or from transient changes in cell membrane permeability as a feature of the cell cycle. The latter explanation is likely, as at times 0, 18 and 24 h, cell membranes were largely intact, with permeability in the control seen only at time points 6 and 12 h (Figure 9, Appendix). To avoid the confounding influence of this observed feature of *Z. bailii* physiology, determinations of antimicrobial-mediated permeability were made at timepoints other than at 6 and 12 h. The effects of SPP alone on cell permeability were similar at both 18 and 24 h, with ~83% of the cell population being permeabilized to PI (Figure 5).

![Figure 5. Comparison of control treatment (no antimicrobial additions, panel A) after 24 h](image-url)
growth in Apple Juice Medium (AJM) and addition of sodium polyphosphate (BekaPlus FS, 0.013%, panel B) on permeability of Zygosaccharomyces bailii ATCC 60483 to the cell integrity probe propidium iodide (PI). Panel A indicates that a small portion of Z. bailii cells (6.5% of the total population) were intrinsically permeable to PI at the 24 h timepoint. However, 84% of the cells grown in the presence of 0.013% SPP for the same amount of time were permeabilized to PI. These data indicate that SPP alone has the ability to permeabilize Z. bailii to exogenous compounds, suggesting its role as an effective component of a multistep hurdle system.

Fraction H with and without SPP appeared similar at both 18 and 24 h, with a dramatic increase in PI fluorescence seen for the combination of H and SPP at both timepoints. As an example, the geometric mean (red fluorescence channel) measurement for fraction H alone at 18 h was 3,185; for fraction H + SPP the geometric mean channel number was 17,693.

The extent of permeability caused by fraction I was similar with and without SPP after 24 h, with a tighter distribution (more homogeneous permeabilization of the population) seen for I + SPP. At 18 h, combination of SPP and fraction I resulted in two populations, with a subpopulation (~50% of the cells) having a greater permeability (geometric mean channel number 1.88 x 10^5) than that seen in the single population obtained with fraction I alone (geometric mean channel number 15,663) (Figure 6).
Figure 6. Effects of PSE fraction I without and with sodium polyphosphate (BekaPlus FS, 0.013%) on permeability of Zygosaccharomyces bailii ATCC 60483 to the cell integrity probe propidium iodide (PI). Cells grown for 18 h in the presence of 2 mg/mL PSE fraction I alone (panel A) and with the addition of 0.013% SPP (panel B) were stained with PI as described in Materials and Methods. While treatment with PSE fraction I resulted in a single population of cells with increased permeability compared to the 18 h control (panel A), the combination of PSE fraction I and SPP (panel B) yielded a bimodal distribution where an additional ~50% of the cells displayed substantially increased fluorescence/permeability. These data clearly demonstrate the cooperative cell-permeabilizing effects of PSE fraction I and 0.013% SPP.

For whole PSE without SPP, approximately 40% of the population at both times 18 and 24 h were permeabilized (Figure 7). With the addition of SPP, a greater portion of the total population was permeabilized. Specifically, at 18 h, ~69% of the total population was permeabilized. This increased to 79% of the total population at 24 h (Figure 7).
Figure 7. Effects of whole PSE without and with sodium polyphosphate (BekaPlus FS, 0.013%) on permeability of Zygosaccharomyces bailii ATCC 60483 to the cell integrity probe propidium iodide (PI). Cells grown for 24 h in the presence of 2 mg/mL whole PSE (panel A) and with the addition of 0.013% SPP (panel B) were stained with PI as described in Materials and Methods. While treatment with whole PSE resulted in uptake of PI by ~40% of the cells (panel A), ~80% of the cells were permeabilized in the presence of both agents, indicating increased permeabilization of Z. bailii cells in the presence of both compounds.

In sum, cell permeability testing demonstrated that whole PSE and fractions H and I were able to permeabilize the cell membrane of Z. bailii to PI, as was SPP alone. The combination of SPP and PSE treatments resulted in enhanced membrane permeability, indicating that these treatments were working in concert against this cellular target. Increased exposure time to the combination of whole PSE and SPP resulted in increased permeability. Because the cell membrane is a fundamental cell structure, agents capable of interfering with its barrier functions can be expected to affect cell essential cell processes, resulting in reduced cellular fitness. The energy-dependent (pump) mechanisms demonstrated for Z. bailii resistance to weak acid preservatives require an intact membrane/membrane potential to function (Erickson and Mckenna 1999). It may be possible that the membrane-permeabilizing effect of SPP and PSE may preclude this or similar pump mechanisms from acting effectively
on this combination of natural antimicrobials, a premise that should be examined in future experiments.

**Calculation of FIC**

The FIC was calculated for 2mg/mL whole PSE plus 0.013% SPP:

\[
FIC_A = \frac{2\text{mg/mL}}{2.97\text{mg/mL}} = 0.673
\]

\[
FIC_B = \frac{0.019\text{mg/mL}}{0.02\text{mg/mL}} = 0.95
\]

\[
FIC = 0.673 + 0.95 = 1.6
\]

Based on the FIC coefficient of 1.6, according to EUCAST, this would fall into the indifference effect for the combination. However, according to Weinkauf, it would be termed as an additive effect since it falls between 0.5 and 4 points (Weinkauf 2009). This is evident in the other data that is presented because of the whole PSE and SPP decreases ~2 log from the original concentration of *Z. bailii* cells vs. just SPP by itself only decreases cells ~1 log and whole PSE by itself increases ~0.5 log.

**pH Measurement Results**

Student’s t-test was used to compare pH data. First, AJM was compared with AJM + 2mg/mL Whole PSE + SPP. Based on these results, it was determined that they were not significantly different (P-value = 0.2567). Next, AJM was compared with AJM + 2mg/mL Whole PSE, these results indicated that there was a significant difference the pH of these two treatments (P-value = 0.0115). Finally, the treatments of AJM + 2mg/mL Whole PSE was compared to AJM + 2mg/mL PSE + SPP. Based on the results from the t-test, there was a significant difference between the two (P-value = 0.0160).
Table 7. pH changes in AJM upon the addition of 2mg/mL whole PSE and 0.013% SPP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH Measurement and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJM + 2mg/mL PSE + 0.013% SPP</td>
<td>3.35a</td>
</tr>
<tr>
<td>AJM + 2mg/mL PSE</td>
<td>3.29b</td>
</tr>
<tr>
<td>AJM</td>
<td>3.39a</td>
</tr>
<tr>
<td>10% SPP Stock Solution</td>
<td>5.82c</td>
</tr>
</tbody>
</table>

*Means with same letter are not significantly different, and α=0.05.

These results points to the thought that SPP has a buffering effect when added to the system. SPP is used in many aspects of the food industry already as a buffer and is displaying that effect in the AJM upon the addition of PSE.

Color Measurement Results

Student’s t-test was applied to the ΔE values (overall color differences between each treatment and the AJM or standard apple juice sample) and there was a significant difference observed among the treatments. There was a significant difference between the AJM and the AJM samples with SPP and PSE (the P value = 0.0006) and a significant difference between the apple juice samples and the apple juice with BekaPlus FS and PSE (P < 0.0001) (Table 8 and 9 below; Appendix, Figure 10 and 11). However, to the researchers there was no apparent color change.

Table 8. Tukey Distribution: Average ΔEab for modified apple juice media (AJM) with peanut skin extract and SPP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ΔEab and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJM + PSE</td>
<td>9.89a</td>
</tr>
<tr>
<td>AJM + PSE + SPP</td>
<td>7.18b</td>
</tr>
</tbody>
</table>

*Means with different letters are significantly different, and α=0.05.
Table 9. Tukey Distribution: Average ΔEab for modified Apple Juice, Peanut Skin Extract and SPP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ΔEab and grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple Juice + PSE</td>
<td>7.28a</td>
</tr>
<tr>
<td>Apple Juice + PSE + SPP</td>
<td>8.94b</td>
</tr>
</tbody>
</table>

*Means with different letters are significantly different, and α=0.05.

Off-Aroma Detection Using Gas Chromatography

The results from the gas chromatography study indicated that there were no off-aromas detected upon the addition of whole PSE, SPP, or a combination thereof. Studies were carried out in both in unmodified apple juice and AJM. This indicates that there will be no undesirable aromas if PSE and SPP are used as a preservative in apple juice.

The retention times of the aromas in PSE match up with the retention times of the aromas found in apple juice. This indicates that the only aromas found in PSE are from the apple juice. The SPP also does not give off any off aromas, alone or in combination with AJM and PSE.

Table 10. Retention times and areas for different aromas for each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Retention Time (min)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple Juice 1</td>
<td>4.06</td>
<td>4000</td>
</tr>
<tr>
<td></td>
<td>18.45</td>
<td>3000</td>
</tr>
<tr>
<td>Apple Juice 2</td>
<td>18.5</td>
<td>6953</td>
</tr>
<tr>
<td></td>
<td>18.6</td>
<td>16784</td>
</tr>
<tr>
<td>Peanut Skin Extract (PSE) 1 and 2</td>
<td>3.23</td>
<td>52037</td>
</tr>
<tr>
<td></td>
<td>18.69</td>
<td>2500</td>
</tr>
<tr>
<td>PSE 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AJM + PSE + SPP</td>
<td>18.71</td>
<td>3000</td>
</tr>
<tr>
<td>AJM + PSE + SPP</td>
<td>21.69</td>
<td>3520</td>
</tr>
<tr>
<td>Apple Juice + PSE</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PSE + SPP</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 10. (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Retention Time (min)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple Juice Medium + SPP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SPP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methanol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.7</td>
<td>9726</td>
</tr>
</tbody>
</table>

CONCLUSION

The addition of 0.013% SPP tested should be considered promising preservative in the juice beverage industry. The 2mg/mL of whole and fractionated samples of PSE are particularly effective against *Z. bailii* and *Z. bisporus* with the SPP, which was not the case in just the 2mg/mL of whole and fractionated samples of PSE with both yeasts. It was determined that cell permeability increases upon the addition of 0.013% sodium polyphosphate to whole PSE as well as two of the HPLC fractions (H and I). Although the instrumental measurements of color detected a significant difference, to researchers determined the color change was not extreme or undesirable. There was no significant difference in pH, possibly due to the buffering effect of the SPP. No undesirable or off-aroma were detected using cryofocusing gas chromatography, although depending on the cultivar or juice this could change. Peanut skin extract with the addition of SPP are a better choice that traditional preservatives such as sorbates and benzoates, to which most spoilage yeasts are resistant.
REFERENCES


Appendix

**Figure 8.** *Z. bailii*: Comparison of 2mg/mL of various treatments of PSE with and without the addition of 0.013% SPP averaged over 120 h. * indicates significant differences

**Figure 9.** *Z. bisporus*: Comparison of 2mg/mL of various treatments of PSE with and without the addition of 0.013% SPP averaged over 120 h. * indicates significant differences
**Figure 10.** Effects of 2mg/mL of various treatments of PSE with and without the addition of 0.013% SPP on CFU/mL of *Z. bailii* after 120 h. * indicates significant differences.

**Figure 11.** Effects of 2mg/mL of various treatments of PSE with and without the addition of 0.013% SPP on CFU/mL of *Z. bisporus* after 120 h. * indicates significant differences.
Figure 12. Impact of growth time on intrinsic permeability of Z. bailii

Figure 13. ∆L*, a*, b* values on full strength apple juice upon the addition of 2mg/mL PSE and 0.013% SPP
Figure 14. $\Delta L^*, a^*, b^*$ values on AJM upon the addition of 2mg/mL PSE and 0.013% SPP
CHAPTER 3: CONCLUSIONS AND FUTURE PERSPECTIVES

Due to issues with both consumer acceptability and microbial resistance, new alternatives to the chemical preservatives (i.e. benzoic and sorbic acids) traditionally used to prevent juice spoilage are needed (Fleet 1992). Naturally-occurring polyphenolic compounds, such as those present in grape seed and peanut skin extracts may have promising applications as hurdles in multicomponent antimicrobial preservative systems for the control of yeasts such as *Z. bailii* and *Z. bisporus*.

Specifically, the present study has demonstrated that the combination of PSE and SPP can inhibit these yeasts in a model juice system. With very slight impacts on juice quality (color, as measured by Hunter L*a*b), this combination was able to increase yeast cell permeability and effectively inhibit growth in this juice system. As PSE is a naturally-derived antimicrobial and sodium polyphosphate has Generally Recognized as Safe (GRAS) status, this combination may be able to serve as a substitute for traditional chemical preservatives to which many *Zygosaccharomyces* spp. are resistant (Erickson and Mckenna 1999; Warth 1977; Praphailong and Fleet 1997; Deak 2008), resulting in a “clean” label.

Depending on the treatment used, growth of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* growth was inhibited and in some cases, the number of surviving cells reduced. Compared to the AJM-only controls, whole PSE in combination with 0.013% SPP resulted in a ~3 log reduction in final CFU/mL for both *Z. bailii* and *Z. bisporus*.

Additional research should focus on optimization of PSE-SPP treatments, to yield a preservative system acceptable to both juice producers and consumers. For example, other
polyphosphates should be evaluated to determine whether they may have different or improved effects against these yeasts and the impact of reduced pH as an additional hurdle variable should be examined. Microscopic observations should be made to determine the gross physiological impact, if any, of these treatments on yeast structure, including those resulting from programmed physiological responses (formation of hyphae, apoptosis). The immunogenic potential of PSE, as isolated here, should be tested to evaluate the risk to sensitive consumers. This is of particular importance to safeguard the safety of children, who are simultaneously the most susceptible to peanut allergens and also the largest consumer of juices.

For the most promising PSE treatments, test periods longer than 5 days should be evaluated to probe the long-term efficacy of this approach and its likely utility as a preservative under realistic shelf storage conditions, under both ambient and refrigeration conditions, where yeasts are expected to grow much more slowly. Although relatively high inocula ($10^5$ CFU/well) were used here, it is expected that naturally-occurring contamination would result in much lower inoculum levels (~1-100 cells). The relatively high inoculum levels used in this study provided a conservative estimation of efficacy for these SPP-PSE combinations; these systems would be expected to have increased efficacy against the low-level inocula resulting from natural routes of contamination (drippage, splashing, aerosolization in liquids or on solid debris such as dust, etc.). The potential for development of resistance against SPP-PSE systems should also be examined. A key resistance mechanism that renders Zygosaccharomyces spp. resistant to weak acid preservatives relies on energy-dependent efflux pumps. Treatments such as SPP-PSE permeabilize the membrane and could interfere with transmembrane potential. SPP-PSE systems may therefore be intrinsically less
susceptible to such resistance mechanisms, an hypothesis that should be experimentally addressed. The degree to which treatment with SPP, PSE or SPP-PSE combinations are able to depolarize yeast cell membranes should be examined via flow cytometry using appropriate fluorescent probes.

Since pathogenic bacteria, such as *E. coli O157:H7*, continue to be problematic in juices and ciders, PSE and SPP should be tested to determine efficacy against these bacteria. Sodium polyphosphates have already been tested against Gram-negative bacteria and proven to be effective permeabilizers of the outer membrane (OM), the principle barrier structure in Gram-negative bacteria. The combination of SPP, PSE and low pH or mild thermal treatment may allow the design of effective, more natural and less harsh treatments for multi-log reductions of pathogenic bacteria in cider systems.

From our research, it is suggested that 2mg/mL of PSE in conjunction with 0.013% SPP may be useful as a replacement for the traditional preservatives used in apple juice to prevent the growth of spoilage yeasts. This approach may also be useful in other juice systems, providing an alternative, natural means for reducing spoilage losses in the juice industry.

References


