Inactivation of enteric pathogens and natural microflora in pineapple juice with added Isoeugenol and Yucca extract

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ABSTRACT

Studies were conducted to determine the effectiveness of isoeugenol (ISO-EU) at 0 (control) and 0.50, 0.75, 1.0 and 1.5 μL mL^{-1} for inactivating three enteric pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*) and indigenous spoilage microflora in Tyndallized and raw pineapple juice (PJ) respectively, during refrigerated (4 °C) storage of the juice. Sub-lethal injury of pathogen survivors in PJ and the effect of ISO-EU on sensory characteristics of PJ were also evaluated. Batches of Tyndallized PJ with added ISO-EU and YEX (0.5% w/v) were each inoculated with a five-strain mixture of one pathogen to obtain ~7.1 log CFU mL^{-1}. Non-inoculated batches of raw PJ were used for sensory analysis and monitoring the indigenous microflora namely, yeast and molds (YM), aerobic plate count (APC), lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT). Cultural methods using non-selective and appropriate selective agar media were used to enumerate microbial survivors. At 4ºC, all three enteric pathogens survived for more than 42 days in the control PJ; however, within 24 h, all ISO-EU treatments tested inactivated >5 log of each pathogen. After 2 h, ISO-EU treatments caused sub-lethal injury in survivors of all three pathogens. Initial populations of all four groups of indigenous microflora decreased significantly ($P<0.05$) in PJ containing ISO-EU and YEX with the most rapid decrease occurring in the ENT group. Although the appearance (color), odor and viscosity of both Tyndallized and raw PJ, each with ISO-EU (0.5 μL mL^{-1}) and YEX, were acceptable, taste and overall acceptance of those juices were unsatisfactory ($P<0.05$). Sensory scores for all characteristics of ISO-EU-containing PJ were similar after 1, 14 and 28 days; however, after 28 days the color, odor, taste and overall acceptance of control (raw PJ) were unsatisfactory. Based on these

results, ISO-EU (0.5 to 1.5 μL mL^{-1}) combined with YEX has good potential for improving both microbial safety and shelf-life of refrigerated PJ although ISO-EU negatively affected taste of the juice. In this respect further research is needed to determine an optimum balance between microbial control and taste acceptability of PJ with added ISO-EU.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Consumption of fresh fruit and vegetable juices has increased substantially over the years. Current trends indicate that consumers are demanding juices that are high in vitamins, antioxidants and are flavorful, fresh-like, low-calorie, natural and free from synthetic preservatives. However, consumption of minimally processed, fresh-like juices increases the risk of microbial foodborne diseases. In addition, a shortened shelf-life due to contamination by spoilage microbes can also occur if a preservation method is not applied to raw juices. Although fresh-like juices provide important health benefits, fruit and vegetable juices may cause foodborne illnesses due to contamination with harmful pathogens such as *Salmonella* and enterohaemorrhagic *Escherichia coli* O157:H7 (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martín-Belloso, 2009).

Microbial contamination of fruits or vegetables destined for production of juices may occur at any point in their movement from farm to consumer. Soil and water harboring pathogenic and spoilage bacteria may come in contact with fruits and vegetables and contaminate them. Pre-harvest sources of contamination may include animals (wild and domestic), dust, insects and manure used as fertilizer. Contamination may also occur after harvest such as during preparation and extraction of the juice. Failures in pasteurization or sanitation processes can compromise the safety of juices. Furthermore, poor storage conditions may contribute to the survival and growth of pathogenic bacteria in juices despite the low pH of many juices especially those from acidic fruits. Some juices such as orange juice and apple juice are no longer considered safe although they have an intrinsically low pH because survival of pathogenic bacteria has been reported in highly acidic juices. In fact,

several juice-related outbreaks have been reported over the years due to the consumption of unpasteurized apple, apple cider and orange juices (Raybaudi-Massilia et al., 2009).

In addition to foodborne illnesses, microbial contamination of fresh-like, minimally processes juices may result in spoilage. Quality losses such as change in appearance, taste or smell in juices due to microbial growth can have serious consequences such as huge economic losses. Fruit and vegetable juices are spoiled primarily by the growth of acid tolerant bacteria, yeasts and molds. Several spoilage microbes are of great concern in the juice industry. Yeasts and molds are the predominant spoilage microorganisms of acidic juices. Yeasts have been reported to be present in fruit juices in numbers ranging from 1.0 to 6.83 $\log_{10}$ CFU mL^{-1} (Tournas, Heeres, & Burgess, 2006). They are able to grow in the low water activity, low pH environment of juices, and produce carbon dioxide and alcohol. Yeasts from the genera *Saccharomyces, Candida, Pichia* and *Rhodotorula* have often been reported as responsible for the spoilage of fruit juices. Molds can also spoil juices by producing mycelial mats and off-flavors. *Penicillium* spp., *Botrytis* spp., and *Aspergillus niger* are examples of dominant molds implicated in juice spoilage. In addition to yeasts and molds, some bacteria such as lactic acid bacteria (*Lactobacillus* and *Leuconostoc*), acetic acid bacteria (*Acetobacter*) and *Erwinia* spp., *Pseudomonas* spp., and *Alicyclobacillus acidoterrestris* are known to spoil juices (Raybaudi-Massilia et al., 2009).

In response to safety concerns posed by pathogenic microbes in juices and economic losses due to juice deterioration by spoilage microbes, the United States Food and Drug Administration (USFDA) in 2001 implemented regulations for juice manufacturers. The USFDA implemented a hazard analysis and critical control point (HACCP) regulation that

now requires juice processors to apply a treatment that achieves at least a 5-log reduction (99.999%) of the pertinent microorganism but does not specify the type of treatment that should be applied.

Pasteurization and canning have been popular among juice manufacturers for achieving the 5-log pathogen reduction; however, thermal treatment can cause significant changes in sensory characteristics such as the color and taste of juices, and substantial loss of vitamins and minerals (Leite et al., 2016). Other antimicrobial interventions to improve the microbial safety of juices include the use of synthetic preservatives such as potassium sorbate and/or sodium benzoate; however, consumers are becoming more health conscious. They are demanding juices that are natural and devoid of chemically synthesized preservatives. More advanced technologies such as pulsed electric field, high hydrostatic pressure and ultrasound have been developed. Although these novel technologies achieve the USFDA inactivation levels without compromising the sensory and nutritional quality of juices, they require high amounts of energy and are extremely costly. Natural antimicrobials such as essential oils (EOs) are therefore excellent alternatives to synthetic preservatives, thermal treatments and high energy consuming technologies. The EOs and their constituents are natural and most of them are generally recognized as safe (GRAS) by the USFDA. Additionally, there is a rapidly growing amount of knowledge on the antibacterial and antifungal activity of EOs in various foods and beverages.

The use of low concentrations of the EO constituent, isoeugenol, combined with yucca extract, a natural surfactant, may be an effective multifunctional preservative system with good potential for dispersing the hydrophobic isoeugenol while inactivating pathogens

and native spoilage microflora in juices. This natural antimicrobial system presents a good alternative that could ensure the microbial safety and improve the microbial shelf life of juices.

Thesis Organization

This thesis comprises of five chapters. The first chapter contains a general introduction. The second chapter is a literature review that includes current knowledge on topics that are relevant to the research presented in this thesis. Chapter 3 and Chapter 4 are comprised of the research completed and are intended to be submitted to the scientific journals namely, *Foodborne Pathogens and Disease* and *Food Microbiology*, respectively. Chapter 5, the final chapter, is comprised of a general conclusion derived from research results presented in chapters 3 and 4. Tables and figures are presented at the end of their respective papers. References also appear at the end of each chapter. Formatting of tables, figures and references follows the specifications set forth by those two previously stated journals. The abstract titled "Antimicrobial Effectiveness of Iso-Eugenol against Human Enteric Pathogens in Refrigerated Raw Pineapple Juice with Added *Yucca schidigera* Extract" was presented in a poster format at the annual meeting of the International Association for Food Protection in Salt Lake City, Utah (July 2018). The abstract titled "Inactivation of Natural Spoilage Microflora in Refrigerated Raw Pineapple Juice with Added Iso-eugenol" was also presented in a poster format at the annual meeting of the International Association for Food Protection in Salt Lake City, Utah (July 2018).

CHAPTER 2. LITERATURE REVIEW

Essential oils

Essential oils (EOs) are plant-derived aromatic, volatile compounds that possess antimicrobial activity (Dorman & Deans, 2000). They are extracted from plant cells and glands from various parts of plant materials such as the leaves, buds, flowers, seeds, roots and barks (Burt, 2004). Methods for obtaining EOs from plant materials include expression, distillation (steam or dry), fermentation or ethanol extraction. However, steam distillation is most commonly used in the production of EOs for commercial purposes (Burt, 2004). The EOs are complex mixtures of approximately 20-80 individual constituents found at different concentrations. Major constituents are typically in concentrations ≥1%, while minor constituents are present in lower concentrations (0.1% to <1%) and traces (<0.1%) (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). They are composed of several compounds including alcohols, terpenes, aldehydes, esters, acids, cetones and phenols (Burt, 2004).

Uses

Although EOs and their constituents have been used in the preservation of foods, they are mainly utilized in the flavorants for foods (Mendonca, Jackson-Davis, Moutiq, & Thomas-Popo, 2018). Most EOs and their individual constituents such as cinnamaldehyde, eugenol and thymol are classified as Generally Recognized As Safe (GRAS) substances by the United States Food and Drug Administration (USFDA) and are approved by the European Commission for use as flavorings in food (Hyldgaard, Mygind, & Meyer, 2012). Other uses of EOs are in perfumes (fragrance), cosmetics and as components in pharmaceutical products (Nychas, Skandamis, & Tassou, 2003).

Essential oils as secondary metabolites of plants

The EOs are secondary metabolites of plants and are known to have antimicrobial properties. They are produced by plants and play an essential role in defense against disease agents (Tajkarimi, Ibrahim, & Cliver, 2010). The EOs have antifungal activities and play an important role as insecticides. They act as a repellant to predators thus offering protection to plants. In addition to defense, EOs are assumed to play an ecological role in plants by helping them to reduce stress from abiotic environmental factors such as UV light, carbon dioxide levels, ozone and drought. They can also inhibit the germination of seeds to perhaps delay reproduction and increase chances of survival, and their characteristic odor plays a role in attracting pollinating insects (Dhifi, Bellili, Jazi, Bahloul, & Mnif, 2016). Due to their strong aroma and flavor, EOs and their constituents are also responsible for giving unique odor and taste to parts of plants, especially those used as herbs or spices.

Mechanisms of action

Due to the complex composition of EOs, several targets in bacterial cells have been proposed for their antibacterial activity. Researchers have reported degradation of the cell wall, damage to the cytoplasmic membrane and membrane proteins, leakage of cytoplasmic contents, depletion of proton motive force and coagulation of cytoplasm as mechanisms of action of EOs in bacterial cells (Burt, 2004; Nychas et al., 2003). Nychas and others (2003) have reported that at low concentrations EOs may inhibit enzymes; however, at higher concentrations they may cause the precipitation of proteins suggesting that EOs' mode of action may be concentration dependent. The precise antimicrobial mode of action of EOs might be challenging to identify due to the complex multi-component characteristics of these natural plant extracts which include major and minor antimicrobial constituents in varied concentrations.

Differences in sensitivity of gram-negative versus gram-positive bacteria

The effectiveness of EOs and their constituents is dependent on the type of microorganism that is exposed to EOs. The EOs and their constituents have antimicrobial activities against several bacterial species, and some yeasts and molds (Burt, 2004). In addition, they have antiparasitic, antiviral, antioxidant, and insecticidal properties. Generally, gram-positive bacteria are more susceptible to EOs and their constituents than gram-negative bacteria. Gram-negative bacteria possess an outer membrane with a hydrophilic lipopolysaccharide layer that is absent in gram-positive bacteria and is believed to act as an impermeable barrier to hydrophobic compounds such as EOs entering the bacterial cell (Burt, 2004; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2008). Although several researchers have shown gram-negative bacteria to be more resistant to EOs and their constituents, this may not be the case for every EO and its constituents as demonstrated by Tassou and others (1995). This research group demonstrated that an EO obtained from mint (*Mentha piperita*) was most effective against the gram-negative *Salmonella* Enteritidis than *Listeria monocytogenes,* a gram-positive bacteria, in Greek appetizers (Tassou, Drosinos, & Nychas, 1995).

Inherent properties that contribute to antimicrobial activity

The EOs and their constituents have been studied for their antimicrobial activity against a wide range of pathogenic and spoilage microbes. The EO constituents such as eugenol, carvacrol and thymol, with phenolic structures have higher antimicrobial activity (Burt, 2004; Dorman & Deans, 2000). This high antimicrobial activity may be due to the presence and relative position of a hydroxyl group in the structure of phenolic compounds (Aziz & Karboune, 2018).

Another very important characteristic of EOs and their constituents that contribute to their antimicrobial activity is their hydrophobicity. Because they are hydrophobic oily compounds, they can partition in the lipid layer of bacterial cell membranes resulting in the

disruption of the cell membrane and increased membrane permeability (Burt, 2004). These lipophilic oily compounds are also able to alter lipid-protein interactions by accumulating in the lipid bilayer (Cosentino et al., 1999).

In addition, the antimicrobial effectiveness of EOs is dependent on the chemical structure and composition of their individual constituents. Efficacy may be due to a major individual constituent only or it may be influenced by interactions between major and minor constituents (Mendonca et al., 2018). Some studies have shown that whole extracts of EOs are more effective against microorganisms in contrast to mixtures of their major individual constituents (Gill, Delaquis, Russo, & Holley, 2002; Mourey & Canillac, 2002). It has been hypothesized that whole EO extracts are more effective because minor and major constituents are likely interacting resulting in an additive or synergistic effect. Though this may be true for some EOs and their constituents, a major constituent of oregano oil, carvacrol, was found to be more effective against yeast than the whole oregano extract (Rao, Zhang, Muend, & Rao, 2010). Therefore, predictions of likely antimicrobial activity should be made cautiously considering the complexity of EOs with regard to types and relative concentrations of EO constituents.

Isoeugenol

Isoeugenol (ISO-EU) (synonym: 2-methoxy-4-propenylphenol) is an essential oil component derived from plant sources such as clove, ylang ylang, nutmeg, and cinnamon. It is an aromatic, volatile and hydrophobic compound which is GRAS and USFDA approved (21 CFR 172.515) for use as a flavorant. Isoeugenol has been used as a flavoring agent or food additive in several foods and beverages and is known for its spice-clove odor.

Chemical structure

The ISO-EU is a small (164.201 g/mol) hydrophobic compound that belongs to a group of EOs known as phenylpropenes. It has a six-carbon aromatic ring and hydroxyl group and the

empirical formula of ISO-EU is $C_{10}H_{12}O_2$. It is a structural isomer of eugenol and differs in structure to eugenol by the positioning of one carbon-carbon double bond in its side chain. The chemical structure of ISO-EU shows that it has double-bonds in the α, β positions of the side chain which is closer to the benzene ring (Figure 1). This EO component also possesses a free hydroxyl group and methyl group in the γ position which together with its double bond in the side chain have been proposed to be responsible for its antimicrobial activity. In fact, due to its structure, it has exhibited antibacterial activity analogous to and at times even greater than its isomer eugenol (Laekeman et al., 1990; Zemek, Kosikova, Augustin, & Joniak, 1979; Zemek, Valent, Pódová, Košíková, & Joniak, 1987).

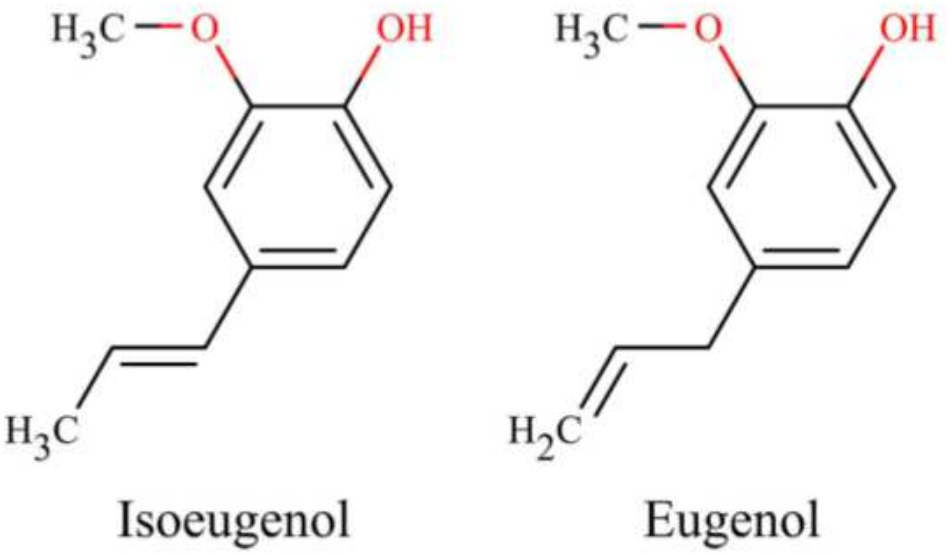

Figure 1. Chemical structures of Isoeugenol and Eugenol. Source: (Morten Hyldgaard, Mygind, Piotrowska, Foss, & Meyer, 2015).

Antibacterial activity

There are limited food-related studies on the antibacterial activity of ISO-EU. Despite the few studies reported, ISO-EU has demonstrated antimicrobial activity against some gram-negative and gram-positive bacteria. Antibacterial activity was demonstrated against *Escherichia*

coli, Pseudomonas aeruginosa, Salmonella, Micrococcus luteus, Bacillus licheniformis and *Staphylococcus aureus* (Laekeman et al., 1990; Zemek et al., 1979; Zemek et al., 1987). In a comparison study on the antibacterial activities of eugenol and isoeugenol against six foodborne pathogens (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051, *Listeria monocytogenes* ATCC 19115, *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 19430, and *Shigella dysenteriae* CMCC (B) 51252) despite ISO-EU's structural similarity to eugenol, ISO-EU exhibited greater efficacy than eugenol against the gram-positive bacteria and *Salmonella* Typhimurium. Those same results showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of eugenol were, 312.5 $\mu g\ mL^{-1}$ for *E. coli* and *S. dysenteriae* and 625 $\mu g\ mL^{-1}$ for the other bacteria tested, while the MIC and MBC values of ISO-EU were lower at 312.5 $\mu g\ mL^{-1}$ for each tested bacterium (Zhang, Zhang, Xu, & Hu, 2017).

Due to the hydrophobicity of ISO-EU, when applied to a food matrix, the antibacterial efficacy may be negatively affected as ISO-EU may interact with the lipid phase of the food system not interact with bacteria present in the aqueous phase (Robins & Wilson, 1994). It was proposed that encapsulation may be a solution to enhancing the antibacterial efficacy of ISO-EU by concentrating them in the aqueous phase to increase its interaction with foodborne bacteria. Encapsulated ISO-EU in spray-dried emulsions was used as a means of enhancing its antibacterial efficacy against two model organisms, *Escherichia coli* K12 and *Listeria monocytogenes* in growth media, milk and carrot juice (Krogsgard Nielsen et al., 2016). The results of this study showed that emulsion encapsulation of ISO-EU enhanced the antibacterial efficacy of ISO-EU against *Escherichia coli* K12 and *Listeria monocytogenes* in growth media and carrot juice but not in milk. Those results demonstrate that the composition of the food

matrix is an important factor in determining the antimicrobial efficacy of EOs and their components. The authors proposed explanation was that the decreased antimicrobial efficacy may be due to interference from fat and protein in milk. In another study, the properties of the food matrix were also found to be an important factor in determining the antimicrobial effect of emulsion-encapsulated ISO-EU. In that study, the antimicrobial effect of emulsion-encapsulated ISO-EU was evaluated against biofilms of food pathogens and spoilage bacteria in a broth system and carrot juice (Krogsgard Nielsen et al., 2017). Although emulsion encapsulation enhanced ISO-EU's efficacy against biofilms in media, it did not enhance ISO-EU's efficacy in carrot juice. However, in that same study, the authors speculated that this difference could be due to the difference in pH of the media and carrot juice. The pH of the culture medium and carrot juice (Biotta, lactofermented, pasteurized, organic) was reported to be 6.0 and 4.4 respectively. Considering that low pH can augment the hydrophobicity of EOs and thus increase their antibacterial activity due to increased interaction with cell membranes, the reported ineffectiveness of encapsulated ISO-EU in low pH (pH 4.4) carrot juice is interesting and warrants further investigation. Those same researchers also demonstrated that ISO-EU had similar antimicrobial effect against biofilms and planktonic bacteria.

The efficacy of ISO-EU alone and in combination with epsilon-poly-L-lysine was also studied against a range of pathogenic and spoilage microbes in fresh turkey meat. Combinations of the natural antimicrobials agents (ISO-EU and epsilon-poly-L-lysine) resulted in an additive effect against all gram-negative strains of bacteria tested (Hyldgaard et al., 2015).

Antifungal activity

To date few studies have been published on the antifungal effect of ISO-EU against yeasts and molds. In one study, ISO-EU resulted in the lowest MIC for certain food fungi when compared to eugenol and most antimicrobial compounds tested. The ISO-EU exhibited the most

pronounced inhibitory effect on the growth of yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) as well as mold (*Aspergillus niger*) (Zemek et al., 1979). Also, ISO-EU was very effective against 31 strains of *Candida* (Bhatia et al., 2011) .

In another study done by (Pizzolitto et al., 2015), the antifungal activity of ten natural phenolic compounds including ISO-EU was investigated against *Aspergillus parasiticus* AF54 strain. *Aspergillus parasiticus* is of importance because it produces carcinogenic secondary metabolites, aflatoxins, which cause economic loss and increase the risk of human disease from consuming contaminated crops such as corn and peanuts. Based on the results of that study, ISO-EU was the most active phenolic component among those tested. It had the lowest MIC (1.26 mM) followed by carvacrol (1.47 mM) and thymol (1.50 mM). Eugenol, an isomer of ISO-EU, had an MIC of 2.23 mM.

Fusarium is another fungus (mold) of major importance; it produces mycotoxins and can infect and destroy crops such as corn and wheat. In an attempt to find a natural antimicrobial to replace toxic synthetic fungicides, (Dambolena, López, Meriles, Rubinstein, & Zygadlo, 2012) evaluated the inhibitory effect of 10 natural phenolic compounds including ISO-EU against *Fusarium verticillioides.* Among the 10 phenolic compounds, ISO-EU was found to be the third most active inhibitor against *Fusarium verticillioides* after carvacrol and thymol with an MIC of 0.40 mM (carvacrol MIC was 0.32 mM and thymol MIC was 0.40 mM).

Mode of action

Despite the antibacterial and antifungal activity exhibited by ISO-EU, there is very limited research on its mode of antibacterial action on the molecular scale. One study proposed that ISO-EU has a non-disruptive detergent-like mechanism of antibacterial action (Morten Hyldgaard et al., 2015). Research done by this research group investigated ISO-EU's antibacterial mechanism of action using *Escherichia coli* K12 and *Listeria innocua*. Cellular

morphology, viability, enzymatic activity and cell membrane integrity, permeabilization and fluidity were evaluated. Morten Hyldgaard et al., 2015 proposed that the cytoplasmic membrane was the primary site of action as the results indicated that ISO-EU did not affect cell morphological changes to the outer membrane or cell wall of *Escherichia coli* K12 and *L. innocua.* However, ISO-EU compromised their cytoplasmic membrane affecting membrane permeability and possibly inactivated intracellular esterase activity within *L. innocua*. In addition, those same authors proposed that ISO-EU increases membrane fluidity and that ISO-EU interacts with *E. coli* cell membranes in a reversible non-disruptive detergent-like manner causing membranes to be destabilized and become leaky.

Although the free hydroxyl group in ISO-EU's structure has shown importance in this EO's antibacterial activity, it did not seem to be associated with ISO-EU's activity against yeast (Laekeman et al., 1990). Research done by Bhatia et al., 2011 showed that ISO-EU has antifungal activity based on experiments involving 31 strains of *Candida* spp. Bhatia et al. (2011) proposed that ISO-EU's mode of antifungal action was the targeting of H^+-ATPase in the membranes. It was demonstrated that ISO-EU inhibited H^+-ATPase, leading to intracellular acidification and hence cell death. Other researchers attributed the hydrophobic properties of EOs and EO constituents such as ISO-EU to be most responsible for their antifungal activity. Due to its lipophilic property, ISO-EU is believed to penetrate the plasma membrane and induce changes in cell wall, cell membrane and cellular organelles (Dambolena et al., 2012; Rasooli & Owlia, 2005).

Despite the importance of the free hydroxyl group, the double-bonds in the α, β positions of the side chain, and methyl group in the γ position to ISO-EU's antimicrobial activity, several other factors most likely impact ISO-EU's efficacy. For example, the type of microbial strain and

experimental conditions such as the intrinsic characteristics of the growth medium and temperature may also affect the antimicrobial activity of ISO-EU (Pauli & Kubeczka, 2010).

Yucca schidigera extract

Yucca schidigera extract (YEX) is obtained from a natural plant source. It is derived from the Mohave Yucca plant, a desert plant that is native to the southwestern United States and Mexico. It is a natural mild surfactant and possesses foaming properties. It is GRAS and is USFDA approved (21 CFR 172.510).

Current applications

Several uses of YEX in the food, cosmetic and feed industries have been documented. In food, YEX has been used as a foaming agent in several beverages such as root beer, foamy cocktail mixes and carbonated foamy drinks. In the cosmetic industry, YEX has been used in natural shampoos, foaming cosmetics and washing soaps. There are several reports of YEX being used in the feed industry for several reasons. This natural surfactant has already been used as a feed additive for livestock and companion animals to control the ammonia and fecal odors in animal excreta (Lowe & Kershaw, 1997). In another study, YEX added to water and shrimp feed significantly reduced the accumulation of ammonia during shrimp farming (Santacruz-Reyes & Chien, 2012). In addition, YEX enhanced the growth of the Pacific white shrimp when 0.2% YEX was added in the shrimps' diet (Yang, Tan, Dong, Chi, & Liu, 2015). In another study, the inclusion of yucca plant powder in the feed of sows resulted in a significant reduction in number of stillbirths (Herpin, Vincent, & Cheeke, 2004). The *Yucca schidigera* plant has also been used for many years by Native Americans as an anti-inflammatory and anti-arthritic agent. Yucca products have been used for prevention and treatment of arthritis in humans, horses and dogs although evidence of its efficacy is not reported (Cheeke, Piacente, & Oleszek, 2006).

Cheeke et al., (2006) also highlighted the use of YEX in the agricultural sector as a nematode and fungi control agent during crop production. It was also used to stimulate growth of crops and as a soil wetting agent.

Surfactant properties

The YEX are known to contain saponins which are natural surfactants containing a lipophilic nucleus and one or more carbohydrate side chains that are hydrophilic. Thus, saponins have both lipid-soluble and water-soluble properties (Cheeke, 2000). This makes YEX an excellent foaming agent in beverages such as root beer that requires the formation of stable foam. The use of YEX in shampoos is also due to its surfactant properties.

Antimicrobial properties

To our knowledge, most of the research on YEX to date has been done in animal nutrition. In addition, there is a scarcity of published reports on the antimicrobial activity of YEX. Inhibition of ruminal protozoa appears to be widely reported due to the presence of saponins in YEX; however, inhibition or inactivation of some bacteria has also been reported (Wallace, Arthaud, & Newbold, 1994). Steroidal saponins from YEX inhibited fungi and ruminal bacteria capable of digesting cellulose but their antibacterial effects on amylolytic bacteria are species dependent (Wang, McAllister, Yanke, & Cheeke, 2000). Saponins have been reported to inhibit protozoal activity by complexing with cholesterol in their membrane resulting in cell lysis (Cheeke, 2000).

The extract from *Yucca schidigera* has also shown antagonistic effects against pathogenic fungi. In a recent publication, silver nanoparticles from *Yucca schidigera* effectively inhibited growth of strawberry soil-borne pathogenic fungi *Fusarium solani* and *Macrophomina phaseolina* (Ruiz-Romero, Valdez-Salas, González-Mendoza, & Mendez-Trujillo, 2018). The yield and quality of strawberries can be reduced due to contamination with these pathogenic

fungi. Because several synthetic fungicides are environmental hazards, the use of green nanotechnology seems like an excellent alternative. The results of that study done by (Ruiz-Romero et al., 2018) reveals the potential use of YEX in agro-nanotechnological applications.

Escherichia coli

Characteristics

One of the major developments in Food Microbiology occurred in 1885, when Theodor Escherich isolated *Escherichia coli* (*E. coli*) (called *Bacterium coli* at that time) from feces. He also made the association between some strains and infant diarrhea. Currently, *Escherichia* is one of the most important genera of bacteria in foods. Bacteria in this genus are gram-negative facultative anaerobes. They are straight rods with dimensions about 1 x 3 μm and can be motile or nonmotile. They are mesophiles and grow optimally between 30 and 40 ºC. Members of this genus are known as indicator organisms and are used as indicators of sanitation in coliform and fecal coliform groups. Although many strains are nonpathogenic, some strains are pathogenic to humans and animals and continue to cause foodborne illnesses. Pathogenic *E. coli* are subdivided into six groups based on how they adhere to and invade epithelial cells as well as their toxin production ability. They are 1) enterotoxigenic *E. coli* (ETEC), 2) enteropathogenic *E. coli* (EPEC), 3) enteroinvasive *E. coli* (EIEC), 4) shiga toxin producing /enterohemorrhagic *E. coli* (STEC/EHEC), 5) enteroaggregative *E. coli* (EAEC) and 6) diffuse-adhering *E. coli* (DAEC). One of the most important species associated with foodborne illness is *E. coli* O157:H7, a principal serotype in the STEC/EHEC group. In my experiments presented in this thesis, five strains of *E. coli* O157:H7 were used and hence discussions below will focus on *E. coli* O157:H7.

Escherichia coli O157:H7

E. coli O157:H7 is a foodborne pathogen of public health concern because it can cause serious human illness. *E. coli* O157:H7 expresses the 157th somatic (O) antigen and the 7th flagellar (H) antigen. It belongs to STEC/EHEC group because of its ability to produce one or more Shiga toxins (verocytotoxins or Shiga-like toxins). The Shiga toxin binds to globotriaosylceramide (Gb3), a specific receptor in intestinal and kidney cells and leukocytes, then inhibits protein synthesis which leads to cells death (Mead & Griffin, 1998). Production of Shiga toxin is known to be the major virulence factor of pathogens in this group. Another virulence factor of *E. coli* O157:H7 and other pathogens in the EHEC group is Intimin, an intimate adhesion factor responsible for attachment-effacement lesion in the intestines (Ray & Bhunia, 2013). Illness with this pathogen is characterized by bloody stool, abdominal cramps and little or sometimes no fever. It was first identified in 1982 as a human pathogen but in 1983 was associated with post-diarrhoeal haemolytic uraemic syndrome (HUS) characterized by acute renal injury and thrombocytopenia (Mead & Griffin, 1998). *E. coli* O157:H7 is generally unable to ferment sorbitol. It grows well at 30-42ºC, grows poorly at 44-45ºC. While many strains might be unable to grow below 10ºC (Ray & Bhunia, 2013), growth of some *E. coli* O157:H7 strains have been reported at temperatures as low as 6 ºC (Tamplin, Paoli, Marmer, & Phillips, 2005).

Sources of contamination

E. coli O157:H7 is typically found in the intestinal tracts of humans, warm blooded animals, birds, reptiles and transiently in the environment. Healthy cattle are a reservoir for the pathogen and it has been isolated from feces of cattle, chicken, goats, pigs, dogs and cats (Ferens & Hovde, 2011). Generally, *E. coli* O157:H7 infection occurs after ingestion of contaminated food or water. The pathogen can also be transmitted directly from person to person in environments such as day-care or chronic-care facilities.

Juice associated outbreaks

Although most *E. coli* O157:H7 foodborne outbreaks have been associated with the consumption of foods derived from cattle such as ground beef and raw milk, outbreaks have also resulted from the consumption of contaminated produce. Fruits and vegetables and subsequently their juices can be contaminated with *E. coli* O157:H7 derived from contaminated soil, irrigation water and untreated manure or fertilizer of animal origin. Contamination of juices can also occur due to inappropriately washing of fruits and vegetables prior to processing and as well as during the manufacturing process. In the United States, low-risk acidic juices such as apple cider and unpasteurized apple juice have been implicated in *E. coli* O157:H7 foodborne outbreaks (Cody et al., 1999; Steele, Murphy, Arbus, & Rance, 1982). Low pH juices are no longer considered safe because some pathogenic bacteria such as *E. coli* O157:H7 are acid tolerant and are capable of surviving in acidic juices (Jordan, Oxford, & O'Byrne, 1999).

Outbreaks caused by *E. coli* O157:H7 associated with fruit juices have been reported as early as 1980 when 14 people were infected due to the consumption of unpasteurized apple juice. All 14 cases were HUS cases. Another 23 cases were reported in 1991 due to consumption of contaminated apple cider while 6 cases were reported in 1992 associated with orange juice. Within the period 1996 to 1999, 172 cases were reported due to consumption of contaminated apple juice or unpasteurized apple cider (Raybaudi-Massilia et al., 2009).

A report on *E. coli* O157 outbreaks in the United States for the period 2003-2012 revealed that there were a total of 390 outbreaks which resulted in 4,928 illnesses with the majority (353) being attributed to *E. coli* O157:H7 (Katherine, Rajal, Shacara, Patricia, & Gould, 2015). The report showed that 6 of the total outbreaks (~2% of all outbreaks) were due to the

consumption of contaminated fruits or their juice. There were 4 outbreaks involving unpasteurized apple cider, 1 outbreak involving fruit salad and 1 outbreak attributed to strawberries.

Listeria monocytogenes

Characteristics

Listeria monocytogenes (*L. monocytogenes*) is a short, nonsporulating, gram-positive, rod-shaped bacterium that is facultatively anaerobic. *L. monocytogenes* has peritrichous flagella that is temperature dependent. At 20-30ºC, the bacterium exhibits high tumbling motility when expression of flagellar is at the maximum. It is pathogenic to both humans and animals. *L. monocytogenes* is one of nine species in the genus *Listeria* and consists of 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7. Serotype 4b was the predominant isolated serotype in foodborne cases in the United States and Canada while serotypes 1/2a and 1/2b were predominantly isolated in European countries. *L. monocytogenes* generally forms short chains in the presence of lots of nutrients but may appear elongated or be present in long chains in harsh conditions such as >10% salt or >45ºC. This pathogen is psychrotrophic and grows optimally at 30-37ºC; however, it can grow between 1º and 44ºC. In this respect a major concern is that it can grow in refrigerated extended shelf-life foods such as processed meats. It is resistant to drying, freezing, pH ≥ 5.0 and salt content >10%. *L. monocytogenes* possess a glutamate decarboxylase enzyme system which is responsible for its acid tolerance. Despite its hardiness it can be destroyed by thermal pasteurization temperature (Ray & Bhunia, 2013).

Sources of contamination

L. monocytogenes is ubiquitous in the natural environment and can grow in several foods and in the environment if conditions are suitable for cell multiplication. It is a saprophyte and has been isolated from dead vegetation, soil, water, silage and sewage. Once ingested, this pathogen

is capable of surviving within the host cell particularly in monocytes if infection progresses beyond the intestinal environment (Freitag, Port, & Miner, 2009). *L. monocytogenes* has been isolated from the intestines of domesticated animals and birds. It poses a serious problem to food manufacturers as it is usually found to contaminate food processing and storage facilities and can persist there for years in food processing facilities. *L. monocytogenes* has the ability to form biofilms in association with other microbes that enables it to survive harsh processing environments (Rodríguez-López, Rodríguez-Herrera, Vázquez-Sánchez, & López Cabo, 2018).

Juice associated outbreaks

L. monocytogenes has been implicated in several cases of listeriosis within the past years. It can cause two forms of the disease, febrile gastroenteritis and the more severe invasive illness. Listeriosis is especially detrimental to the immunocompromised, pregnant women and their fetuses, the elderly and children (Ray & Bhunia, 2013). Most outbreaks from *L. monocytogenes* have been linked to ready-to-eat meats and soft cheeses. Several foods have been implicated including pasteurized milk, raw milk and dairy products, cold cut meats, turkey franks, coleslaw, and soft cheeses such as Mexican style, Brie and Liedderkranz. The most recent outbreak involved deli hams. As of October 3rd, 2018, four people were reported to be infected with *L. monocytogenes* from 2 states (North Carolina and Virginia). Ready-to-eat deli hams by Johnson County Hams, Inc. were likely the source of the outbreak and were recalled (CDC, 2018a).

The consumption of contaminated raw foods from animal and plant origin may also cause listeriosis. Although this pathogen has not been implicated in outbreaks related to the consumption of unpasteurized juices, fruits and vegetables were involved in outbreaks. In 2011, there was a multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. A total of 147 persons were infected from 28 states and 33 deaths were reported (CDC, 2012). Because *L. monocytogenes* has been previously isolated from apple juice (pH

3.78) and apple and raspberry juice blend (pH 3.75) (Sado, Jinneman, Husby, Sorg, & Omiecinski, 1998), and because studies have shown the survival and growth of *L. monocytogenes* in fruit juices (Ingham, Schoeller, & Engel, 2006; Yuste & Fung, 2002), there is a potential for juices to harbor this pathogenic bacteria and result in a juice outbreak.

Salmonella enterica

Characteristics

Bacterial cells from the genus *Salmonella* are gram-negative, facultative anaerobic rods that are nonsporulating. They are about 2-5 microns long and 0.5-1.5 microns wide and are usually motile due to the presence of peritrichous flagella. This pathogen of major public health concern belongs to the family *Enterobacteriaceae* (Andino & Hanning, 2015). Although *Salmonella* can grow between 5ºC and 46 ºC, its optimum growth temperature is between 35º and 37ºC and it is therefore mesophilic. *Salmonella* cells are sensitive to pasteurization temperatures, and low pH (≤4.5). They can multiply at a water activity (a_w) between 0.99 and 0.94 but can survive at a_w as low as 0.2 in dried foods such as in peanuts, peanut butter, wheat flours, almonds and spices.

There are two species in the genus *Salmonella* namely, *Salmonella enterica* (*S. enterica*) and *Salmonella bongori*. There are six subspecies and greater than 2, 579 serovars of *S. enterica*. The six subspecies of *S. enterica* are: *S. enterica* subsp. *enterica, S. enterica* subsp. *arizonae, S. enterica* subsp. *houtenae, S. enterica* subsp. *salamae, S. enterica* subsp. *diarizonae and S. enterica* subsp. *indica*. Two of the serotypes of *S. enterica* subsp. *enterica* implicated frequently in foodborne illness are *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium.

Sources of contamination

Salmonella can colonize the intestinal tract of humans, farm animals, birds, reptiles (turtles, lizards, snakes), insects, frogs and pets. The pathogen can be excreted through feces for

a long time and could contaminate soil and water. The use of manure as fertilizer or washing food products with polluted water can subsequently contaminate food with this enteric pathogen. Several foods from both animal and plant origin have been linked to salmonellosis. Foods such as contaminated eggs, poultry, meat, cheese, unpasteurized juice or milk as well as raw fruits and vegetables such as melons, spices and nuts have all been involved in *Salmonella* outbreaks. Inadequate cooking of meats, eggs and other animal products as well as insufficient pasteurization of milk and juices may lead to *Salmonella* outbreaks (Ray & Bhunia, 2013). Therefore, animals and their environment are considered major sources of *Salmonella* contamination. The numerous reservoirs for *Salmonella* in the natural environment make it challenging to control contamination of food by this pathogen. Additionally, cross contamination at food services, commercial kitchens, and homes of cooked foods with raw foods through utensils, equipment, cutting boards and hands contribute to the spread of this organism to increase the risk of foodborne infection. Direct contact with infected people can also be a source of infection.

Juice associated outbreaks

In the United States, foodborne salmonellosis has been recognized as the major cause of all foodborne diseases. Infection with *Salmonella* causes about 1.2 million illnesses annually in the United States with 23,000 hospitalizations and 450 deaths. Between 2006 and 2017 *Salmonella* was responsible for more than half (53.4%) of all foodborne disease outbreaks (Liu, Whitehouse, & Li, 2018). Consumption of contaminated food and water as well as direct fecal-oral transmission can cause salmonellosis. Several outbreaks have been associated with foods of animal origin such as eggs, milk, beef, chicken and turkey. Foods harboring *S. enterica* could result in illness if consumed raw, inadequately heated, became contaminated after heat treatment or was contaminated directly with fecal matter (Andino & Hanning, 2015; Ray & Bhunia, 2013).

Recently, in October 2018, 92 cases of people infected with the outbreak strain *S. enterica* ser. Infantis in 29 states were reported (CDC, 2018b). A variety of sources of contaminated raw chicken products was implicated in this outbreak.

Even though most *Salmonella* outbreaks are associated with land animals, outbreaks linked to foods of plant origin have also been implicated. Fruits and nuts were among foods from plants that caused the most outbreaks from 2006 to 2011 (Andino & Hanning, 2015). Juices may become contaminated by using fruits and vegetables tainted with *Salmonella* from animal fecal matter in the soil. Contamination of juices could also occur during processing. Other ways of contamination include inadequate washing of fruits and vegetables and the use of manure as fertilizer. Several cases of outbreaks of *Salmonella* spp. associated with the consumption of unpasteurized juices have been reported. In 2005, there were 157 cases of *S. enterica* ser. Typhimurium and *S. enterica* ser. Saintpaul outbreaks associated with the consumption of unpasteurized orange juice. In 2000, 74 cases were reported due to the consumption of *S. enterica* ser. Enteritidis contaminated orange, grapefruit and lemonade juices. In 1999, there were several *S. enterica* outbreaks linked to orange juice, unpasteurized orange juice and unpasteurized mamey juice (Raybaudi-Massilia et al., 2009).

Natural spoilage microflora of raw juices

Fruit and vegetable-based beverages are rich sources of vitamins, minerals, fiber and antioxidants and can be consumed by many as they do not contain dairy allergens (Luckow & Delahunty, 2004). However, because fruit and vegetable juices are nutrition-rich, they can support the survival or growth of some microorganisms that can compromise the safety and/or quality of the juices. Microbial spoilage can lead to substantial financial losses. Several acid-tolerant bacteria, yeasts and molds can survive and grow in juices. The microbiota of these juices however depends on the composition of the juice. Intrinsic factors such as pH, water activity,

nutrient content and presence or absence of natural antimicrobials can determine the type of microbes present in a juice. Microbes such as yeasts and molds, *Enterobacteriaceae*, lactic acid bacteria and non-lactic-acid-producing acid tolerant bacteria can contaminate fruits and vegetables at pre- and post-harvest. Sources of contamination include soil, irrigation water, feces, wild and domestic animals, dust, insects, manure, human handling, harvest equipment, processing equipment, transport containers and transport vehicles (Beuchat, 2002).

Yeasts and molds

Yeasts and molds (fungi) are typically predominant on foods in which bacteria are unable to grow and are the major spoilage microbes of acidic fruit juices. They grow in a wide pH range of 1.5-9.0 thus low acid and high acidic juices are susceptible to yeast and mold spoilage. Yeasts and molds are widely distributed in the environment and when the surface tissues of fruits and vegetables are bruised, yeasts and molds that are present on the surface can rapidly multiply.

Molds are multicellular, fuzzy filamentous fungi that are distinguished by hyphae, mycelium color, and spore size. The sub-surface hyphae of molds produce extracellular enzymes that digest external substrates enabling molds to easily absorb nutrients usually through hyphal cell walls. Molds grow readily in acidic low-moisture environments and can spoil foods with low water activity. Molds, along with yeasts, are the main causal agents of microbial spoilage in juices. Common molds such as *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp. and *Botrytis* spp. can spoil fruit juices (Raybaudi-Massilia et al., 2009; Tournas et al., 2006).

Yeasts are generally unicellular, colored, and spherical or oval fungi. Many are useful in several food applications such as making of bread, wine, beer, cheese and enzymes; however, some are harmful and are of importance in the clinical area. Yeasts are major spoilage microbes of juices with low pH and high sugar content. *Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Pichia membranifaciens, Saccharomyces* spp., *Candida* spp. and *Rhodotorula* spp. are the

yeasts generally isolated from spoiled juices. The production of ethanol or formation of films on the surface of spoilt juice is generally due to *Saccharomyces* and *Pichia* (Bevilacqua, Speranza, Campaniello, Sinigaglia, & Corbo, 2014).

Enterobacteriaceae

This group of microbes consist of a large family of gram-negative bacteria. The main habitat of some species in this group is in the intestinal tract of animals. However, some are naturally found in the environment such as on plants and in water and soil. They are rod-shaped, facultative anaerobes. *Salmonella* spp. and *E. coli* O157:H7 are pathogens that belong to this group that are of public health significance in citrus fruit juices. Members of this group are indicators to the hygienic quality of foods. They are typically useful in indicating post-processing contamination in foods (N.R.C., 1985).

Lactic acid bacteria

Lactic acid bacteria are gram-positive, non-spore forming rods or cocci that produce a large quantity of lactic acid from carbohydrates. Some are used in the fermentation of yogurt, cheese, sausage, fruits and vegetables, few are pathogenic such as *Streptococcus pneumoniae* and *Streptococcus pyogenes* and some can spoil juices. Some lactic acid bacteria such as homofermentative (lactic acid is primary by-product) *Lactobacillus plantarum* can spoil juices producing slime as a result of the production of dextran and other exopolysaccharides (Ray & Bhunia, 2013). Another homofermentative lactic acid bacteria is *Pediococcus acidilactici*. Some lactic acid bacteria are heterofermentative and can produce ethanol or acetic acid and carbon dioxide in addition to lactic acid as by-products. These include *Leuconostoc mesenteroides* and *Lactobacillus fermentum*. Lactic acid produced by members of this group can lower the pH of the juice hence antagonizing other bacteria (Gram et al., 2002).

Aerobic plate count

Aerobic plate count (APC) also known as standard plate count, is an estimate of number of microbes able to grow in an aerobic environment at a minimum growth temperature of 15ºC - 20ºC and maximum of 45ºC (mesophile). This microbial group is an indicator of quality not safety; however, unusually high initial aerobic plate counts may be indicative of poor handling history of a food product for example temperature-abuse or use of poor microbial quality food ingredients. In fact, the APC provides information about the microbial load of the food product and can give an idea of the sanitary quality. The general quality and hence potential shelf life of a juice can be determined by the APC. Contrary to popular belief, the APC does not measure the total count of all microbes in a sample of food. Instead, it measures only a fraction of the microbial population that can produce colonies in a specific non-selective medium under specific conditions of agar plate incubation (N.R.C., 1985).

Sub-lethal injury

Definition of sub-lethal injury

Microbes in food and the environment are subjected to several chemical and physical stresses. Some microbes are unable to withstand the effects of such agents or treatments and may completely lose viability and die while others may be damaged but not killed. Damage may include membrane damage, cell wall damage, protein and enzyme damage, as well as DNA damage. Sub-lethal injury is complex because the exposure to stresses may produce a wide spectrum of sub-lethal effects. (Gilbert, 1984) described sub-lethal injury of microorganisms as damage to structures within the cell which may result in some transient or permanent loss of cell function. Different stresses can cause different types of cell damage due to the many inactivation mechanisms of these stresses. Likewise, the physiological state of a microbe determines the degree of injury it experiences.

When microbial cells are subjected to different levels of stress, the degree of injury may vary from mild to moderate to extreme. Microbial growth is generally not affected by very low levels of stress as microorganisms are usually able to adapt and even develop increased stress tolerance. Moderate and extreme stress however may cause a range of injuries in cells some of which may be very severe. Sub-lethal injury therefore can be defined as any injury that causes a microbe to adapt to the change (Wesche, Gurtler, Marks, & Ryser, 2009).

Sub-lethal injury is typically identified by the ability of the microbe to form visible colonies on selective media versus nonselective media. Selective media typically consist of several salts and inhibitory agents such as bile salts, bismuth sulfite, sodium chloride and selective antibiotics that can be harmful to sub-lethally injured microbes. Sub-lethally injured microbes are very sensitive to those agents and are unable to repair, resuscitate and grow on selective agar. The difference in numbers of survivors on non-selective and selective media is used to measure the percentage of the surviving population that is injured (Wesche et al., 2009).

Stresses that lead to injury

Stress has been used extensively to refer to agents or treatments causing injury. Food however, based on its intrinsic properties may also be stressful to microbes. Therefore, stress can be defined as a physical, chemical or nutritional condition that is insufficient to kill a microorganism but can sub-lethally injure the microorganism (Hurst, 1977; Murano & Pierson, 1993). The use of acids and bases (organic and inorganic), heat, cold and freezing temperatures, and antimicrobial agents such as benzoate are examples of physical and chemical stresses experienced by microbes in food (Wesche et al., 2009). Novel non-thermal technologies such as high hydrostatic pressure have also been reported to cause sub-lethal injury (Wuytack et al., 2003). Another example of stress is starvation stress. Starvation stress refers to the survival of

the microorganism in an environment with but low or no available nutrients for the microorganism's metabolic functions and growth (Dickson & Frank, 1993).

Microbial stress response, adaptation and repair mechanisms

Some microbes when exposed to stress may be severely injured but when provided enough time and the right conditions (nutrients, temperature) are able to repair their lesions and survive. These microbes undergo several metabolic processes during repair such as ATP, DNA, RNA, protein and lipid synthesis. Some bacteria produce intracellular compounds that can protect the cell membrane and macromolecules. Others produce general or specific stress-induced proteins. One such example is the production of "heat-shock proteins" when microbial cells are exposed to mild heat or other stresses. Some microbes can gain thermal resistance to mild heat because they have a heat-shock response and it has been proposed that exposure to some stress conditions may enhance microbial resistance to not only that specific stress but other stresses as well. In one study, the effect of heat shock and incubation atmosphere on injury and recovery of *E. coli* O157:H7 was studied (Murano & Pierson, 1993). The results indicated that heat shock enhanced the ability of *E. coli* O157:H7 to better repair themselves and survive hydrogen peroxide, a toxic substance, and heat. In addition, that same study found that an anaerobic gaseous atmosphere results in better recovery of injured microbes after a heat treatment. The same results were obtained by (Knabel, Walker, Hartman, & Mendonca, 1990) when they too recovered higher numbers of heat-stressed *L. monocytogenes* in an anerobic environment versus an aerobic environment. Additionally, severe heat injury in *L. monocytogenes* can cause that pathogen to be highly sensitive to oxygen and therefore require strictly anaerobic nutrient-enriched conditions for growth (A. F. Mendonca & Knabel, 1994). Other protective proteins produced by some bacteria are cold shock proteins, acid shock proteins, and starvation proteins (Wesche et al., 2009).

Some microbes can enter the viable but non-culturable state as a stress response. Severely sub-lethally injured cells of foodborne pathogens such as *Salmonella, E. coli, Shigella* and *Campylobacter* that are metabolically active but are unable to be resuscitated on culture media can enter a viable but non-culturable state and still retain their pathogenicity. However, once the appropriate nutrients, temperature, a_w and other conditions are provided, those sub-lethally injured cells may repair and regain the characteristics of a healthy cell with increased stress tolerance (Wesche et al., 2009).

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CHAPTER 3.

ISOEUGENOL SIGNIFICANTLY INACTIVATES *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* IN REFRIGERATED PINEAPPLE JUICE WITH ADDED *YUCCA SCHIDIGERA* EXTRACT

A paper to be submitted to *Foodborne Pathogens and Disease*

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Abstract

The antimicrobial effectiveness of isoeugenol (ISO-EU) against *Escherichia coli* O157:H7, *Salmonella enterica,* and *Listeria monocytogenes* in refrigerated (4ºC) pineapple juice (PJ) with added 0.5 % (w/v) yucca extract (YEX) was investigated. Additionally, sub-lethal injury of pathogen survivors in PJ and the effect of ISO-EU on sensory characteristics (appearance, odor, taste, aftertaste, viscosity and overall acceptance) of PJ were evaluated. Also, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ISO-EU were performed in brain heart infusion (BHI). Samples of PJ with added YEX and ISO-EU (0.50, 0.75, 1.0, or 1.5 μL mL^{-1}) were each inoculated with a five-strain mixture of one pathogen to obtain an initial viable count of ~7.1 log CFU mL^{-1}. Inoculated PJ with YEX but without added ISO-EU served as the control. The PJ samples were stored at 4°C for 70 days. During storage, numbers of non-injured and sub-lethally injured pathogens were determined by plating diluted juice samples on selective (sorbitol MacConkey agar (SMAC), xylose lysine tergitol 4 (XLT 4) agar or modified oxford agar (MOX); Difco) and non-selective media agar (tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE). The MIC of ISO-EU for *E. coli* O157:H7 or *S. enterica* was 0.5 μL mL^{-1} in brain heart infusion broth and the MIC of ISO-EU for *L. monocytogenes* was 0.2 μL mL^{-1}. The MBC of ISO-EU for *E. coli* O157:H7 or *S. enterica* was 0.6 μL mL^{-1} while the MBC for *L. monocytogenes* was 0.4 μL mL^{-1}. All three pathogens survived for more than 42 days in the control juice. Within 24 h, all ISO-EU treatments achieved greater than 5 log reduction of each pathogen. The ISO-EU at 1.5 μL mL^{-1} completely inactivated *E. coli* O157:H7 and *S. enterica* after 4 h and 6 h, respectively ($P<0.05$). After 2 h, ISO-EU treatments inflicted sub-lethal injury in survivors of all three pathogens. Sensory scores for appearance, odor and viscosity for non-inoculated PJ containing ISO-EU (0.5 and 0.75 μL mL^{-1}) were acceptable; however, changes in taste, aftertaste and overall acceptance

of PJ containing ISO-EU were unsatisfactory. The ISO-EU (0.5 to 1.5 μL mL^{-1}) combined with YEX has good potential for enhancing the microbial safety of refrigerated PJ although taste of the juice was negatively affected. Additional research on strategies to simultaneously achieve microbial safety and acceptable taste of PJ with added ISO-EU is needed.

1. Introduction

Currently, there is a growing consumer demand for fruit and vegetable juices that are nutritious, healthy, fresh-like and devoid of synthetic chemicals (Raybaudi-Massilia et al., 2009). Consumers' preference for minimally processed juices have therefore led to an increase in the consumption of raw juices (Leite de Souza, Almeida, & Guedes, 2016). Raw fruit and vegetable juices are low-calorie, healthy and are natural sources of several antioxidants, vitamins and minerals. Scientific evidence suggests that the consumption of raw juices may help prevent heart diseases and cancer (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Despite their several health benefits, raw juices can be contaminated by foodborne pathogens thus increasing the risk of foodborne diseases.

Many fruits such as pineapples grown in fields and orchards are exposed to several sources of microbial contamination and their outermost layer can become easily contaminated by soil or water harboring microbes (Leite et al., 2016). Environmental sources of microbial contamination include soil, feces, windblown dust, irrigation water, insects, wild and domestic animals, and inadequately composted manure (Beuchat, 2002). During juice preparation and extraction, harmful microbes can be transferred from the outermost layer of the fruit to the juice resulting in contamination of the raw juice. Furthermore, other postharvest conditions such as improper storage of raw juices may influence the survival and growth of these pathogens. In past decades, several disease outbreaks linked to the consumption of raw juices have been reported (Harris et al., 2003). The consumption of low pH raw juices can result in foodborne

diseases as some human enteric pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* can survive in such unfavorable conditions of high acidity (Duan & Zhao, 2009; Mosqueda-Melgar et al., 2008). In 2001, the United States Food and Drug Administration (USFDA) responded to outbreaks associated with the consumption of unpasteurized juices by imposing regulations that require juice processors to apply a treatment that achieves at least a 5-log reduction (99.999%) of the pertinent microorganism. Regulations also require that 100% fruit and vegetable juices to be produced under a Hazard Analysis and Critical Control Point (HACCP) plan (USFDA, 2001).

Juice manufacturers have used several strategies for controlling microorganisms in juices. Traditional synthetic preservatives such as sodium benzoate and potassium sorbate are used to extend the shelf-life of juices by inhibiting the growth of spoilage microorganisms (Amirpour, Arman, Yolmeh, Akbari Azam, & Moradi-Khatoonabadi, 2015). However, they are no longer widely accepted by consumers who are becoming increasingly concerned over the long-term effect of synthetic food preservatives on their health. Other commercially available juice preservation interventions such as heat pasteurization and canning have been used to achieve the 5-log reduction required by the USFDA; however, heat treatment can be detrimental to sensory attributes such as taste, color, flavor and nutritional compounds in juices (Elez-Martínez, Soliva-Fortuny, & Martín-Belloso, 2005). One potential alternative is the use of natural antimicrobials such as plant essential oils (EOs) and their constituents.

The EOs and their constituents are gaining widespread consumer acceptance as an alternative to synthetic chemicals and thermal processes because they are derived from natural sources. They are aromatic, oily liquids derived from the leaves, seeds, roots, buds, flowers, and bark of plants (Gutierrez, Rodriguez, Barry-Ryan, & Bourke, 2008; Nychas et al., 2003). Most

are generally recognized as safe (GRAS) by the USFDA and are approved by the European Commission for use as flavoring additives in the food industry (Hyldgaard et al., 2012). Isoeugenol (ISO-EU), is an example of an EO constituent that is GRAS and approved as a flavoring agent in foods (21 CFR 172.515). It is a structural isomer of eugenol and is extracted from essential oils of clove, nutmeg and cinnamon. Although several EOs and their constituents have shown antimicrobial activity against numerous foodborne pathogens and spoilage microbes, their use as a food preservative has been limited by several factors. Sensory changes due to high concentrations needed to achieve inhibitory effects, interactions with food components such as proteins and fats, temperature and pH dependency as well as poor miscibility in hydrophilic solutions are major limitations to their use (Mendonca et al., 2018). To overcome poor solubility of EOs, researchers have used synthetic surfactants such as Tween 20 or Tween 80 (Budzynska, Wieckowska-Szakiel, Kalemba, Sadowska, & Rozalska, 2009; Remmal, Bouchikhi, Tantaoui-Elaraki, & Ettayebi, 1993). Emulsion encapsulation was useful in dispersing ISO-EU and improving its antimicrobial efficacy against foodborne pathogens and spoilage bacteria in biofilms (Krogsgard Nielsen et al., 2017). To enhance the dispersion of EOs in hydrophilic liquids such as fruit juices while satisfying the increasing consumer demand for more natural food additives, the use of a natural surfactant such as yucca extract (YEX) seems to be a very promising alternative. The YEX from the Mohave Yucca plant (*Yucca schidigera)* is FDA approved for use in the food, cosmetic, and feed industries (21 CFR 172.510). It is a mild surfactant with potential for emulsifying EOs in hydrophilic liquids such as juices and therefore may result in better dispersion and enhanced antimicrobial efficacy of EOs.

While there is a fast-growing body of knowledge on the efficacy of EOs and their constituents against pathogenic and spoilage microorganisms in juices, there is currently limited

food-related research on the antimicrobial efficacy of ISO-EU. Furthermore, to our knowledge, there are no publications on the antibacterial effectiveness of ISO-EU against human enteric pathogens in juices with added YEX. Accordingly, the purpose of this study was to investigate the efficacy of ISO-EU for killing *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes* in pineapple juice (PJ) with added YEX at refrigeration temperature (4ºC) and at abusive temperatures (30ºC for 2h and 18ºC for1 h). An additional objective was to evaluate sub-lethal injury in surviving populations of *E. coli* O157:H7, *S. enterica*, *L. monocytogenes* in refrigerated PJ (4ºC) with added ISO-EU and YEX. The effect of ISO-EU on sensory characteristics of PJ was also evaluated.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Five strains of *Escherichia coli* O157:H7 (ATCC 35150, ATCC 43894, ATCC 43895, FRIK 125 and 93-062), five serotypes of *Listeria monocytogenes* (Scott A NADC 2045 serotype 4b, H7969 serotype 4b, H7962 serotype 4b, H7596 serotype 4b and H7762 serotype 4b) and five strains of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14028, Gaminara-8324, and Oranienburg- 9239) were obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. Stock cultures were maintained frozen (-80 °C) in brain heart infusion (BHI) broth (Difco; Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol. The frozen stock cultures were thawed and activated in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; pH 7.2; Difco; Becton Dickinson) at 35 °C. Before preparing the cells for inoculation of BHI or PJ for each experiment, two consecutive 24-h transfers of each stock culture in TSBYE were performed.

2.2. Preparation of inoculum

For *Escherichia coli*, *Listeria monocytogenes* or *Salmonella enterica*, 6 mL from each of the five strains (working cultures) were combined to obtain a 5-strain mixture. Cells were harvested by centrifugation (10,000 x g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). Cells were washed twice in 0.85% (w/v) saline, harvested by centrifugation (as previously described) and the pelleted cells were suspended in 0.85% (w/v) NaCl (saline) to obtain a viable cell concentration of ~10^9 colony-forming units (CFU) mL^{-1}. To obtain the colony counts of the cell suspensions, serial dilutions (10-fold) were prepared and the diluted cell suspensions were evaluated by surface plating on selective agar (sorbitol MacConkey agar (SMAC), xylose lysine tergitol 4 (XLT 4) agar or modified oxford agar (MOX); Difco) and on tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE). Bacterial colonies were counted after aerobic incubation at 35 ºC for 48 h.

2.3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of Isoeugenol

Certified food grade ISO-EU was purchased from Sigma-Aldrich, Milwaukee, WI. The MIC and MBC of ISO-EU were determined by the broth dilution method as described by (Lopez-Malo Vigil, Palou, Parish, & Davidson, 2005). The ISO-EU was sterilized by filtration using 0.45 µm pore size Luer-Lok filter attached to a syringe (Fisher Scientific, Chicago, Illinois). Concentrations of sterile ISO-EU at ranging from 3.0 to 0.023 µL mL^{-1} (in two-fold increments) and at 0.4, 0.5, 0.6 and 0.7 µL mL^{-1} were aseptically prepared in tubes of sterile BHI (pH 7.54) with 0.5% (w/v) *Yucca schidigera* extract (YEX; Garuda International, Inc., Exeter, CA). The tubes of broth were each inoculated with 0.05 mL (50µL) of an overnight (20-h) culture of the test pathogen (*Escherichia coli*, *Listeria monocytogenes* or *Salmonella enterica)* to

obtain an initial viable cell concentration of Log 5.0 CFU mL^{-1}. The positive controls were tubes of the inoculated growth medium (BHI + 0.5% YEX) without any added ISO-EU. Negative controls were tubes of non-inoculated growth medium (BHI + 0.5% YEX) without added ISO-EU. All tubes were incubated at 35º C and observed for turbidity after 24 h. The MIC was determined based on the lowest ISO-EU concentration at which no visible growth occurred (absence of turbidity).

The MBC was determined by surface plating on non-selective media (TSAYE) 10-µl aliquots (in duplicate) of broth medium with the lowest concentration of ISO-EU at which the pathogen exhibited growth and broth from all the tubes showing no growth. The lowest concentration of ISO-EU that produced ≥ 99.9% (3 log) kill of the pathogen was determined as the MBC (NCCLS, 2002). Tests for MIC or MBC were performed in triplicate.

2.4. Preparation and inoculation of pineapple juice

Del Monte Gold extra sweet pineapples from the same production batch were purchased from a local grocery store in Ames, Iowa. Cores were removed manually with an OXO pineapple corer and slicer and the pineapples were cut into pieces. The juice was extracted, and particulates were removed by first using two double layers then one double layer of cheesecloth clamped over a stainless-steel strainer with five 2-inch metal binder clips. Two-hundred milliliters of strained PJ was measured and poured into sterile Erlenmeyer flasks. Flasks containing raw PJ were Tyndallized at 80ºC for 1 h on three consecutive days in a water bath. The flasks were kept at ambient room temperature for 24 h after each heating (Valero & Salmeron, 2003). A stock solution of 0.2 g mL^{-1} (20% w/v) *Yucca schidigera* extract (YEX; Garuda International, Inc., Exeter, CA) in PJ was prepared by adding 10g YEX to 40 mL PJ in a sterile Erlenmeyer flask and filter sterilized using a vacuum pump and 0.45 µm pore size sterile Corning® filtration

system (Fisher Scientific, Chicago, Illinois). Filter sterilized stock YEX was aseptically added to Tyndallized PJ to obtain a final concentration of 0.5% YEX. The capped flasks were vigorously shaken manually to mix their contents and 39.6 mL Tyndallized PJ with added YEX was aseptically dispensed into each of fifteen 50mL sterile centrifuge tubes (VWR International, Batavia, IL).

The ISO-EU was filter sterilized using 0.45 μm pore size Luer-Lok filter and syringe (Fisher Scientific). Tyndallized PJ with added YEX in centrifuge tubes were mixed thoroughly by vortexing and appropriate volume of ISO-EU was added to each tube to obtain the desired concentrations (0.50, 0.75, 1 or 1.5 μL mL^{-1}) of ISO-EU. The contents of the tubes were mixed by vortexing.

Control PJ and juice with added 0.5% YEX and 0.50, 0.75, 1.0 or 1.5 μL mL^{-1} ISO-EU was inoculated with 0.4 mL (400 μL) of a 5-strain mixture of *E. coli* O157:H7*, L. monocytogenes* or *S. enterica* to obtain an initial viable count of ~ 7.1 log CFU mL^{-1}. Inoculated juice without ISO-EU (0 μL mL^{-1} ISO-EU) served as the control. Appropriately labeled centrifuge tubes were held at 4ºC ± 1ºC until used for microbiological analysis at 2, 4, 6, 8 h and days 1, 3, 7, 14, 28, 42, 56 and 70.

2.5. Microbiological analysis

At set intervals during 70 days of refrigerated storage, survivors were determined by surface plating juice samples diluted (10-fold) in buffered peptone water (BPW) (Difco) on TSAYE and on appropriate selective agar (SMAC, XLT 4 or MOX) followed by counting bacterial colonies to determine numbers of survivors after incubation (35°C, 48 h).

2.6. Evaluation of temperature abuse on numbers of viable E. coli O157:H7, *S. enterica*, and *L. monocytogenes*

Samples of PJ with added YEX and ISO-EU (0.50, 0.75, 1 or 1.5 µL mL^{-1}) were temperature abused at 30ºC for 2 h on day 3 and at 30ºC for 2 h followed by 18ºC for 1 h on day 14. Numbers of viable pathogens were determined after 3, 7, 14, 28, 42, 56 and 70 days by performing serial dilutions (10-fold) of PJ in BPW and surface plating appropriate aliquots (in duplicate) on TSAYE and selective agar (SMAC, MOX or XLT 4). Inoculated agar plates were incubated at 35°C for 48 h and survivors were counted after incubation.

2.7. Determination of sub-lethal injury of E. coli O157:H7*, L. monocytogenes* and *S. enterica*

Based on recovery of bacterial colonies on selective agar (SMAC, MOX or XLT 4) and non-selective agar (TSAYE), survival curves were prepared. Percent sub-lethal injury was determined for survivors of each pathogen by using the following equation:

% Injury = [(CFU/mL TSAYE - CFU/mL selective agar)/CFU/mL TSAYE] x 100

2.8. Measurement of pH and degrees BRIX

The pH of juice samples was measured using an Orion model 525 pH meter (Orion Research Inc, Boston, MA) and their degree BRIX (º BRIX) was measured using a digital pocket refractometer PAL (ATAGO, USA, Inc., Bellevue, WA). Both measurements were performed on tempered (23±1ºC) juice samples before and after Tyndallization as well as on Tyndallized juice with or without added YEX.

2.9. Analysis of sensory characteristics of pineapple juice

Sensory analysis was conducted using acceptability tests to identify concentrations of ISO-EU in PJ with added YEX that would be acceptable to consumers. Evaluations were performed by twenty-eight panelists ranging in age from 22 to 43 years and who frequently drank PJ. All panelists signed written consent forms confirming their voluntary participation in

the sensory study. Due to a strong "clove" odor detected in PJ with 1.5 μL mL^{-1} ISO-EU, that treatment was omitted from sensory evaluation. The PJ with added ISO-EU (0, 0.5, 0.75, 1.0 μL mL^{-1}) and YEX were prepared and held refrigerated (4 ± 1 °C) for ~ 24 hours before evaluation. Juice with YEX but no ISO-EU was used as control. Panelists were seated in individual booths with controlled lighting and temperature (23 ± 1 °C). Within five minutes of removing the juice from the refrigerator, each panelist was given four juice samples (30 mL per sample) in disposable plastic cups coded with randomized three-digit numbers. The samples in each set of juice were served together using a blind method of random sequence. Bottled water and unsalted crackers were provided for panelists to clear their palates between tasting of samples as instructed. Panelists were asked to use a 9-point hedonic scale ranging from 1 (dislike very much) to 9 (like very much) to evaluate the following characteristics: appearance, odor, taste, after taste, viscosity, and overall acceptance (Stone & Sidel, 1993).

2.10. Statistical analysis

Three replications of the experiment were performed. Mean numbers of survivors were analyzed by using JMP Pro statistical software version 14 (JMP business unit of SAS Institute, Inc., Cary, NC). Treatment means were evaluated for significant differences ($p < 0.05$) by using the student's t-test. The significant differences between selective and non-selective media were determined with a two-sided T-test.

3. Results and Discussion

3.1. MIC and MBC of ISO-EU in BHI broth

The ISO-EU exhibited antibacterial activity against all three enteric pathogens tested with marked differences on efficacy of ISO-EU among the Gram-positive and Gram-negative pathogens. The MIC of ISO-EU for the gram-negative pathogens, *E. coli* O157:H7 and *S.*

enterica was 0.5 μL mL^{-1}; however, the MIC of ISO-EU for gram-positive *L. monocytogenes* was 0.2 μL mL^{-1}. The MBC (0.6 μL mL^{-1}) of ISO-EU for *E. coli* O157:H7 and *S. enterica* was slightly higher than its MIC. The MBC (0.4 μL mL^{-1}) of ISO-EU for *L. monocytogenes* was less than the MBC of the gram-negative bacteria, suggesting a greater sensitivity of *L. monocytogenes* to ISO-EU compared to *E. coli* O157:H7 or *S. enterica*. Generally, it has been hypothesized that EOs are more effective against Gram-positive than Gram-negative bacteria (Burt, 2004) and our results support this hypothesis. Gram-negative bacteria possess a lipopolysaccharide layer in their outer membrane that is hypothesized to prevent the passage of some antimicrobials such as some EOs (Burt, 2004; Mosqueda-Melgar et al., 2008). While some studies demonstrated that ISO-EU has higher antimicrobial activity against gram-negative bacteria compared to gram-positive bacteria, several factors including differences in bacterial strains, use of different surfactants for dispersing ISO-EU and variations in culture conditions and preparation could contribute to such observed discrepancies in antimicrobial effect (M. Hyldgaard et al., 2012; Morten Hyldgaard et al., 2015; Krogsgard Nielsen et al., 2016, 2017). In addition, in the present study, YEX may not only be functioning as a natural surfactant but may also be exerting an antimicrobial effect. It could be working additively or synergistically with ISO-EU to enhance the antimicrobial effect of ISO-EU against Gram-positive bacteria. However, since to date there are no studies on the antimicrobial efficacy of YEX against foodborne pathogenic bacteria and its mode of action in food systems, further research on YEX is warranted.

3.2. pH and degrees BRIX of PJ

The pH PJ with YEX but no ISO-EU was 3.47 ± 0.05 and 3.48 ± 0.06 respectively. Addition of ISO-EU did not significantly result in a change of the pH of the PJ ($P>0.05$) (data

not shown). Tyndallization did not significantly alter the pH of the juice (P>0.05). The mean pH of Tyndallized juice with YEX was 3.48 ± 0.09. Tyndallized juice without YEX had a mean pH of 3.46 ± 0.07.

The PJ samples with or without YEX presented no difference (P>0.05) in ºBRIX. The mean ºBRIX of raw PJ without YEX was 13.17 ± 1.78 and the mean ºBRIX of PJ with YEX was 13.58 ± 1.71. Tyndallization did not result in any significant differences in the mean ºBRIX of PJ with (14.48 ± 0.92) or without (14.12 ± 0.97) YEX (P>0.05). Since ºBRIX is an index of total soluble solids content which is directly related to viscosity, the use of 0.5% YEX as an emulsifier for EOs in juices will not alter juice viscosity which is an important sensory characteristic.

3.3. Viability of enteric pathogens in PJ at 4ºC

The effect of ISO-EU (0.50, 0.75, 1 or 1.5 μL mL^{-1}) with added 0.5% YEX on the viability of *L. monocytogenes, E. coli* O157:H7 and *S. enterica* in PJ stored at 4ºC is shown in figures 1-3. The initial viable count of *L. monocytogenes, E. coli* O157:H7 and *S. enterica* in the control and treated samples at 0 h were 7.18, 7.09 and 7.02 CFU mL^{-1} respectively. None of the enteric pathogens multiplied in the refrigerated (4°C) juice with or without added ISO-EU. The mean pH of the raw PJ without YEX or ISO-EU was 3.47 ± 0.05. The raw PJ alone, due to its low pH and high acidity presented a hostile environment for the pathogens hence no growth was observed in any of the treated or un-treated PJ samples.

Nevertheless, all three pathogens survived for more than 42 days and none of them were detected after 56 days in the control juice (0 μL mL^{-1}) (figures 1-3). Survival of the pathogens up to 56 days indicates that even in the acidic PJ, enteric pathogens could survive for a relatively long time to pose a food safety hazard to consumers. The *E. coli* O157:H7 and *Salmonella* spp. can survive in acidic environment of fruit juices due to acid stress response. In this regard several

juice-borne outbreaks of involving *E. coli* and *S. enterica* were associated with the consumption of similar low pH juices such as unpasteurized orange juice and apple juice (Raybaudi-Massilia et al., 2009).

The addition of ISO-EU to PJ + YEX at all concentrations tested (0.50, 0.75, 1 or 1.5 μL mL^{-1}) caused substantial decreases in viable counts of *L. monocytogenes, E. coli* O157:H7 and *S. enterica.* All ISO-EU treatments achieved greater than 5 log reduction of each pathogen within 24 h. These results are promising as the lowest concentration of ISO-EU tested (0.5 μL mL^{-1}) can be used to meet the FDA requirement of at least 5 log reduction in the pertinent microorganism and this can be achieved within 24 hours. Use of low concentrations of ISO-EU would minimize any sensory changes to the juice. After 4h, the > 5 log reduction of *L. monocytogenes* requirement was exceeded for 1.0 and 1.5 μL mL^{-1} ISO-EU. Although to date *L. monocytogenes* has not been implicated in unpasteurized fruit juice outbreaks, this pathogen is ubiquitous in the environment and has been isolated from an unpasteurized apple juice and an apple/raspberry juice blend (Gabriel & Nakano, 2009; Sung, Song, Kim, Ryu, & Kang, 2014). This organism is of public health significance in juices because it poses a threat to the immunocompromised persons including pregnant women, neonates, organ transplant patients and the elderly. In addition, *L. monocytogenes* is psychrotrophic and can survive very well or grow in refrigerated foods or beverages depending on the concentration of intrinsic or added antibacterial substances. The ability of ISO-EU (1.0 and 1.5 μL mL^{-1}) to achieve > 5 log reduction in PJ in just 4 h suggest its potential to ensure the safety of this popular beverage. The *E. coli* O157:H7 was very sensitive to 1.0 and 1.5 μL mL^{-1} ISO-EU which achieved a >5 log reduction of that pathogen after just 2 h. ISO-EU (1.5 μL mL^{-1}) completely inactivated *E. coli* O157:H7 and *S. enterica* after 4 h and after 6 h, respectively ($P<0.05$). After 8h, 1.0 μL mL^{-1} ISO-EU completely

inactivated *S. enterica.* Although the target site and mode of action of ISO-EU is not yet fully understood, it has been reported that the free hydroxyl groups of the EO component are responsible for its antimicrobial activity (Hyldgaard et al., 2012). The ISO-EU's antimicrobial activity may be attributed to the double bond in the α, β positions of the side chain and a methyl group in the γ position (G. Jung & Fahey, 1982). The inhibitory effects of EOs or EO components are a likely consequence of the increase in their hydrophobicity which improves their dissolution in membrane lipids of bacteria (Juven, Kanner, Schved, & Weisslowicz, 1994). Therefore, the low pH of the PJ in addition to the emulsifying characteristic of YEX could have enhanced the antimicrobial activity of ISO-EU against *L. monocytogenes, E. coli* O157:H7 and *S. enterica.*

3.4. Efficacy of ISO-EU on viability of enteric pathogens in temperature-abused PJ with YEX

The PJ with added YEX and ISO-EU at all concentrations tested (0.50, 0.75, 1 or 1.5 μL mL^{-1}) prevented the survival of all three enteric pathogens under temperature-abuse conditions. For each pathogen the initial viable count in refrigerated PJ was ~7.1 $\log_{10}$ CFU mL^{-1} and; based on non-selective plating after PJ samples were temperature-abused at 30ºC for 2 h following 3 days of refrigeration, viable cells of *E. coli, L. monocytogenes and S. enterica* were not detected even at the lowest concentration of ISO-EU tested (data not shown). The PJ was held at 30ºC for 2 h to simulate temperature-abuse of juice during loading and unloading from a delivery truck on a hot day. In this respect the results suggest that even under temperature-abuse conditions ISO-EU can maintain a strong antibacterial effect on enteric pathogens in PJ. Additionally, after temperature-abuse of 14-day refrigerated PJ samples at 30ºC for 2 h followed by storage at 18ºC for 1 h, none of the enteric pathogens were detected (data not shown). This type of temperature-abuse juice simulated potential mishandling by a consumer whereby purchased juice is held in a

hot car in transit from a retail store to the consumer's home where it is further abused by holding it at room temperature before placing into the refrigerator.

The FDA requires juice manufacturers to demonstrate that under temperature-abuse conditions, antimicrobial interventions for juices are able to maintain at least 5 log reduction of the pertinent pathogen throughout the shelf-life. The results of the present study indicated that ISO-EU can be very effective against human enteric pathogens to ensure the microbial safety of PJ under temperature-abuse conditions due to consumer's negligence. Warm temperatures have been shown to facilitate enhanced antimicrobial activity of EOs due to higher microbial metabolic activity, growth and death rates (Yuste & Fung, 2004). In addition, higher temperatures are known to result in the increased fluidity of the cell membrane to facilitate the diffusion of EOs into microbial cells (Mendonca et al., 2018).

3.5. Determination of sub-lethal injury in surviving populations of E. coli, L. monocytogenes and S. enterica

Table 1 shows the *p*-values (T-test, α=0.05) for the three enteric pathogens in control PJ based on colony counts on selective and non-selective media. There was no significant difference between counts on selective and non-selective media in control juices for *L. monocytogenes* and *E. coli* O157:H7 ($P>0.05$). However, for *S. enterica* a significant difference ($P<0.05$) was observed in survivors on TSAYE compared to XLT 4. The results indicate that XLT 4 significantly recovered less survivors than TSAYE indicating that some *S. enterica* cells were sub-lethally injured in the control juice and were unable to grow on the selective media. Bacterial cells in the control PJ with added YEX could be impaired by sub-lethal injury from exposure to low pH and acidity of the PJ in addition to the added YEX. The *Yucca schidigera* plant contains steroidal saponins which can be injurious to some microbes (Chapagain, Wiesman, & Tsror,

2007; Wulff, Zida, Torp, & Lund, 2011). Therefore, low pH, acidity and YEX most likely exerted multiple stresses on the pathogens in PJ to the extent where some stressed Salmonellae were incapable of multiplying on XLT 4. Selective ingredients such as sodium chloride, bile salts, sodium deoxycholate, tergitol, and antibiotics in selective media are known to be detrimental to the resuscitation of sub-lethally injured cells (Adams, 2005; Wesche et al., 2009).

Tables 2-4 show the mean value of percentage sub-lethal injury ± standard error of the mean for *L. monocytogenes, E. coli* O157:H7 and *S. enterica* survivors following exposure to various concentrations of ISO-EU in PJ after 2, 4 and 6 h. The surviving populations of all three pathogens in PJ exhibited sublethal injury after just 2 hours of exposure to ISO-EU at 0.5 or 0.75 μL mL^{-1}. In this respect, there was a significant difference in percent sub-lethal injury of *E. coli* O157:H7 cells exposed to 0.5 and 0.75 μL mL^{-1} ISO-EU ($P<0.05$). Almost the entire population of *E. coli* O157:H7 survivors (99.99 ± 0.01%) was sub-lethally injured by 0.75 μL mL^{-1} ISO-EU compared to 35.30 ± 18.02% sub-lethal injury caused by 0.5 μL mL^{-1} ISO-EU. The higher concentrations of ISO-EU (1.0 and 1.5 μL mL^{-1}) inflicted severe injury in all three pathogens in PJ especially after 4 or 6 hours (Tables 2 to 4).

In general, there was consistently high variability in percent injury data across all replications of the experiment and several factors are likely associated with this observation. For example, the extent of sub-lethal injury inflicted on a population of microorganisms by any antimicrobial treatment may be structural and/or metabolic and result in a spectrum of injury that ranges from mild to severe (Wesche et al., 2009). Within that spectrum substantial variations can occur in numbers of survivors that fit into the mild, medium or severe categories of sub-lethal injury. Also, variations in the proportion of sub-lethally injured survivors depend on different factors such as the target organism(s), the type and intensity of the antimicrobial treatment, and

the type and concentration of selective agents present in the selective culture medium (Wuytack et al., 2003). In some instances of severe sub-lethal injury, the target organism may suffer from oxygen toxicity and not grow aerobically on either non-selective or selective agar media but only on non-selective agar incubated anaerobically (A. F. Mendonca & Knabel, 1994).

Determination of sub-lethal injury in pathogens caused by antimicrobial treatments is important for two main reasons: i) the inability to detect sub-lethally injured organisms can lead to an erroneous overestimation of the antimicrobial efficacy of a treatment and ii) antimicrobial treatments that cause sub-lethal injury can be used in combination with other hurdles or interventions to prevent repair of cellular lesions and totally inactivate sub-lethally injured organisms. The results of the present study indicate that relatively low concentrations of ISO-EU in PJ with added YEX and short storage times inflict significant sub-lethal injury in the surviving populations of *L. monocytogenes, E. coli* O157:H7 and *S. enterica.* This EO constituent can be potentially combined with other non-thermal technologies such as high pressure or ultra sound to inactivate enteric pathogenic microbes. At very low concentrations it may be used to sensitize the microbial cells before the application of those technologies which could lead to effective microbial inactivation while achieving energy efficiency and low cost.

3.6 Sensory analysis of pineapple juice

The mean scores for sensory characteristics of PJ are shown in Table 5. Juice samples with YEX but no ISO-EU received the highest scores for taste, aftertaste and overall acceptance ($P<0.05$). For those control samples the mean scores for all sensory characteristics evaluated were “like moderately”. These results indicate that YEX at 0.5 % (w/v) did not negatively affect the juice characteristics evaluated in the present study. The highest concentration of ISO-EU (1.0 $\mu L\ mL^{-1}$) tested, negatively affected odor, taste, aftertaste and overall acceptance ($P<0.05$).

Although ISO-EU at 0.5 or 0.75 μL mL^{-1} did not significantly affect juice appearance or odor ($P>0.05$), the taste, aftertaste and overall acceptance were negatively affected ($P<0.05$). Sensory scores for viscosity of PJ with added ISO-EU were acceptable and significantly higher than those for control juice ($P < 0.05$). Based on these results, the overall acceptance scores for juice samples that contained ISO-EU (0.5 or 0.75 μL mL^{-1}) was likely impacted by the panelists' perceptions of taste and aftertaste. Leite et al., 2016 also reported similar findings for PJ with added lemongrass essential oil at 1.25 or 2.5 μL mL^{-1} whereby taste and aftertaste of the juice were scored as unsatisfactory. In this regard, further research on reducing the negative impact of ISO-EU or other EO components on some sensory characteristics of PJ is warranted. Such research should focus on combining lower concentrations of EO components with non-thermal antimicrobial processes for enhancing the microbial safety of raw juices.

4. Conclusions

This study demonstrates that *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes* can survive in PJ (pH 3.46) with added YEX for more than 42 days at refrigeration temperature (4ºC). This relatively prolonged survival of pathogens in the acidic environment of PJ confirms the microbial food safety risk posed by contaminated fruit juices devoid of an antimicrobial treatment. ISO-EU (0.5 to 1.5 μL mL^{-1}) combined with YEX has good potential for destroying all three pathogens in PJ at refrigeration temperature (4ºC) and at abusive temperatures (30ºC for 2h and 18ºC for1 h). Addition of ISO-EU (0.5 to 1.5 μL mL) to PJ provides a non-thermal kill step that can achieve the 5-log pathogen reduction in juice as stated in the juice HACCP regulations. Substantial ISO-EU-induced sub-lethal injury in pathogen survivors observed in the present study presents an opportunity to combine low concentrations of ISO-EU with other

antimicrobial agents to completely inactivate pathogens in juices. Further research is needed on approaches to reduce the negative effects of ISO-EU on taste and aftertaste of PJ while maintaining effective control of pathogenic microorganisms in PJ.

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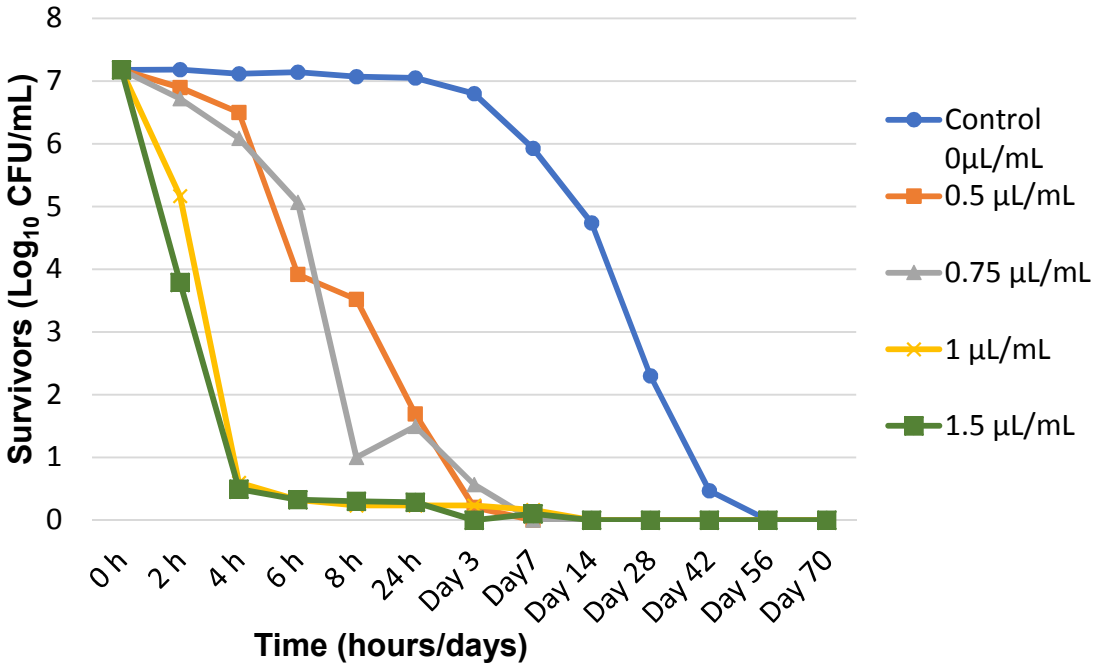

Figure 1. Effect of Isoeugenol on the Viability of *Listeria monocytogenes* in Pineapple Juice with added Yucca extract stored at 4ºC.

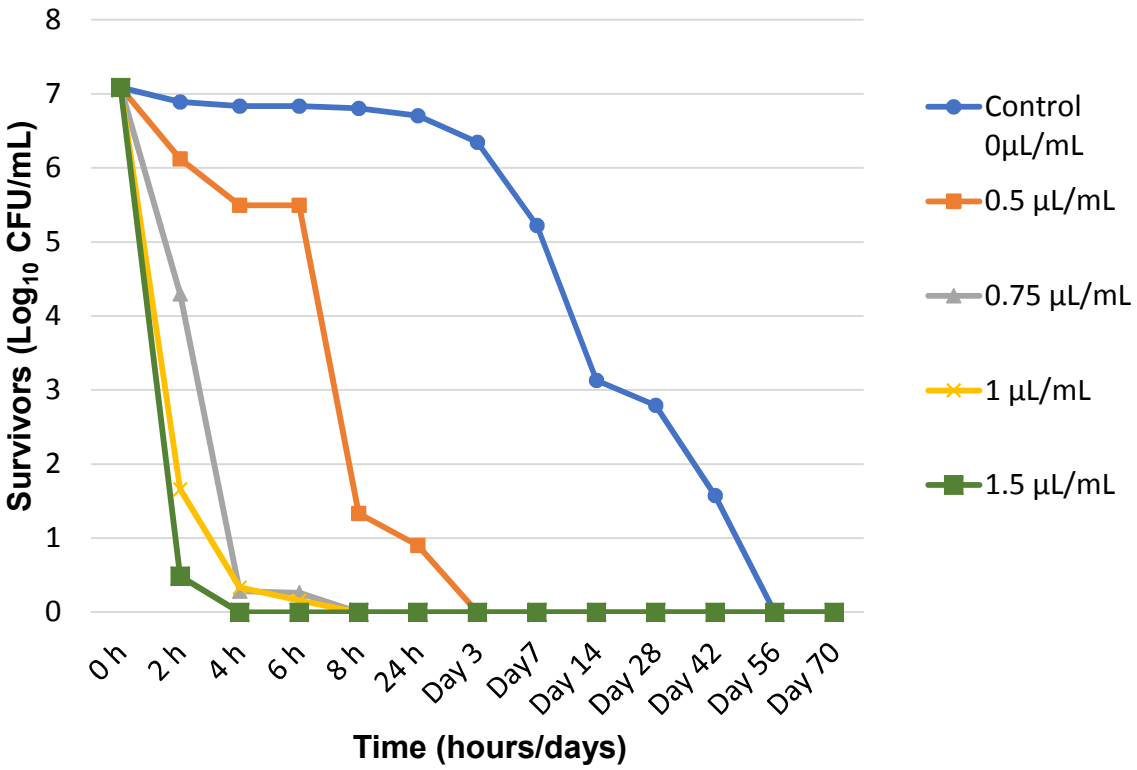

Figure 2. Effect of Isoeugenol on Viability of *Escherichia coli* O157:H7 in Pineapple Juice with added Yucca extract stored at 4ºC.

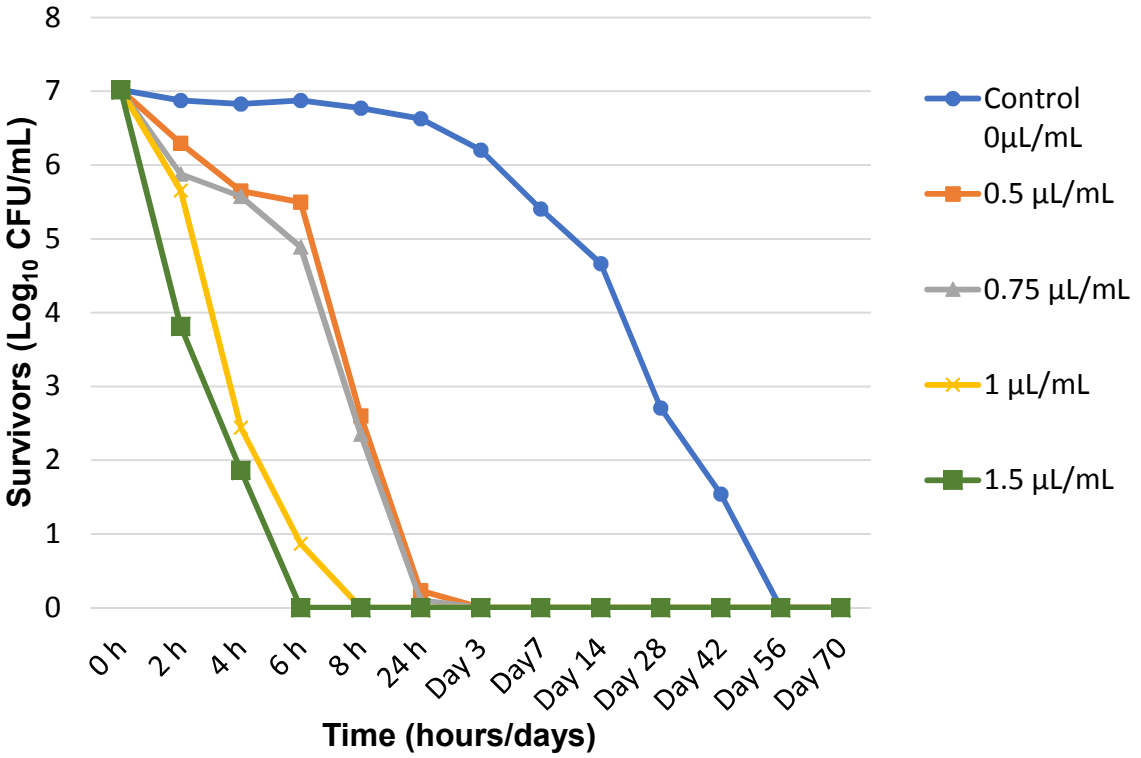

Figure 3. Effect of Isoeugenol on the Viability of *Salmonella enterica* in Pineapple Juice with added Yucca extract stored at 4 ºC.

Table 1. The *p*-values (two-sided T-test, α=0.05) analyses for three enteric pathogens enumerated on selective and non-selective media for the control juice (PJ + YEX).

Pathogen	Media	*p*-value	Difference
L. monocytogenes	TSAYE and MOX	0.4149	No
E. coli O157:H7	TSAYE and SMAC	0.5756	No
S. enterica	TSAYE and XLT 4	0.0482	Yes

Table 2. Mean value of percentage sub-lethal injury ± standard error of the mean for *Listeria monocytogenes* survivors after 2, 4, and 6 h of exposure to ISO-EU in tyndallized pineapple juice (4 C) with added yucca extract.

	Exposure time		
Treatment (µL mL^{-1})	2 hours	4 hours	6 hours
ISO-EU (0.5)	36.37 ± 28.45[b]	36.52 ± 18.86[b]	5.34 ± 5.34[b]
ISO-EU (0.75)	36.71 ± 18.28[b]	35.34 ± 24.28[b]	63.23 ± 31.74[b]
ISO-EU (1.0)	4.13 ± 4.13[b]	-[a]	-[a]
ISO-EU (1.5)	4.76 ± 4.76[b]	-[a]	-[a]

-[a] survivors not detected on non-selective or selective agar medium in 2 or more replications
[b]Means with a different letter within a column differ significantly (P<0.05)

Table 3. Mean value of percentage sub-lethal injury ± standard error of the mean for *Escherichia coli* O157:H7 survivors after 2, 4, and 6 h of exposure to ISO-EU in tyndallized pineapple juice (4 C) with added yucca extract.

	Exposure time		
Treatment (µL mL^{-1})	2 hours	4 hours	6 hours
ISO-EU (0.5)	35.30 ± 18.02[b]	19.88 ± 14.44	21.16 ± 10.60
ISO-EU (0.75)	99.99 ± 0.01[c]	-[a]	-[a]
ISO-EU (1.0)	-[a]	-[a]	-[a]
ISO-EU (1.5)	-[a]	-[a]	-[a]

-[a] survivors not detected on non-selective or selective agar medium in 2 or more replications
[b,c]Means with a different letter within a column differ significantly (P<0.05)

Table 4. Mean value of percentage sub-lethal injury ± standard error of the mean for *Salmonella enterica* survivors after 2, 4, and 6 h of exposure to ISO-EU in tyndallized pineapple juice (4 C) with added yucca extract.

	Exposure time		
Treatment (µL mL^{-1})	2 hours	4 hours	6 hours
ISO-EU (0.5)	76.59 ± 21.45[b]	99.59 ± 0.41[b]	99.99 ± 0.01[b]
ISO-EU (0.75)	59.44 ± 29.99[b]	93.66 ± 5.44[b]	71.37 ± 28.63[b]
ISO-EU (1.0)	94.37 ± 4.49[b]	100 ± 0[b]	-[a]
ISO-EU (1.5)	98.07 ± 1.93[b]	-[a]	-[a]

-[a] survivors not detected on non-selective or selective agar medium in 2 or more replications
[b]Means with a different letter within a column differ significantly (P<0.05)

Table 5: Sensory evaluation of attributes of PJ (tyndalized) with or without ISO-EU and added YEX

Concentration ISO-EU (µL mL^{-1})	Scores for Sensory Characteristics, mean ± SD					
	Appearance	Odor	Taste	After taste	Viscosity	Overall Acceptance
Control	7.61 ± 0.88^{a}	7.68 ± 0.77^{a}	7.89 ± 0.74^{a}	7.07 ± 1.02^{a}	7.07 ± 0.90^{b}	7.32 ± 0.94^{a}
0.5	7.57 ± 0.92^{a}	7.75 ± 0.84^{a}	5.79 ± 1.17^{b}	5.75 ± 1.08^{b}	7.61 ± 0.79^{a}	5.86 ± 0.65^{b}
0.75	7.54 ± 0.79^{a}	7.64 ± 0.78^{a}	4.68 ± 0.94^{c}	4.14 ± 0.76^{c}	7.50 ± 0.75^{a}	4.04 ± 0.74^{c}
1	7.43 ± 0.63^{a}	3.36 ± 0.78^{b}	3.07 ± 0.72^{d}	2.79 ± 0.63^{d}	7.57 ± 0.63^{a}	2.82 ± 0.67^{d}

^{a,b,c,d}Means with a different letter within a column differ significantly ($P<0.05$)

PJ, pineapple juice; ISO-EU, isoeugenol; YEX, yucca extract; Control has no ISO-EU but has YEX; Acceptability of each characteristic was evaluated using a 9-point hedonic scale ranging from1 (dislike very much) to 9 (like very much)

CHAPTER 4.

DESTRUCTION OF INDIGENOUS SPOILAGE MICROFLORA BY ISOEUGENOL IN REFRIGERATED RAW PINEAPPLE JUICE WITH ADDED *YUCCA SCHIDIGERA* EXTRACT

A paper to be submitted to *Food Microbiology*

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Keywords: Natural spoilage microflora**,** isoeugenol**,** natural surfactant**,** juice

Abstract

The effectiveness of isoeugenol (ISO-EU) for killing the indigenous microflora in raw pineapple juice (PJ) (pH 3.64) with added yucca extract (YEX) was investigated. Effects of ISO-EU on the sensory characteristics of PJ were also assessed. The PJ with 0.5% (w/v) YEX containing 0 (control), 0.50, 0.75, 1.0, or 1.5 μL mL^{-1} ISO-EU was stored at 4°C for 32 days. At set intervals, counts of viable yeast and mold (YM), aerobic plate count (APC), lactic acid producing bacteria (LAB) and *Enterobacteriaceae* (ENT) were determined using cultural methods and counting microbial colonies on agar plates after incubation. The YM grew well in PJ without added ISO-EU and YEX and reached 7.19 log_{10} colony forming units (CFU) mL^{-1} after 32 days. Viable YM in juice with YEX alone were 1.86 log CFU mL^{-1} lower than that of control juice (no YEX) after 32 days ($P< 0.05$). In juice with YEX and ISO-EU, YM counts decreased by 3 to 4 log CFU mL^{-1} after 32 days ($P<0.05$). Similarly, the APC decreased in juice with added ISO-EU and YEX. Growth of LAB was completely inhibited in PJ with ISO-EU and YEX. The ENT were eliminated after 4 h of exposure to ISO-EU at 0.75, 1.0, or 1.5 μL mL^{-1} and after 12 h by 0.5 μL mL^{-1}. After 14 days, the color, odor and viscosity of PJ with added YEX and ISO-EU (0.5 μL mL^{-1}) were not significantly different ($P>0.05$) from those of control (Raw PJ); however, taste and overall acceptance of PJ with ISO-EU were unsatisfactory. Scores for all characteristics of ISO-EU-containing PJ were similar after 1, 14 and 28 days; however, after 28 days the color, odor, taste and overall acceptance of Raw PJ were unsatisfactory. Based on these results, ISO-EU combined with YEX is an effective natural preservative system with good potential for killing the native microflora and extending the microbial shelf life of refrigerated

(4°C) PJ. Further research on approaches to control juice spoilage organisms using lower concentrations of ISO-EU to prevent negative effects on sensory characteristics of PJ is warranted.

1. Introduction

Current trends indicate a growing consumer demand for minimally processed, fresh-like fruit and vegetable juices that are devoid of preservatives of synthetic origin. There is a rapidly growing demand for colorful, flavorful, natural, low-calorie juices that contain several nutrients such as antioxidants, vitamins and minerals. Most importantly, the demand for natural juices has increased because consumers are now more aware of the relationship between consumption of fresh fruits and vegetable juices and prevention of several types of cancers and cardiovascular diseases (Rico et al., 2007). According to the United States Department of Agriculture Economic Research Service, Americans consumed 23.7 pounds (2.7 gallons) of orange juice per person in 2015, leading the list of the most consumed fruit juices. Apple juice consumption was second at 14 pounds (1.6 gallons) per person, followed by grape juice and pineapple juice (USDA, 2017).

Pineapple (*Ananas comosus* (L.) Merril) juice is one of the most popular consumed fruit juices worldwide and in 2016, the world production of pineapples was 25.8 million tons with Costa Rica, Brazil and Philippines accounting for nearly one-third of the world's production (FAO, 2017). The pineapple fruit is known for its attractive aroma, strong flavor and is an excellent source of fiber, minerals such as calcium, iron and magnesium, and vitamins such as vitamin C. It is also a great source of phenolic compounds and antioxidants (Couto, Cabral, Matta, Deliza, & Freitas, 2011). Pineapples also contain a natural source of bromelain, a protein-digesting enzyme that has demonstrated several health benefits (Aiyegbusi, Olabiyi, Duru, Noronha, & Okanlawon, 2011; Hale, Chichlowski, Trinh, & Greer, 2010). However, despite the several health benefits of the consumption of pineapple juice, the juice may inevitably become

contaminated with spoilage and pathogenic microorganisms from the outer rind surface of the pineapple fruit (Leite et al., 2016). During cutting of the pineapple fruit and juice extraction, microbes on the highly contaminated leaves of the crown or outside of the peel can be transferred to the juice. In addition, the pineapple fruit has several blossom cups which extend from the peel into the actual edible part of the fruit that are capable of harboring pathogenic and spoilage microbes. The total microbial contamination load in juices has been reported to be as high as 3 to 5 $\log_{10}$ CFU mL^{-1} (Leite de Souza et al., 2016)

Microbial contamination of fruit juices can result in spoilage, deterioration in organoleptic characteristics, and economic losses. Signs of microbial spoilage of juices include cloud loss, development of unpleasant aroma, gas production and changes in color and texture (Sospedra, Rubert, Soriano, & Mañes, 2012). In this respect, fungi (yeasts and molds) are known as the main spoilage microbes responsible for microbiological spoilage in fruit juices due to their ability to grow in the high acidic environment of most fruit juices. Common molds such as *Penicillium* spp., *Aspergillus* spp., *Botrytis* spp., and *Alternaria* spp. and yeasts such as *Saccharomyces* spp. and *Rhodotorula* spp. cause spoilage of fresh fruits and juices. Some bacteria such as *Erwinia* spp., *Pseudomonas* spp., *Enterobacter* spp. and lactic acid bacteria have also been implicated in spoilage of fresh-cut fruit and fruit juices (Raybaudi-Massilia et al., 2009). Juice spoilage microorganisms can deteriorate juices due to microbial growth or by the production of enzymes that decompose various constituents of juices. Without a preservation intervention, fruit juices are subjected to a shortened shelf-life due to spoilage microbes.

Traditionally, juice manufacturers have used thermal processing, notably canning or heat pasteurization, to reduce or inhibit growth of spoilage microbes in juices; however, heat treatments may alter flavor, color and heat-labile nutrients in juices. Synthetic preservatives such

as potassium sorbate and sodium benzoate have also been used extensively to extend the shelf-life of juices but are highly unacceptable to consumers (Amirpour et al., 2015). In recent times, emerging non-thermal processes such as high hydrostatic pressure processing, pulsed electric field and ultrasound have been explored but may be considered too costly or energy expensive (Raybaudi-Massilia et al., 2009). In addition, further research is required on some novel processes such as cold plasma before they become practical in the food industry. On the other hand, natural antimicrobials such as essential oils (EOs) and their constituents provide an excellent alternative. They are natural and are derived from different parts of plants (flowers, seeds, bark, leaves) and so are widely accepted by consumers (Mendonca et al., 2018). Most EOs and their constituents are generally recognized as safe (GRAS) by the United States Food and Drug Administration (USFDA) (Hyldgaard et al., 2012). The EOs and their constituents have been used as flavoring agents in beverages because of their strong aroma and flavor; however, in recent years, their use as natural preservatives has been extensively studied. The EO constituent, ISO-EU, is extracted from EOs of nutmeg, clove and cinnamon and has a distinct clove-like flavor. Although it is approved for use in food as a flavoring agent (21 CFR 172.515), it has antibacterial and antifungal activity (Morten Hyldgaard et al., 2015) and therefore should be exploited for shelf-life extension of juices.

Despite the antimicrobial properties of EOs and their constituents, their use as juice preservatives may be limited by their hydrophobicity and poor solubility in hydrophilic environments (Mendonca et al., 2018). Although synthetic surfactants Tween 20 or Tween 80 were used by previous researchers to overcome this poor miscibility (Budzynska et al., 2009; Mendonca et al., 2018; Remmal et al., 1993), in the present study, a promising alternative, Yucca extract, was used. Yucca extract from the Mohave Yucca plant (*Yucca schidigera)* was used as a

natural dispersion aid. It is FDA approved for use in the food industry (21 CFR 172.510) and to our knowledge there are no publications on its use as a natural surfactant in PJ or other fruit or vegetable juices to which EOs or EO constituents have been added.

Traditionally, PJ infused with assorted spices such as clove buds has been consumed for centuries in the Caribbean region. This tropical juice has a distinct clove-like flavor that has already been widely accepted in Caribbean countries. Because ISO-EU is known to have a distinct clove-like flavor, a combination of PJ with ISO-EU could be exploited for ISO-EU's antimicrobial activity against natural spoilage microflora. To our knowledge, there are currently no publications on the antibacterial efficacy of ISO-EU against natural spoilage microflora in PJ with added YEX. Therefore, in the present study, the effectiveness of ISO-EU was evaluated for control of naturally occurring microflora in refrigerated (4ºC) PJ (with added YEX) over a 32-day period. The effects of ISO-EU on the sensory characteristics of PJ were also evaluated.

2. Materials and Methods

2.1. Preparation of pineapple juice

Fresh Del Monte Gold extra sweet pineapples were purchased from a local grocery store in Ames, Iowa. Pineapples that were uniform in size were selected from the same variety free from mechanical injuries. An OXO pineapple corer and slicer was used to manually remove cores and the pineapple rings were chopped into pieces. The juice was extracted using a juice extractor (Model #67608Z, Hamilton Beach Big Mouth Pro Juice Extractor, Glen Allen, Virginia), and particulates were removed by using cheesecloth clamped over a stainless-steel strainer. Juice samples were held refrigerated (4 ± 1°C) in 195-mL portions in sterile Erlenmeyer flasks.

2.2. Addition of yucca extract and isoeugenol to pineapple juice

A stock solution of 0.2 g mL^{-1} (20% w/v) *Yucca schidigera* extract (YEX; Garuda International, Inc., Exeter, CA) was prepared by adding 10g YEX to 40 mL PJ in a sterile Erlenmeyer flask. The stock solution was sterilely filtered using a vacuum pump and 0.45 µm pore size sterile Corning® filtration system (Fisher Scientific, Chicago, Illinois). Five milliliters of the filter sterilized stock YEX was aseptically added to the 195-mL aliquots of PJ in sterile 250-mL screw capped Erlenmeyer flasks to obtain a final concentration of 0.5% (w/v) YEX.

Ten milliliters ISO-EU was sterilely filtered using 0.45 µm pore size Luer-Lock filter and syringe (Fisher Scientific). The PJ with added YEX in each Erlenmeyer flasks was mixed thoroughly by manual shaking and the appropriate volume of ISO-EU was aseptically added to each flask to obtain the desired concentrations (0.50, 0.75, 1 or 1.5 µL mL^{-1}) of ISO-EU. The contents of the flasks were mixed by shaking. A flask containing PJ without ISO-EU (0 µL mL^{-1} ISO-EU) but with 0.5% YEX served as the control. However, in the experiments evaluating the effect of ISO-EU on the viability of YM, an additional flask containing 200 mL raw PJ without YEX and ISO-EU served as another control. Appropriately labeled Erlenmeyer flasks were held at 4ºC ± 1ºC until used for microbiological analysis at 0, 4, 12, 24 h and days 4, 8, 16, 24, 28, and 32.

2.3. Microbiological analysis

At set intervals during 32 days of refrigerated storage, microbial survivors were determined by plating juice samples diluted (10-fold) in buffered peptone water (BPW) (Difco) on appropriate agar growth media. For yeast and molds (YM), samples of PJ were plated on dichloran rose Bengal chloramphenicol (DRBC) agar and fungal colonies were counted after incubation (25ºC, 5 days). The aerobic plate count (APC) of PJ was obtained by plating juice

samples on tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE) and enumerating colonies after 48 h of incubation (35 ºC). Populations of lactic acid producing bacteria (LAB) were determined by surface plating PJ samples on deMann Rogosa Sharpe agar with 0.02 % (w/v) of added potassium sorbate (MRS-PS) and counting bacterial colonies on MRS-PS plates following 72 h of anaerobic incubation at 35ºC. The *Enterobacteriaceae* (ENT) were enumerated after plating juice samples on tryptic soy agar supplemented with 0.6 % yeast extract (TSAYE, BBL) with an overlay of violet red bile agar (VRB, Difco Laboratories) and counting colonies after incubation (35°C) for 48 h (Hartman, Hartman, & Lanz, 1975). Glucose was not added to the VRB because TSA has fermentable carbohydrates other than lactose in the papaic digest of soybean meal component (Rhode, 1969).

2.4. Measurement of pH and degrees BRIX

All juice samples were tempered to 23 ± 1ºC before pH and degrees BRIX (ºBRIX) were measured at set times. The pH of juice samples with or without ISO-EU was measured using an Orion model 525 pH meter (Orion Research Inc, Boston, MA). The º BRIX of juice samples was measured using a digital pocket refractometer PAL (ATAGO, USA, Inc., Bellevue, WA).

2.5. Sensory analysis

Sensory analysis was performed using acceptance tests to identify concentrations of ISO-EU in PJ with added YEX that would be acceptable to consumers. Twenty-five panelists (age range 22 to 43 years) who frequently consumed PJ participated in evaluating juice samples. All panelists provided signed written consent forms confirming their voluntary participation in the sensory study. Only juice samples containing the two lower concentrations of ISO-EU (0.5 and 0.75 μL mL^{-1}) were included along with control PJ (with or without YEX) for sensory analysis. Batches of PJ with or without added YEX alone or with ISO-EU (0.5 and 0.75 μL mL^{-1}) and

YEX were prepared and stored under refrigeration (4 ± 1 ºC) for 32 days. Sensory evaluation tests of PJ samples were performed after 1, 14 and 28 days of storage. Panelists performed evaluations in individual booths with controlled lighting and temperature (23 ± 1 ºC). Immediately after removing the PJ from the refrigerator, each panelist was served with four juice samples (30 mL per sample) in disposable plastic cups coded with randomized three-digit numbers. The juice samples within each set of juice were presented to the panelists using a blind method of random sequence. Panelists were instructed to use bottled water and unsalted crackers to clear their palates between tasting of juice samples. The panelists used a 9-point hedonic scale ranging from 1 (dislike very much) to 9 (like very much) to evaluate the color, odor, taste, viscosity, and overall acceptance of the juice samples (Stone & Sidel, 1993).

2.7. Statistical analysis

Three replications of the experiment were performed. Mean numbers of survivors were analyzed by using JMP Pro statistical software version 14 (JMP business unit of SAS Institute, Inc., Cary, NC). Treatment means were evaluated for significant differences ($P<0.05$) by using the Tukey-Kramer test.

3. Results and Discussion

3.1. pH and degrees BRIX of pineapple juice

There was no significant difference in pH of the control PJ with or without YEX at 0 h ($P>0.05$). The mean pH of the control juice without YEX was 3.64 ± 0.02 and the mean pH of the control PJ with added YEX was 3.63 ± 0.04. The addition of YEX to the PJ appeared to slightly lower the pH of the PJ but not significantly. Overall, the addition of ISO-EU at all concentrations to PJ with added YEX did not significantly ($P>0.05$) alter the pH of the juice. The mean pH of the treated PJ ranged from 3.63 – 3.65. The values for ºBRIX of the control PJ samples (with or without YEX) at 0 h were significantly different from each other and from

those for the ISO-EU-treated samples ($P<0.05$). The mean ºBRIX of the control PJ without YEX was 12.3 ± 0.05 and the mean ºBRIX of control PJ with YEX was 12.8 ± 0.12. The addition of YEX to the PJ appeared to significantly increase the ºBRIX of the PJ. It has been reported that YEX has at least 90% carbohydrates (Garuda International, 2013). This high percentage of carbohydrates might be contributing to the total soluble solids of the PJ when YEX is added, hence resulting in an increase in ºBRIX. Addition of ISO-EU (0.50, 0.75, 1 or 1.5 μL mL^{-1}) to the PJ with YEX resulted in a significant difference in the mean ºBRIX of PJ ($P<0.05$). However, there were no significant differences in the mean ºBRIX among PJ samples containing various concentrations of ISO-EU ($P>0.05$). The mean ºBRIX of the PJ samples containing various concentrations of ISO-EU ranged from 12.9 – 13.0.

3.2. Viability of natural spoilage microflora groups in pineapple juice with added ISO-EU and YEX at 4ºC

Figure 1 shows the viable populations of YM in PJ during refrigerated (4ºC) storage for 32 days. The initial viable count of YM in the controls (0 μL mL^{-1} ISO-EU with or without YEX) and treated samples at 0 h was 4.09 ± 0.25 $\log_{10}$ CFU mL^{-1}. That YM count remained relatively stable for about 4 days but steadily increased and reached 7.19 $\log_{10}$ CFU mL^{-1} in 32 days. The YM counts in the control juice with YEX and no ISO-EU increased to 5.23 $\log_{10}$ CFU mL^{-1} on day 32, representing a substantial difference (1.86 log CFU mL^{-1}) in counts. This result indicates that YEX, added at 0.5% (w/v) to PJ for emulsification of ISO-EU, exerts an inhibitory effect on YM growth in PJ. The *Yucca schidigera* plant is a rich source of steroidal saponins and polyphenols (Cheeke et al., 2006) both of which have been shown to have antibacterial and antifungal properties (Killeen et al., 1999; Wulff et al., 2011). Considering that YM are the predominant spoilage microorganisms in acidic fruit juices (Tournas et al., 2006; Raybaudi-

Massilia et al., 2009) further research on YEX is warranted to explore the antifungal effects of this natural surfactant at various concentrations in other fruit juices with added EOs or EO components.

No growth of YM occurred in the presence of ISO-EU; therefore, none of the PJ samples with added ISO-EU reached the spoilage detection limit of ~7.0 $\log_{10}$ CFU mL^{-1} (Ray & Bhunia, 2013) during the 32 days of refrigerated storage. In contrast to the control juice, PJ with added ISO-EU exhibited significant ($P<0.05$) reductions in populations of YM. After just 4 h, the viable count of YM in PJ with 1.5 μL mL^{-1} ISO-EU was significantly different from both controls ($P<0.05$). This indicates that this EO component works quickly to inactivate YM. After 24 hours, 1.0 and 1.5 μL mL^{-1} ISO-EU reduced the initial viable YM count by ~1.31 and 2.60 $\log_{10}$ CFU mL^{-1} respectively. A concentration of 1.5 μL mL^{-1} ISO-EU in PJ completely inactivated YM after 24 days with no YM survivors detected in juice samples on days 28 and 32.

Common molds such as *Penicillium* spp. and *Aspergillus* spp. as well as yeasts such as *Candida* spp, *Geotrichum* spp, *Saccharomyces* spp. and *Rhodotorula* are often associated with spoilage of fresh fruits and their juices (Tournas et al., 2006; Raybaudi-Massilia et al., 2009). Tournas et al. (2006) reported the capability of some of the yeast isolated from fruit salads and fruit juices to grow at refrigeration temperatures and spoil those food products before their expiration date. Based on the inactivation of YM in PJ as shown in the present study the addition of ISO-EU to acidic fruit juices seems to be a promising approach for effective control of YM to prevent economic losses due to fungal spoilage of juice resulting in poor or unacceptable sensory characteristics.

The antibacterial effect of ISO-EU on the APC of PJ with added YEX and held at 4ºC for 32 days is shown in table 1. The initial APC of the control (0 μL mL^{-1} ISO-EU with YEX) and

treated juice samples at 0 h was 3.98 ± 0.37 $\log_{10}$ CFU mL^{-1}. This initial count is consistent with the 3 to 5 $\log_{10}$ CFU mL^{-1} of microbial contamination levels typically reported in juices (Leite de Souza et al., 2016). Pineapples grow close to the ground and their outer skin surfaces can be easily contaminated with pathogenic and spoilage microbes from sources such as soil, water, wind-blown dust, insects, and animal fecal material. Further contamination of the flesh can occur during processing thus increasing the possibilities of spoilage and foodborne illness. The initial APC in the control juice decreased, reaching 3.02 ± 0.76 $\log_{10}$ CFU mL^{-1} on day 16. That observed initial decrease in APC in the control juice is likely due to the inactivation of some bacteria that are sensitive to the hostile high acidic environment of the PJ. However, after day 16, there was an increase in APC in the control juice to reach approximately 5.0 log CFU mL^{-1} after 28 days (table 1). This increase could be attributed to the growth of aerobic acid-tolerant bacteria and yeast that are more resistant to the high acidity of the juice (Raybaudi-Massilia et al., 2009; Bevilacqua et al., 2011).

The addition of ISO-EU to PJ with added YEX at all tested concentrations (0.50, 0.75, 1.0 or 1.5 µL mL^{-1}) caused a decrease in the APC. After 4 h, ISO-EU at 1.0 and 1.5 µL mL^{-1} reduced the initial APC in the PJ by ~1.85 and 1.94 $\log_{10}$ CFU mL^{-1}, respectively. There were significant differences in the APC between the control and 1.5 µL mL^{-1} or 1.0 µL mL^{-1} and 0.5 µL mL^{-1} and 1.5 µL mL^{-1} ISO-EU treated juices after 4 h ($P<0.05$). At all days after day 8, (except day 16) there were significant differences in APC between the control and all concentrations of ISO-EU in PJ ($P< 0$). All ISO-EU treatments achieved ~3.0 $\log_{10}$ CFU mL^{-1} reduction on day 32. However, on day 32, the APC of control juice reached 5.00 ± 0.09 $\log_{10}$ CFU mL^{-1}. These results are promising and demonstrate that even the lowest concentration of

ISO-EU tested (0.5 μL mL^{-1}) in combination with YEX is effective in reducing the APC related to microorganisms that can grow in the PJ stored at refrigeration temperature (4°C).

The addition of ISO-EU to PJ with added 0.5% YEX at all concentrations tested (0.50, 0.75, 1 or 1.5 μL mL^{-1}) caused a decrease in the initial viable counts of LAB (Table 2). The initial viable count of LAB in the control (0 μL mL^{-1} ISO-EU with 0.5% YEX) and treated samples at 0 h was 2.43 ± 0.43 log_{10} CFU mL^{-1}. The initial viable count of this group of spoilage microbes was the least when compared to the other microbial groups evaluated in the present study. After just 4h, 0.50, 0.75, 1 and 1.5 μL mL^{-1} ISO-EU decreased viable LAB counts to 1.36, 1.39, 1.31 and 1.23 log_{10} CFU mL^{-1} respectively. However, LAB counts in the control juice was 2.26 log_{10} CFU mL^{-1}. The LAB counts in control PJ remained relatively constant during 8 days of refrigeration but increased to 2.99 log CFU mL^{-1} after 24 days followed by a decrease to 0.35 log CFU mL^{-1} after 32 days (Table 2). Interestingly, that decline in LAB counts on control PJ coincided with a marked increase in populations of YM and APC in control PJ (Fig 1 and Table 1). Although LAB are acid tolerant and certain strains can produce metabolites that inhibit growth of YM (Govaris, Botsoglou, Sergelidis, & Chatzopoulou, 2011), the reduction of LAB counts observed in the present study might have been a result of competitive inhibition from growth of indigenous microorganisms in the PJ. It has been reported that some yeasts can have an inhibitory effect on growth of LAB due to competition and production of ethanol or other inhibitory agents (Dierings, Braga, Silva, Wosiacki, & Nogueira, 2013). Another plausible explanation is that our refrigerated juice samples in Erlenmeyer flasks were held under aerobic conditions. Since LAB are anaerobes, the continual diffusion of air (from the head space in the flasks) into the PJ could have resulted in oxidative stress in the LAB especially during manual swirling of the PJ to mix it before taking samples for microbiological analysis.

The LAB survivors in all ISO-EU treated samples decreased after day 16 and were not detected after 28 days (Table 2). In addition to YM, the LAB is an important microbial group that can cause spoilage of fruit juices (Raybaudi-Massilia et al., 2009; Govaris et al., 2011; Bevilacqua et al., 2011); therefore, controlling the growth of this group of spoilage microbes using ISO-EU can extend the shelf-life of PJ. Overall, 1.5 μL mL^{-1} ISO-EU was most effective against the LAB throughout the refrigeration of the juice for 32 days. Because there were no significant differences in LAB survivors among treatments ($P>0.05$) at each time, the lowest concentrations of ISO-EU (0.5 μL mL^{-1}) could be used to reduce the growth of LAB in PJ while reducing negative effects on the sensory characteristics of the juice.

Figure 2 shows the effect of ISO-EU (0.50, 0.75, 1 or 1.5 μL mL^{-1}) with added 0.5% YEX on the viability of ENT in PJ stored at 4ºC. The initial viable count of ENT in the control (0 μL mL^{-1} ISO-EU with 0.5% YEX) and treated samples at 0 h was 3.00 ± 0.48 $\log_{10}$ CFU mL^{-1}. This microbial group was very sensitive to all concentrations of ISO-EU tested in PJ. The ISO-EU completely inactivated ENT after 12 h at the lowest concentration of ISO-EU tested (0.5 μL mL^{-1}); at higher concentrations, 0.75, 1 or 1.5 μL mL^{-1} ISO-EU complete inactivation occurred after just 4 h. Although members of this group did not multiply in the refrigerated (4°C) juice with or without added ISO-EU, viable cells survived for up to 24 days in the control PJ. On days 28 and 32, viable cells of ENT were not detected. The decrease in viable numbers of ENT in the control juice during refrigerated storage could be due to the low pH of the juice (pH 3.63) and/or the presence of saponins and polyphenols in the YEX. Survival of ENT up to day 24 in the control juice indicates that some members of the ENT may possess acid tolerance mechanisms than contribute to their survival in low pH environments (Bearson, Bearson, & Foster, 1997). Considering that the ENT contains organisms of public health significance including *Salmonella*

and shiga toxin producing *E. coli*, the effective control of that microbial group in PJ by added ISO-EU can enhance the microbial safety of that popular juice.

The ENT are a large family of gram-negative, facultative anaerobes that are non-spore-forming. This group includes pathogenic bacteria such as *Escherichia coli*, *Klebsiella* spp., *Salmonella enterica* serotype Typhi, and *Shigella* as well as harmless microbes. Several members of this group are found in the lower gastrointestinal tract of animals but some are also commonly found in the environment such as in the soil, on animals, plants and water (Donnenberg, 2015). Contamination of the PJ by microbes from this group may cause deterioration of the juice which will result in quality loss but most importantly may also lead to foodborne illness as this group contains pathogenic microbes.

3.3. Sensory analysis of pineapple juice

Tables 3, 4, and 5 show the mean scores for sensory characteristics of control PJ and PJ with added YEX and ISO-EU based on evaluations performed after 1 day, 14 days and 28 days, respectively. After day 1 and day 14, control PJ with or without added YEX received the highest scores for taste and overall acceptance ($P<0.05$). For those controls the mean of scores for all sensory characteristics evaluated fell between “like moderately” (score =7) to “like strongly” (score = 8). For all juice characteristics evaluated, there were no significant differences in sensory scores for the two controls (on days 1 and 14) ($P > 0.05$). Those results suggest that the YEX did not negatively affect the sensory characteristics of PJ. The PJ samples with ISO-EU at 0.5 μL mL^{-1} did not differ from control (raw PJ) with regard to juice color, odor or viscosity ($P > 0.05$) on days 1 and 14; however, the taste and overall acceptance were negatively affected ($P < 0.05$). Also, taste and overall acceptance of juice with 0.75 μL mL^{-1} ISO-EU were negatively affected ($P < 0.05$). The scores for all sensory characteristics of ISO-EU-containing PJ were

similar after 1, 14 and 28 days; however, after 28 days scores for color, odor, taste and overall acceptance of control (raw PJ) without YEX were unsatisfactory. After 28 days those reductions in sensory scores for raw PJ without YEX coincide with a marked increase in the YM population in the juice (Fig 1). Panelists reported a cloudy appearance of the juice along with a slight alcoholic odor and "fizzy" mouth feel. Such changes in fruit juices typically result from growth of yeast, a major group usually responsible for spoilage of acidic fruit juices (Tournas et al., 2006).

Those sensory evaluation results suggest that the overall acceptance scores for PJ with added ISO-EU (0.5 or 0.75 μL mL^{-1}) were most likely influenced by the panelists' perception of taste. Our results are consistent with those of Leite et al. (2016) who reported that PJ with added lemongrass EO at 1.25 or 2.5 μL mL^{-1} received unsatisfactory scores for taste and aftertaste; however, appearance, odor and viscosity of the juice were scored as satisfactory. Accordingly, there is need for further research to decrease the negative effect of ISO-EU or other EO components on organoleptic characteristics of PJ. Such research should focus on exploiting additive or synergistic antimicrobial effects of combined EO components at low concentrations to achieve effective microbial control while minimizing negative sensory changes in raw juices.

4. Conclusions

The ISO-EU at 0.5 to 1.5 μL mL^{-1} combined with YEX at 0.5% (w/v) in refrigerated (4ºC) raw PJ can destroy YM and LAB which are major spoilage microorganisms in acidic fruit juices. The YEX alone in PJ is inhibitory to growth of YM and does not alter the sensory characteristics of PJ. Therefore, this natural surfactant could potentially be used as a multifunctional ingredient to readily increase the miscibility of EOs while retarding the growth of fungal contaminants in juices. Also, further research on the potential antimicrobial activity of YEX used alone against other microbial groups in acidic juices is warranted. The ENT can

persist for more than 24 days in refrigerated raw PJ but can be rapidly destroyed in PJ containing ISO-EU. In this respect ISO-EU could potentially improve the microbial safety of PJ by killing juice-borne gram-negative enteric pathogens of the ENT family. The PJ containing ISO-EU at 0.5 or 0.75 μL mL^{-1}can maintain acceptable color, odor and viscosity for 28 days at 4ºC; however, those same concentrations negatively affect the taste of the PJ that in turn can decrease the overall consumer acceptance of PJ with added ISO-EU at levels evaluated in the present study.

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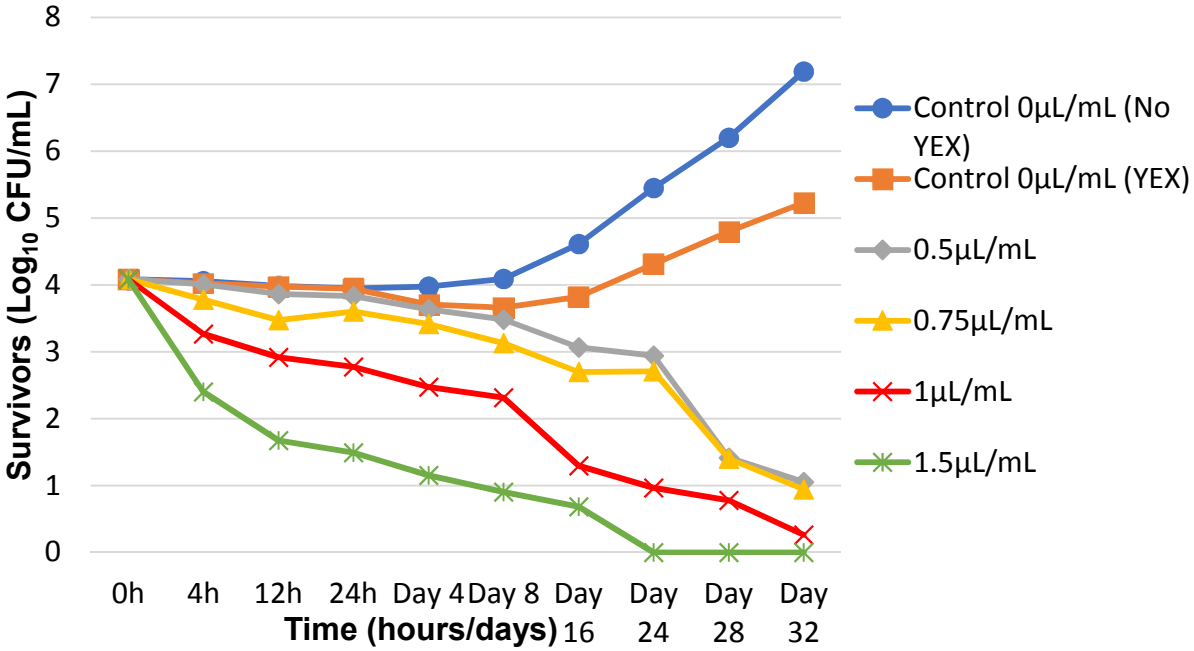

Figure 1. Effect of Isoeugenol on viability of Yeasts and Molds in Pineapple Juice stored at 4ºC.

Table 1. Aerobic plate count of refrigerated (4ºC) raw Pineapple Juice (with added Yucca Extract) as affected by concentrations of iso-eugenol during storage for 32 days

	Treatment (µL mL^{-1})				
Time (hours/days)	*Control	ISO-EU (0.5)	ISO-EU (0.75)	ISO-EU (1.0)	ISO-EU (1.5)
	Viable count (log$_{10}$ CFU mL^{-1})[a]				
4h	3.89 ± 0.32^{b}	3.62 ± 0.85^{b,c}	2.93 ± 0.75^{b,c,d}	2.13 ± 0.31 ^{c,d}	2.04 ± 0.48^{d}
12h	3.82 ± 0.54^{b}	3.44 ± 0.99^{b,c}	2.56 ± 0.29^{b,c}	2.08 ± 0.21^{c}	1.94 ± 0.40^{c}
24h	3.64 ± 0.31^{b}	3.06 ± 0.88^{b,c}	2.08 ± 0.43^{c}	2.12 ± 0.30^{c}	2.08 ± 0.56^{c}
Day 4	3.51 ± 0.16^{b}	2.34 ± 0.19^{b,c}	2.30 ± 0.77^{c}	1.88 ± 0.19^{c}	1.84 ± 0.54^{c}
Day 8	3.56 ± 0.82^{b}	2.17 ± 0.27^{c}	2.06 ± 0.32^{c}	1.95 ± 0.46^{c}	1.95 ± 0.42^{c}
Day 16	3.02 ± 0.76^{b}	2.08 ± 0.45^{b}	2.00 ± 0.54^{b}	2.03 ± 0.50^{b}	1.89 ± 0.48^{b}
Day 24	4.32 ± 1.05^{b}	1.93 ± 0.65^{c}	1.83 ± 0.67^{c}	1.67 ± 0.70^{c}	1.71 ± 0.77^{c}
Day 28	5.09 ± 0.14^{b}	1.14 ± 0.23^{c}	0.80 ± 0.46^{c}	1.23 ± 0.27^{c}	0.81 ± 0.22^{c}
Day 32	5.00 ± 0.09^{b}	1.08 ± 0.30^{c}	1.07 ± 0.21^{c}	0.89 ± 0.36^{c}	0.94 ± 0.28^{c}

Initial viable count: 3.98 ± 0.37 log$_{10}$ CFU mL^{-1}.

[a]Each value for viable count is the mean (standard deviation) of three replicate experiments.

[b,c,d]Means with a different letter within a row differ significantly (p <0.05)

CFU, colony-forming unit; ND = no colonies detected; detection limit =1 CFU mL^{-1}

*Pineapple juice with added 0.5% (w/v) YEX

Table 2. Populations of lactic acid bacteria in refrigerated (4ºC) raw pineapple Juice (with added Yucca Extract) as affected by concentrations of iso-eugenol during storage for 32 days

	Treatment (μL mL^{-1})				
Time (hours/days)	*Control	ISO-EU (0.5)	ISO-EU (0.75)	ISO-EU (1.0)	ISO-EU (1.5)
Viable count (log_{10} CFU mL^{-1})[a]					
4h	2.26 ± 0.44[b]	1.36 ± 1.34[b]	1.39 ± 1.22[b]	1.31 ± 1.18[b]	1.23 ± 1.15[b]
12h	1.91 ± 0.51[b]	1.52 ± 0.72[b]	1.82 ± 0.40[b]	1.47 ± 0.88[b]	1.44 ± 0.92[b]
24h	1.76 ± 0.56[b]	1.58 ± 0.88[b]	1.47 ± 0.71[b]	1.24 ± 1.15[b]	1.18 ± 1.10[b]
Day 4	2.05 ± 0.89[b]	1.36 ± 0.91[b]	1.41 ± 0.82[b]	1.39 ± 0.84[b]	1.06 ± 1.09[b]
Day 8	2.02 ± 0.35[b]	1.38 ± 0.79[b]	1.20 ± 0.88[b]	1.00 ± 0.97[b]	0.89 ± 1.15[b]
Day 16	2.84 ± 0.12[b]	1.60 ± 1.00[b]	1.38 ± 0.94[b]	1.45 ± 0.94[b]	1.27 ± 1.06[b]
Day 24	2.99 ± 1.40[b]	0.92 ± 1.11[b]	0.94 ± 1.15[b]	0.98 ± 1.09[b]	0.87 ± 1.12[b]
Day 28	0.75 ± 0.78	ND	ND	ND	ND
Day 32	0.35 ± 0.60	ND	ND	ND	ND

Initial viable count of Lactic acid bacteria: 2.43 ± 0.43 log_{10} CFU mL^{-1}.

[a]Each value for viable count is the mean (standard deviation) of three replicate experiments.

[b]Means with a different letter across a row differ significantly (p <0.05)

CFU = colony-forming unit; ND = no colonies detected; detection limit = 1 CFU mL^{-1}

*Pineapple juice with added 0.5% (w/v) YEX

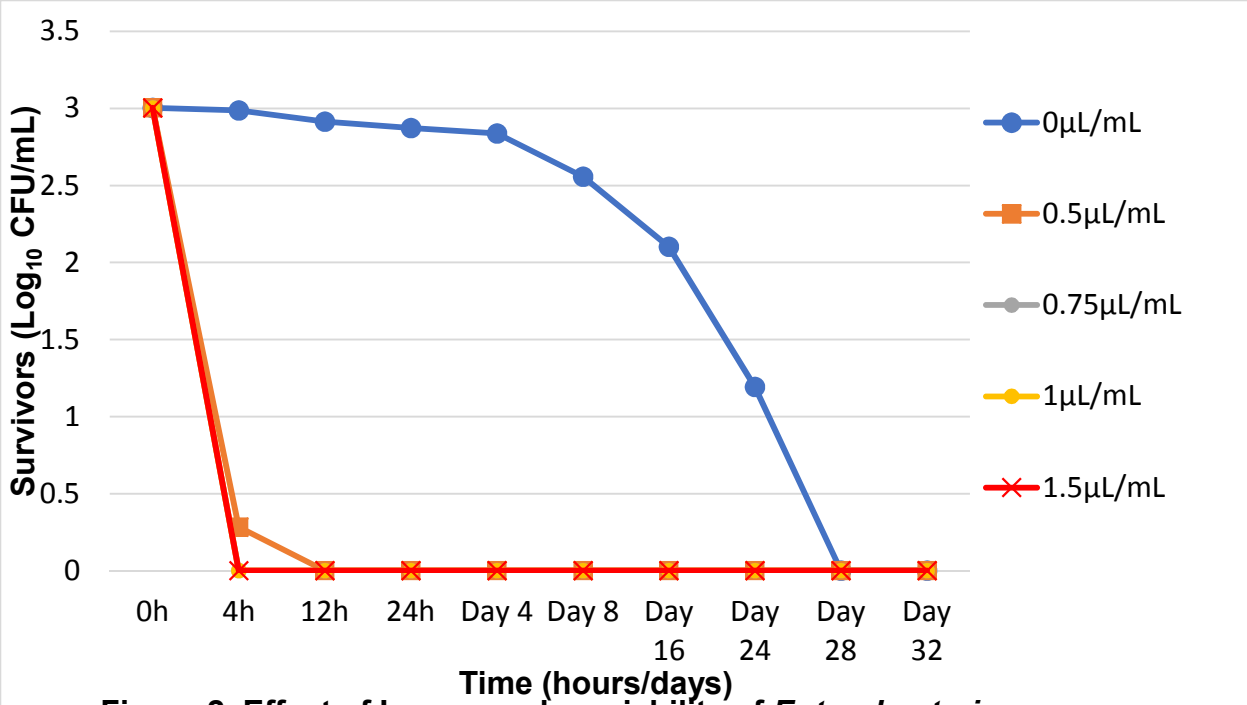

Figure 2. Effect of Isoeugenol on viability of *Enterobacteriaceae* in Pineapple Juice with added yucca extract stored at 4ºC.

Table 3: Day 1 Sensory evaluation of attributes of control Raw PJ and PJ with added YEX and ISO-EU

Concentration ISO-EU (µL mL^{-1})	Scores for Sensory Characteristics, mean ± SD				
	Color	Odor	Taste	Viscosity	Overall Acceptance
Control (Raw PJ)	7.76 ± 0.72^{a}	8.08 ± 0.81^{a}	8.32 ± 0.75^{a}	7.52 ± 0.82^{a}	8.12 ± 0.78^{a}
Control (PJ + YEX)	7.84 ± 0.62^{a}	8.00 ± 0.71^{a}	8.04 ± 0.61^{a}	7.76 ± 0.78^{a}	8.16 ± 0.69^{a}
0.5	7.88 ± 0.53^{a}	8.04 ± 0.54^{a}	5.68 ± 1.03^{b}	7.80 ± 0.76^{a}	5.52 ± 1.00^{b}
0.75	7.92 ± 0.49^{a}	7.48 ± 0.51^{b}	4.44 ± 0.82^{c}	7.80 ± 0.76^{a}	4.08 ± 0.64^{c}

^{a,b,c}Means with a different letter within a column differ significantly (p <0.05)
PJ, pineapple juice; ISO-EU, isoeugenol; YEX, yucca extract; Raw PJ has no ISO-EU and no YEX; PJ + YEX has no ISO-EU but has YEX; Acceptability of each characteristic was evaluated using a 9-point hedonic scale ranging from1 (dislike very much) to 9 (like very much)

Table 4: Day 14 Sensory evaluation of attributes of control Raw PJ and PJ with added YEX and ISO-EU

Concentration ISO-EU (µL mL^{-1})	Scores for Sensory Characteristics, mean ± SD				
	Color	Odor	Taste	Viscosity	Overall Acceptance
Control (Raw PJ)	7.80 ± 0.58^{a}	7.76 ± 0.66^{a}	7.96 ± 0.61^{a}	7.60 ± 0.65^{a}	7.72 ± 0.54^{a}
Control (PJ + YEX)	7.88 ± 0.53^{a}	7.92 ± 0.57^{a}	7.76 ± 0.60^{a}	7.80 ± 0.71^{a}	7.68 ± 0.69^{a}
0.5	7.84 ± 0.62^{a}	7.92 ± 0.49^{a}	5.64 ± 0.91^{b}	7.76 ± 0.72^{a}	5.60 ± 0.82^{b}
0.75	7.84 ± 0.69^{a}	7.40 ± 0.65^{b}	4.04 ± 0.73^{c}	7.64 ± 0.76^{a}	4.04 ± 0.73^{c}

^{a,b,c}Means with a different letter within a column differ significantly (p <0.05)
PJ, pineapple juice; ISO-EU, isoeugenol; YEX, yucca extract; Raw PJ has no ISO-EU and no YEX; PJ + YEX has no ISO-EU but has YEX; Acceptability of each characteristic was evaluated using a 9-point hedonic scale ranging from1 (dislike very much) to 9 (like very much)

Table 5: Day 28 Sensory evaluation of attributes of control Raw PJ and PJ with added YEX and ISO-EU

Concentration ISO-EU (µL mL^{-1})	Scores for Sensory Characteristics, mean ± SD				
	Color	Odor	Taste	Viscosity	Overall Acceptance
Control (Raw PJ)	3.48 ± 1.00^{b}	2.52 ± 1.36^{b}	2.64 ± 0.57^{d}	7.52 ± 0.82^{a}	2.08 ± 0.64^{d}
Control (PJ + YEX)	7.44 ± 0.65^{a}	7.60 ± 0.50^{a}	7.56 ± 0.51^{a}	7.68 ± 0.85^{a}	7.64 ± 0.76^{a}
0.5	7.64 ± 0.76^{a}	7.44 ± 0.77^{a}	5.32 ± 1.22^{b}	7.32 ± 0.85^{a}	5.52 ± 0.92^{b}
0.75	7.68 ± 0.80^{a}	7.56 ± 0.87^{a}	3.84 ± 0.75^{c}	7.48 ± 0.82^{a}	3.52 ± 0.59^{c}

^{a,b,c,d}Means with a different letter within a column differ significantly (p <0.05)
PJ, pineapple juice; ISO-EU, isoeugenol; YEX, yucca extract; Raw PJ has no ISO-EU and no YEX; PJ + YEX has no ISO-EU but has YEX; Acceptability of each characteristic was evaluated using a 9-point hedonic scale ranging from1 (dislike very much) to 9 (like very much)

CHAPTER 5. GENERAL CONCLUSIONS

Results of the present study demonstrate that *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes* can survive in pineapple juice (PJ; pH 3.48) with added YEX (0.5% w/v) for more than 42 days at refrigeration temperature (4ºC). This survival of pathogens in the acidic PJ confirms the microbial food safety risk posed by contaminated fruit juices devoid of an antimicrobial treatment.

Based on ISO-EU inactivation of all three pathogens in PJ with YEX at 4ºC and at 30ºC for 2h and 18ºC for1 h, this EO constituent can substantially reduce the risk of foodborne disease to consumer who might temperature-abuse raw juice.

Addition of ISO-EU (0.5 to 1.5 µL mL^{-1}) to PJ containing YEX provides a non-thermal kill step to achieve the 5-log pathogen reduction in PJ as stated in the FDA juice HACCP regulations. This approach to microbial control in PJ can assist juice manufacturers to improve the microbial safety of the raw juice without application of thermal treatments or synthetic antimicrobials.

Substantial sub-lethal injury in pathogen survivors in PJ containing ISO-EU and YEX presents an opportunity to combine ISO-EU with other hurdles such as essential oil components or non-thermal treatments to completely inactivate pathogens in juices. This strategy can facilitate the use of lower concentrations of ISO-EU that in turn could decrease negative changes in sensory characteristics of PJ.

The observed decrease in populations of major spoilage microorganisms (YM and LAB) in refrigerated PJ with added ISO-EU (0.5 to 1.5 µL mL^{-1}) combined with YEX suggests that ISO-EU can extend the microbial shelf-life of refrigerated raw PJ. Since YEX alone in PJ exhibited antifungal activity without affecting the sensory characteristics of PJ, this natural

surfactant shows good promise for use as a multifunctional ingredient to improve the solubility of EOs in juices while inhibiting the growth of YM juices. Also, further research on the antimicrobial activity of YEX against other microbial groups in acidic juices is warranted.

Rapid death of the ENT in PJ containing ISO-EU and YEX highlights the potential of ISO-EU to enhance the microbial safety of this popular juice because several gram-negative enteric pathogens such as *E. coli* O157:H7 and *Salmonella* belong to the ENT family.

Acceptable color, odor and viscosity can be maintained for 28 days in refrigerated PJ with ISO-EU (0.5 or 0.75 μL mL^{-1}); however, negative effects of those ISO-EU concentrations on the taste of the PJ can decrease the overall consumer acceptance of the popular fruit juice.

Plant-derived antimicrobials such as EOs and their constituents such as ISO-EU are gaining widespread consumer acceptance because they are natural and are viewed as "healthy" alternatives to synthetic antimicrobial preservatives. Further studies are needed on strategies to use lower concentrations of ISO-EU in hurdle technology to control pathogenic and spoilage microorganisms in the juice and simultaneously mitigate the negative effects of ISO-EU on taste of PJ.

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