The Effects of Using Cinnamon Leaf and Bark Essential Oils on Listeria Monocytogenes (L.M.), Salmonella Typhimurium (S.T.), In Model System, Strawberry Shake and Fresh Celery, and Sensory & Shelf Life Studies

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The Effects of Using Cinnamon Leaf and Bark Essential Oils on Listeria Monocytogenes (L.M.), Salmonella Typhimurium (S.T.), in Model System, Strawberry Shake and Fresh Celery, and Sensory & Shelf Life Studies

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Food Science

by

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Abstract

Essential oils derived from the bark and leaves of the cinnamon plant have long been used as natural preservatives and flavoring agents in different types of foods. In this study, we evaluated the antimicrobial effects of cinnamon essential oils (CEOs), obtained from cinnamon leaf or bark, against two foodborne pathogens i.e., *Salmonella Typhimurium* (S.T.) and *Listeria monocytogenes* (L.m.). Two different concentrations of microbial loading were used i.e., $10^9$ and $10^4$, cultured in nutrient media broth, strawberry shakes, and on celery sticks. Both CEOs of leaf and bark at 0.5% and 1% were found to completely inhibit S.T. and L.m., immediately after addition to the cultures. Based on the results of the broth culture experiments, we also investigated the antimicrobial effects of the CEOs at 0.5% and 0.1% in strawberry shake, against S.T. and L.m. Again when 0.5% (v/v) of the CEOs were added to the strawberry shake and stored at 4°C, both bacteria were found to be completely inhibited after a storage period of 8 days. The strawberry shakes containing CEOs from bark had higher ratings of sensory acceptability compared to those containing leaf CEOs, with or without the addition of masking agents. When 0.5% (v/v) of CEOs from leaf were applied on celery, the results showed a higher log reduction for L.m. (1.0 and 3.9 CFU/mL) for both $10^9$ and $10^4$, as compared to S.T for which the reductions were 0.88 and 2.85 CFU/mL, after a storage period of 7 days at refrigeration temperature. For celery samples applied with 0.5 % (v/v) CEO of bark, the reductions were 1.1 and 3.8 CFU/mL for L.m and 1.2 and 3.5 CFU/mL for S.T, at low and high bacterial concentrations, respectively. Overall, this study demonstrates that CEOs derived from bark are better than those derived from leaf with respect to antimicrobial activity and sensory aspects. Hence cinnamon essential oils of leaf and bark can be used as a potential antimicrobial agent to keep fresh produce and milk beverages safe for human consumption.
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CHAPTER 1

1.1 INTRODUCTION

1.1.1 Outbreaks of Foodborne Illness

Foodborne illnesses are increasing in the United States, with several outbreaks occurring every year (Scallan et al., 2011; Callejon et al., 2015). According to the Centers for Disease Control and Prevention CDC (2016a), 48 million illnesses occurred annually and contaminated food was estimated to cause 128,000 hospitalizations, and 3,000 deaths per year. Although several of the recent multiple hurdle technologies have been used to inhibit growth of food-borne pathogens (Tan et al., 2013; Chawla et al., 2006; Kumar et al., 2014), the number of illnesses caused by these microorganisms continue to increase thereby raising the demand for effective prevention technologies that control the microbial growth in food products (CDC, 2015; Crim et al., 2014). For more than a century, many outbreaks have been associated with Salmonella Typhimurium, and Listeria monocytogenes in food products (Callejon et al., 2015; CDC, 2016a), both of which have resulted in the increased concerns for food contamination (Erickson, 2012).

1.1.2 Salmonella Typhimurium and Listeria monocytogenes in Fresh Produce

Fresh produce usually undergoes minimal thermal treatment, which makes it susceptible to foodborne pathogens. Salmonella is in the lead cause for the outbreaks in fresh produce (Callejon et al., 2015). According to the CDC (2016c) several cases and recalls have been reported; in 2016, the contamination of fresh produce by Salmonella resulted in the death of 6 people in several states and 204 hospitalizations. Like Salmonella, Listeria has also been reported as a common causative agent of foodborne illnesses. CDC in 2014 estimated that 1600 illnesses and 260 deaths were caused Listeria infection. According to the CDC (2016d) a multistate outbreak of Listeria strains from the consumption of fresh produce resulted in the...
sickness of 19 people and one death. *Listeria* can affect frozen vegetables as well. Although several researches have indicated some success in reducing foodborne pathogens in fresh produce, there are several foodborne illnesses related to bacterial contamination that needs investigation (Cálix *et al.*, 2014).

### 1.1.3 *Salmonella Typhimurium* and *Listeria monocytogenes* in Milk Beverages

Beverages such as milk can be contaminated by *Listeria* for several reasons. Pasteurization temperature is one of the main reasons for contamination, which resulted in diseases. According to the CDC (2016b) two people were hospitalized and one died from consuming milk products that were pasteurized improperly. Goff reported that 37% of outbreaks in milk beverages were associated with *Salmonella*. Since milk beverages such as the flavored kind, either fluid or raw, are considered as the main vehicles for *Salmonella* and *Listeria* borne illnesses, the need to investigate these microorganisms to prevent the contamination is necessary (Goff *et al.*, 2006).

### 1.1.4 Current methods for Microbial control in Fresh produce & Beverages

Several food processing and safety procedures have been developed in order to inhibit or completely destroy the microbes in both processed foods and fresh produce (Theivendran *et al.*, 2006; Ganesh *et al.*, 2010; Parish *et al.*, 2003). However, some of these techniques are insufficient, inconvenient, and include the over usage of chemicals. Most of the outbreaks associated with food are due to the poor manufacturing practices or ineffective preservation techniques, during processing and handling. In addition, inappropriate storage temperatures also result in the spoilage of food products (Labbe *et al.*, 2001). Similar to food safety, hygiene is also important in preventing foodborne pathogens (Egan *et al.*, 2007; Soon *et al.*, 2012; Hayes and Forsythe 2013; Lelieveld *et al.*, 2014). Maintaining a clean environment during processing, as
well as cleaning is essential for preventing cross contamination in food manufacturing plants (Farkas, 1998). Moreover, it is challenging to develop effective control techniques against foodborne pathogens due to the complex nature of the food matrices (Labbe et al, 2001).

1.1.5 Hurdle Technology

Hurdle technology is a process that is used to decrease the growth of pathogens in food products using different techniques such as, sanitization, thermal treatments, UV irradiation, antimicrobial agents, etc. A study conducted by Huang and Chan (2011) to decrease the growth of foodborne pathogens in fresh produce determined that, food sanitizers such as chlorine was not effective in reducing the bacteria loads compared to the DI water control. However, when hydrogen peroxide was used in combination with organic acids such as, lactic acid and various heat treatments, a very high log reduction of E. coli was observed. Thus the hurdle technology strives to decrease microbial loadings and the probability of microbial survival on fresh produce by using a sequence of compatible antimicrobial techniques.

1.1.6 Treatment with Antimicrobial agents

Antimicrobial agents are one such techniques used in the hurdle technology to reduce food-borne illnesses related to Salmonella and Listeria. Several chemical preservatives have been examined for their antimicrobial properties and were determined to increase the shelf life of food products. Organic acids such as, acetic and maleic acids, were found to be safe alternatives for synthetic chemicals and effective in inhibiting food-borne pathogens (Mani-Lopez et al, 2012; Eswaranandam et al, 2006; Cruz et al, 2013). Although chemicals have promoted the safety and quality of food products, consumer demand for natural sources has led to an increase in the investigation of the antimicrobial activity of plant extracts (Juneja and Sofos, 2001; Mau, 2001).
1.1.7 Plant extracts and Essential oils

Plant extracts have been used for many centuries and have several advantages. Their natural antimicrobial properties have enticed researchers to investigate their use in food products (Jayaprakasha et al., 2003; Corrales et al., 2009; Hosni et al., 2013; Nascimento et al., 2000; Ganesh et al., 2010). There is a potential for plant extracts to be used as antimicrobial agents against food-borne pathogens (Mathlouthi et al., 2012; Kotzekidou et al., 2008). Terpenes and Terpenoids found in plant biomass have been mainly evaluated for their inhibitory action against pathogens (Solórzano-Santos and Miranda-Novales, 2012). Essential oils can be obtained by various processes such as expression, extraction, fermentation and distillation and are known to possess antimicrobial properties against various bacteria, yeasts and mold from long back (Bassole and Juliani, 2012). Essential oils are usually concentrated in leaves, bark and fruits (Burt, 2004). These essential oils were used against both gram positive and gram negative bacteria such as Listeria, E. coli and Salmonella (Delaquis et al., 2002; Singh et al., 2003; Oussalah et al., 2007; Si et al., 2006). The essential oils have historically been used for their desirable aroma and flavor in foods (Burt, 2004; Buckle, 2014; Raut and Karuppayil, 2014). Many spices such as cinnamon oil have exhibited antimicrobial activity (Matan et al., 2006; Mau et al., 2001; Sathishkumar et al., 2009). Cinnamaldehyde, the main ingredient in cinnamon bark oil, has been examined for its antimicrobial activity (Mau et al., 2001). Investigators have found that the cinnamon essential oil, extracted from the bark and leaves, had considerable ability to inhibit the growth of foodborne pathogens (Hill et al., 2013; Singh et al., 2007; Chang et al., 2001).
1.1.8 Sensory attributes of Natural Antimicrobial agents

Qualitative characteristics such as color, flavor, taste, and texture can greatly affect the consumers’ choice of beverages (Tepper, 1993; Piqueras et al, 2012; Kim et al, 2013; Damodaran et al, 2008). According to Rampersaud et al (2003) fruit flavored beverages are highly preferred by children. The consumption of flavored milk beverages is increasing and some of the most popular flavors are chocolate, vanilla and especially, strawberry. Strawberry shake is considered a dairy product, because it is a mix of fruit and milk (Goff et al, 2006). Flavor is important in the consumption of beverages. Harwood et al (2012), conducted a study in chocolate milk, which revealed that the high alkaloids and phenolic content of dark chocolate caused bitterness, resulting in the rejection of the product by the consumers. The study also indicated that bitterness was a negative attribute in food products. Therefore, enhancing the flavor of a product containing natural preservatives is considered as a highly important aspect for consumer satisfaction and increasing the sales.

1.1.9 Shelf life Stability of Beverages

Color is a major attribute for beverages and it has major influence on the acceptance of the product (Shenoy et al, 2014; Mollov et al, 2007; Kerth et al, 2007). According to Goff et al (2006) one of the major concerns of dairy products is their shelf life. The popularity of strawberry shakes makes extending their shelf life highly necessary. Several methods have been used to extend the shelf life of beverages such as, thermal processing, UV irradiation and novel packaging techniques (Polydera et al, 2003; Sampedro et al, 2009). Although using antimicrobial agents such as, essential oils is significant in enhancing the shelf life of milk beverages, it is important to maintain the sensory quality of strawberry shakes, after applying the essential oils treatment.
1.2 HYPOTHESIS

Cinnamon leaf and bark essential oils can inhibit common foodborne pathogens such as *Salmonella* Typhimurium, and *Listeria monocytogenes* that are usually found in milk beverages and fresh produce and as well as preserve the overall consumer acceptance of the food products, along with ensuring the stability of the product under typical storage conditions.

1.3 OBJECTIVES

The objectives of this study are to:

**Objective 1:** Investigate the antimicrobial effects of cinnamon leaf and bark essential oils against *Salmonella* Typhimurium (S.T.), and *Listeria monocytogenes* (L.m.) in broth culture and determine their Minimum Inhibitory Concentration (MIC).

**Objective 2:** Investigate the effects of cinnamon leaf and bark essential oils on *Salmonella* Typhimurium (S.T.), and *Listeria monocytogenes* (L.m.) in strawberry shake

**Objective 3:** Evaluate the inhibitory effects cinnamon leaf and bark essential oils *Salmonella* Typhimurium (S.T.), and *Listeria monocytogenes* (L.m.) in fresh celery.

**Objective 4:** Investigate the shelf life stability of cinnamon leaf and bark essential oils in strawberry shake and conduct sensory evaluation and consumer acceptability of the product.
References:


2 LITERATURE REVIEW

2.1.1 Foodborne illness concerns

Contaminated foods result in foodborne illness (Callejon et al., 2015). Foodborne illness is a significant cause of death every year. Contaminated food is estimated to cause 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths per year (CDC, 2014). These outbreaks are caused by a long list of pathogens that contaminate meat products and fresh produce (CDC, 2016a). Although the government of the United States attempts to prevent the contamination of food products by ensuring its safety, foodborne pathogens are considered a primary concern (Buzby et al., 2001). The effort to prevent foodborne pathogens by the United States government is high since foodborne illnesses are common and sometimes fatal, producing an increasing need for food safety assurance.

Foodborne illnesses cause economic loss due to the necessary funding for medical assistance and hospitalization (Scharff et al., 2012). According to Hoffmann et al 2015 around $15.5 billion is spent yearly on food borne illness prevention. The estimated loss caused by 31 pathogens in the United States is 37.2 million dollars (Scallan, 2011). In 2016a, the Centers for Disease Control and Prevention (CDC) reported, illnesses, hospitalizations and deaths result from known agents in the United States. The numerous costs that results in recalls, treatments and hospitalization, have led to increase in research to control the microbial growth of pathogens in food products (Scharff et al, 2010).

Several pathogens can result in death after consuming contaminated foods. Pathogens such as *Listeria monocytogenes* and *Salmonella Typhimurium* are often responsible for food related death in the United States (CDC 2016b, 2016c). *Salmonella* is in the lead causing illnesses by
11% while *Listeria monocytogenes* caused 19% of illnesses. Since the infection by pathogens is highly increasing, the need to decrease the microbial growth of microorganisms is necessary (Scallan, 2011).

### 2.1.2 *Salmonella Typhimurium*

*Salmonella Typhimurium* is a non-spore former; a gram-negative, anaerobic rod shaped bacterium (Franz *et al.*, 2008). Salmonella Typhimurium is one of the oldest bacteria, which was discovered 100 years ago by the United States scientists Smith and Salmon, after whom Salmonella took its name (Galiş *et al.*, 2013; CDC, 2015) Salmonella has been associated with outbreaks in food sources such as meat, vegetables, poultry, peanut butter, and eggs (CDC, 2015).

In 2015, the CDC reported, 1.2 million Salmonellosis illnesses cases in the United States. In 2015, the CDC reported, 38% of *Salmonella* cases that resulted in hospitalization in the United States. Symptoms of infection of *Salmonella* last 4 to 7 days and some patients require hospital care. Children and elderly people are at greater risk of being affected by the *Salmonella*. Approximately 450 deaths occur in the U.S due to *Salmonella* every year (CDC, 2015). According to the CDC (2016) 138 people became ill due to the outbreak of *Salmonella* strains. *Salmonella* grows in temperatures ranging from 20-47°C (CDC, 2016). In addition, *Salmonella* effectively grows at a pH range of 3.6-9.5 (Labbe *et al.*, 2001). *Salmonella* can be killed during cooking but it is not enough to ensure that the food is free from bacteria. Several studies have been reported to prevent the effect of *Salmonella* in food (Over *et al.*, 2009; Cálix *et al.*, 2014; Shen *et al.*, 2013).
2.1.3 *Listeria monocytogenes*

*Listeria monocytogenes* unlike salmonella is a gram-positive, facultatively anaerobic bacterium that has a rod shape. The disease related to *Listeria* called *Listeriosis* usually occurs as a result of contaminated food consumed by people (CDC, 2016e). *Listeria* can grow in food products whether air is present or not. It can also grow at very low temperatures between 2-4°C, which indicates that the bacteria can thrive even if the food products are refrigerated (Doyle *et al.*, 2001; Swaminathan *et al.*, 2007). In addition, the harmful bacteria, requires a high salt concentration to grow (Nørrung, 2000). There are many places where *Listeria* can be found such as soil, water, and animal products (Ryser and Marth, 2007). The primary source for infection by *Listeria* is food, because the contaminated food with *Listeria* can result in major health risks (Nørrung, 2000). The bacteria, has the ability to grow in several food products when the required environment is suitable. Fresh products and ready to eat food can be contaminated by *Listeria*. Furthermore, fish, meat, vegetable, and low acid products such as milk beverages are exposed to the pathogen due to the lack of thermal treatments, temperature abuse, or ineffective use of antimicrobial agents (Doyle, 2001; Nørrung 2000).

*Listeria* can also cause several diseases such as *Listeriosis*, and has a higher risk of death than other food pathogens such as *Salmonella* by 20% to 30% (Ryser and Marth, 2007). *Listeria* affects people of all ages especially newborns, elderly and pregnant women. Pregnant women can be easily infected with *Listeria*, which causes several symptoms such as fatigue, fever, and headache. In addition, infected pregnant women with *Listeria* results in miscarriage (Cossart, 2007; Farber, 1991; CDC, 2016e). The CDC reported (2015) three people infected by *Listeriosis* died as result of contaminated foods.
2.1.4 Foodborne pathogens associated with dairy products

Beverages such as milk can be contaminated by *Listeria* for several reasons. Pasteurization temperature is one of the main reasons of contamination, which results in diseases. According to CDC (2016) two people were hospitalized and one died from consuming milk products that were pasteurized. The current methods to reduce and kill *Listeria* should enhanced due to the ability of the bacteria to grow during storage and in low temperature (Doyle, 2011). There are multiple hurdle technologies that can be used to kill or reduce *Listeria* such as modifying the temperature and time treatments. Pasteurization at temperature of 72°C for 15 minutes is more effective in preserve milk beverages (Lecuit, 2007). In addition, several antimicrobial agents were used to inhibit the presence of *Listeria* in milk. The presence of antimicrobial agents in the food product is required to work effectively with pasteurization to prevent the microbial growth. This is important because food can be contaminated during cooking and before packaging (Lecuit, 2007).

Flavored milk beverages are becoming more popular in the United States, due to its popularity among people especially children. Several studies have been conducted to evaluate the nutritional value of flavored milk (Goff and Griffiths, 2006). The study indicated that the consumption of milk beverages with different flavor such as vanilla, chocolate and strawberry is increasing. However, foodborne illnesses in flavored milk beverages still persists despite pasteurization and packaging. Goff and Griffiths reported that 37% of outbreaks in milk beverages were associated with *Salmonella*. Since milk beverages such as flavored, fluid or raw, are considered main vehicles of *Salmonella* diseases, the need to investigate *Salmonella* to prevent the contamination is necessary (Goff and Griffiths, 2006). Like milk beverages,
foodborne pathogens associated with vegetables have been a major concern in United States (Natvig et al, 2002).

2.1.5 Foodborne pathogens associated with fresh produce

Vegetable and fruit consumption in the U.S has grown significantly (Olaimat and Holley 2012, Abadias et al, 2008). Lebbe et al., (2001) reported that the consumption of fruits and vegetables has increased since 1970 by 50%, vegetables play a significant role in a healthy diet (Folchetti et al, 2014). Vegetables are an important source of vitamins and minerals, and they contain phytochemicals (Folchetti et al, 2014, De Souza et al, 2014). Vegetables have also been known for their ability in reducing the risk of serious diseases such as cancer, and cardiovascular and disease (Wang et al, 2014). Minimum processing has been used to eliminate microorganisms growth in fresh produce. However, this method is insufficient due to the lack of thermal processing.

Food products contamination, including fresh produce contamination, is a continued concern to people, causing illnesses in the United States yearly (CDC, 2014). In addition, every year there is an estimation of 48 million people of sickness associated with pathogens (CDC, 2014). Commonly, food products are processed to minimize the risk of contamination by foodborne pathogens. However, minimally processed foods have an increased chance of contamination due to the absence of heat treatment (Baier et al, 2014). Although vegetables are healthy and important for wellness, vegetables have high potential of being associated with human foodborne illnesses due to bacterial contamination (Ziuzina et al, 2014).

Listeria has been reported as a common cause of foodborne illness. According to the CDC (2016g) a multistate outbreak due to the consumption of fresh produce resulted in the sickness of 19 people and one death occurred due to the outbreak of Listeria strains. Listeria can
affect frozen vegetable as well. A multistate outbreak in 2016 resulted in recall of the product, 9 hospitalized people, and 3 deaths. Although several researches have indicated some success in reducing foodborne pathogens in fresh produce, there are several foodborne illnesses related to bacteria that need to be investigated (Cáliz et al, 2014).

According to CDC several cases and recalls have been reported. In 2016, contamination of fresh produce by Salmonella resulted in the death of 6 people in several states and 204 of the people were hospitalized. Although washing of fresh produce is a common method that have been used to eliminate microbial growth, wastewater is commonly used in developed countries for irrigation. A study conducted by Melloul et al (2001) to evaluate Salmonella contamination of vegetables with untreated wastewater have shown that 80% of 35 samples were positive to the Salmonella. The contamination for Salmonella is higher in vegetables that can be used in salad such as lettuce and parsley. The continuous contamination of vegetables with has resulted in the need to use antimicrobial agents to prevent the microbial growth of foodborne pathogens.

2.1.6 Common methods to control the growth of food pathogens

The increasing concern of foodborne illnesses has led to the enhancement of used techniques to control microbial growth (Scallan et al, 2011; Larsen et al, 2014). Sanitation of equipment and personal hygiene of personal are important (Lee and Lee, 2005). The lack of hygiene increases the chances of contamination (Mattoni and Sullivan, 1962; Marriott and Gravani, 2006). During processing, handling, and marketing safety regulations must be applied to minimize the possibility of spoilage and prevent health hazards (Juneja and Sofos, 2001). Hazards may result in any steps during processing, and production of food products. In addition, techniques such as heat treatment, irradiation, and reducing water activity and chemicals as
antimicrobial agents are common in inhibiting microorganisms (Kou et al., 2007; Li et al., 2002). Appropriate packaging is an effective method to decrease the contamination of food products (Eswaranandam et al., 2004).

**Pasteurization**

Pasteurization is one of the common methods used to control microbial growth (Wang et al., 2006; Terpstra, et al., 2007). It is primarily used in dairy products such as milk. Milk is a common vehicle for pathogens. Therefore, pasteurization of milk beverages is important. Pathogens have a certain type of temperature needed for growth. Usually the common temperature for microorganisms to grow is no higher than 40°C (Juneja and Sofos, 2001). Pasteurization is used for non-spore forming microorganisms and to prevent the spoilage of food products. For a long time, pasteurization has been used in milk products to expand the shelf life by applying a heat treatment of 72°C for 15 minutes. Although pasteurization destroys the microorganisms of milk beverages, the technique is used in combination with other preservative method such as the addition of chemicals to prevent pathogens (Juneja and Sofos, 2001).

**Chemical compounds**

Chemical compounds at certain levels for each type of pathogen can be added to preserve food products and beverages. Chemicals can be added to food products such as antimicrobial agents for safety purposes. Chemicals are also added for enhancing the quality of food products and beverages (Juneja and Sofos, 2001). Organic acids are commonly used as chemicals to prevent microbial growth. Several organic acids can be found in plants such as benzoic, malic and acetic acids (Eswaranandam et al., 2004).

Acetic acid is a well-known antimicrobial agent used to inhibit pathogens such as *Listeria monocytogenes* and *Salmonella Typhimurium* (Shin et al, 2006; Mani-Lopez and López-Malo,
A study showed that acetic acid with 0.1% (w/v) resulted in the inhibition of *Listeria* and *Salmonella* in food products. Acetic acid has also resulted in preventing microbial growth and extending the shelf life. Like acetic acid, sulfites have proved their ability in preventing bacterial growth (Juneja and Sofos, 2001). Similar to acetic acid, malic acid is an organic acid that several studies have shown its ability to control microbial growth and reduce pathogens (Sagong *et al*, 2011; Choi *et al*, 2012). Eswaranandam *et al* (2004) reported that malic acid has shown effective results on reducing several bacteria such as *Salmonella gaminara*, *Escherichia coli*, and *Listeria monocytogenes*. Malic acid (2.6%) has reduced the strain of *Listeria* on soy protein film by 5.5 log number CFU/mL. Although several studies have indicated the ability of chemicals to inhibit microbial growth and enhance quality, consumers’ concerns about chemical preservatives have led to the need to investigate natural antimicrobial agents (Mau *et al*, 2001).

**Washing and Sanitizing**

Fruits and vegetables are processed with a minimum amount of heat, which makes it less safe for consumption. Outbreaks and recalls are a major reason for enhancing and studying current techniques to reduce the population of microbial growth in fresh produce (CDC, 2015). The continuous number of foodborne illnesses is a primary concern. Usually with fresh produce, industries use sanitation agents to remove soil, dirt and pesticide from the surface of fresh produce (Sapers and Yousef, 2006). Chemical treatments are usually used to eliminate the growth of pathogens such as peroxycetic (Beuchat, 2004). Several studies have evaluated the efectivity of sanitizers such as chlorine and ozone (Yeoh *et al*, 2014; Karaca and Velioglu, 2014; Shen *et al*, 2013; Neo *et al*, 2013).
Chlorine is one of the most common chemicals used in industries (Goodburn and Wallace, 2013). Chlorine has been used as a sanitizer agent in different types of vegetables, leafy and roots. Chlorine can be added to water in different forms such sodium and calcium hypochlorite (Sapers and Yousef, 2006). The FDA approved to have the amount of chlorine used for sanitizing vegetables not more than 2% (Sapers and Yousef, 2006). It also found that chlorine can be effective against bacteria such as salmonella, listeria and E-coli (Van et al, 2013; Wulfkutehelr et al, 2013). Chlorine has disadvantages such as its insufficiency to eliminate bacteria on the surface of fresh produce. However, chlorine is not expensive in comparison to other sanitizing agents (Sapers and Yousef, 2006). Although chlorine has been widely used to sanitize fresh produce, it is not reliable to reduce or kill pathogens such as Listeria (Zhang et al, 2005).

Like Chlorine, Ozone ($O_3$) has the ability to reduce microbial growth. Several studies have studied the effectiveness of ozone sanitizer (Alwi and Ali, 2014; Miller et al, 2013). A study conducted by Alexopoulos et al 2013 compared between chlorine and ozone against fresh lettuce and bell peppers have shown dipping the produce in chlorinated water has resulted in 1 log reduction. However, when ozonated water was used, it decreased the microbial growth by 2 log reductions. A study investigated the effectiveness of ozone in fresh celery to determine the preservation ability of ozonated water in reducing the microbial population. The study found that the treated water with ozone was more effective for reducing bacterial growth count on fresh celery at 4°C storage temperature in comparison to other chemical treatments. A 0.18 ozone concentration was effective in eliminating microbial growth by 1.69 log reduction (Zhang et al, 2005). Another study conducted by Alexandre (2011) using ozonated water against a strain of listeria on fruits and vegetables have found that ozone at high concentration was able to reduce
microbial growth by 1.7 log. However, using water by itself has less influence on the produce. Although several sanitizing agents have the ability in decreasing microbial growth, consumers’ interests in natural product led to the need to investigate natural antimicrobial against foodborne pathogens

Hurdle technology

Hurdle technology is a process to decrease the microbial growth of pathogens from food products using several methods, such as sanitizer, thermal heat treatment, and UV light with antimicrobial agents. A study conducted by Huang and Chan (2011) to decrease the microbial growth of foodborne pathogens on fresh produce have found that, sanitizers such as chlorine is not effective in reducing the microbial loads of bacteria comparing to DI water. However, when hydrogen peroxide along with organic acids and various heat treatments were used, the highest log reduction of E-coli was observed. Lactic acid decreased the microbial growth of E-coli was at 22°C, 40°C, and 50°C. Another study using multiple technologies to reduce the microbial growth of foodborne pathogens was conducted by Sagong et al (2011) ultra sound along with other organic acids was used against Listeria, E.coli and Salmonella on lettuce. The combinations of ultra sound and organic acids had more ability to decrease the microbial growth of foodborne pathogens compered to organic acids by itself, which indicates the ability of using different methods to significantly decrease the microbial growth of foodborne pathogens.

2.1.5 Plant extracts as antimicrobial

The antimicrobial properties of plant extracts such as cilantro, coriander, dill, eucalyptus and cinnamon have been examined in several studies (Lee et al, 2015; Souza et al, 2013; Cava et al, 2007; Delaquis et al, 2002). Despite chemicals’ effectivity to inhibit the microbial growth in food products, consumers’ interest in natural sources, like plant extracts, is increasing. Plant
extracts have several advantages, in addition to their inhibitory properties. Plant extracts have been an important source for humans in food and therapy. Studies have examined the use of plants for therapeutic purposes (Gorelick and Bernstein, 2014). Plant extracts like cilantro and coriander, have bioactive phytochemical compounds, which have many health benefits (Delaquis et al, 2002).

Several researchers have also investigated the antimicrobial properties of plant extracts and many plants have been examined so far (Banerjee, et al 2014; Abdollahzadeh et al, 2014; Jagtap and Bapat, 2013; Kim et al, 2013). Plant extracts have shown their ability to decrease various foodborne pathogens. Cava et al (2007) reported that clove and cinnamon oils were effective in inhibiting Listeria monocytogenes. The plant extract was able to inhibit Listeria monocytogenes at low concentrations, which resulted in considering plant extracts as effective antimicrobial that can be used with hurdle technology such as pasteurization. Although many plant extracts have shown their antimicrobial agents abilities, several plants have not yet been examined to evaluate their antimicrobial properties (Molva and Baysal, 2015; Perumalla and Hettiarachchy, 2011; Rababah et al, 2004).

- **Essential Oils**

  Essential oils also called as volatile or ethereal oils and are aromatic oil compounds obtained from different parts of plants such as bark, twigs, leaves, wood, roots, flowers, fruits, buds and seeds (Burt, 2004; Hyldgaard et al, 2012; Solórzano-Santos and Miranda-Novales, 2012). Essential oils are found as liquids in plants and sometimes are semi solid or solid at ambient temperatures. As the name suggests they are oily in consistency and often consists of terpenes, terpenoids, and other aromatic and aliphatic compounds which can vary with the source from which they are extracted (Bakkali et al, 2008; Jayasena and Jo, 2013).
Essential oils have been used in foods due to their desirable aroma and flavor. The antimicrobial composition of essential oils depends on the type of oil (Fisher and Phillips 2008; Gutierrez, and Bourke, 2009). Several techniques can be followed to obtain the essential oils from plant parts such as expression, extraction, fermentation and distillation. However, commercially, essential oils can be derived using steam distillation. Approximately 3000 types of essential oils have been discovered, but only 300 are commercially important for their desirable flavor and attractive aroma (Burt, 2004). Essential oils are also known to possess antimicrobial properties against various bacteria, yeasts and mold from long back (Bassole and Juliani, 2012). Many investigations have been carried out in the past to evaluate the antimicrobial activity of EOs and their components (Solomakos et al, 2008). The scientific investigations on EOs have increased as result of consumers’ interest in natural additives in food products.

Essential oils consist of phenolic compounds, which function as antibiotics in plants (DU et al, 2009). According to Solórzano-Santos and Miranda-Novales (2012) several compounds are responsible for the activity of essential oils against bacteria. Terpenes and Erpenoids are the main components that have shown their ability to inhibit bacterial growth in food products. Essential oils have been examined on gram-positive bacteria as well as in gram-negative bacteria (Solórzano-Santos and Miranda-Novales, 2012). Which indicates that essential oils can be used in different types of bacteria.

- **Cinnamon essential oils**

  Cinnamon oil is an essential oil, which as opposed to the conventional cooking oil is a concentrated hydrophobic liquid, containing volatile aromatic compounds, that is extracted from different parts of a plant. Cinnamon oils, for example, can be extracted from the flower, buds, bark and leaves of the plant and it has been around for several years. According to Singh and
Catalan (2007) cinnamon is also used as a flavoring substance. Singh and Catalan, reported that in addition to flavor, it could also be used as antioxidant agent to prevent food oxidation.

Furthermore, cinnamon has the ability to reduce the amount of cholesterol and triglyceride, which indicates the high health benefits of cinnamon extract (Jakhetia et al., 2010). Cinnamon oils can be usually found in the bark and leaf (Chang et al., 2001; Paranagama, et al, 2010) and it have the ability to preserve food products, (Khadem et al, 2010). Commonly, cinnamon leaf and bark essential oils consists of several components. Table (1-2) shows compounds that can found in both oils with approximate concentrations.

**Table 1: Compounds commonly found in cinnamon leaf essential oils**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (%)</th>
<th>Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-Thujene</td>
<td>0.1</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>Singh and Catalan, 2007</td>
</tr>
<tr>
<td>b-Caryophyllene</td>
<td>1.9</td>
<td><img src="image2.png" alt="Structure" /></td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>87.3</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>trans-Cinnamaldehyde</td>
<td>15.9</td>
<td><img src="image4.png" alt="Structure" /></td>
<td></td>
</tr>
<tr>
<td>β-Linalool</td>
<td>0.16</td>
<td><img src="image5.png" alt="Structure" /></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1: Compounds commonly found in cinnamon leaf essential oils (Cont.)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (%)</th>
<th>Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Caprolactone</td>
<td>0.02</td>
<td><img src="image1" alt="γ-Caprolactone structure" /></td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0.10</td>
<td><img src="image2" alt="Vanillin structure" /></td>
<td></td>
</tr>
</tbody>
</table>

### Table-2 Compounds commonly found in cinnamon bark essential oils

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (%)</th>
<th>Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Cadinene</td>
<td>0.9</td>
<td><img src="image3" alt="d-Cadinene structure" /></td>
<td>Singh and Catalan, 2007</td>
</tr>
<tr>
<td>l-cinnamaldehyde</td>
<td>97.7</td>
<td><img src="image4" alt="l-cinnamaldehyde structure" /></td>
<td></td>
</tr>
<tr>
<td>α-Copaene</td>
<td>0.8</td>
<td><img src="image5" alt="α-Copaene structure" /></td>
<td>Abdelwahab et al, 2014</td>
</tr>
<tr>
<td>α-Thujene</td>
<td>0.3</td>
<td><img src="image6" alt="α-Thujene structure" /></td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td>36.0</td>
<td><img src="image7" alt="Linalool structure" /></td>
<td></td>
</tr>
</tbody>
</table>
Table-2 Compounds commonly found in cinnamon bark essential oils (Cont.)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (%)</th>
<th>Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-caryophyllene</td>
<td>0.4</td>
<td></td>
<td>Abdelwahab et al, 2014</td>
</tr>
<tr>
<td>α-copaene</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>borneol</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerol</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The chemical compounds and their concentrations in cinnamon leaf and bark essential oils are different depending on the type of cinnamon species. A study conducted by Wang et al (2009) evaluated the volatile components of five different types of cinnamon leaf essential oils from *Cinnamon pauciflorum* and *C. cassia*. Both oils had eugenol as the major component, which contributes to its flavor and aroma. *C. cassia* leaf had 79.75% of eugenol and trans-cinnamaldehyde was at 16.25%. However, *C. pauciflorum* had 54.74% of eugenol and 12.80% of trans-cinnamaldehyde. Although the concentrations of eugenol varied, it was still considered the main component of cinnamon leaf essential oils. The study also concluded that, in addition to eugenol and trans-cinnamaldehyde there were around 22 different volatile compounds in *C. cassia*, and *C. pauciflorum* leaf essential oils. Trans-cinnamaldehyde was found to be the major
component of cinnamon bark essential oils and it was reported to constitute 65% of the bark essential oils (Burt, 2004). Moreover, a study conducted by Mau et al (2001) found that cinnamon essential oils from bark and leaf have shown antimicrobial properties and were used to inhibit harmful pathogenic growth in various food products.

**Antimicrobial activity of cinnamon oils**

Cinnamon oils consist of several components such as “α-Terpineol Terpinolene, α-Terpineol, α-Cubebene, and α-Thujene.” (Jakhetia et al, 2010). Bark and leaf oils are describes as essential oils, which is a natural source that has antimicrobial ability in food products. Bark oil is more commonly used than leaf oils. Bark and leaf oils are used in the food industry to flavor colas, candies and other products as natural preservatives and flavoring substances (Matan et al, 2006).

Some of the compounds listed in the tables 1 and 2 have been known to exhibit antimicrobial activity, both when used alone and in combination with other antimicrobial agents. *Trans*-cinnamaldehyde, or cinnamaldehyde, is the main component of cinnamon bark essential oils and its chemical structure is illustrated in table 2. The aldehyde group of cinnamaldehyde contributes to the cinnamon flavor. Ravishankar et al (2010) reported that cinnamaldehyde alone can act as an antimicrobial agent and that, it inhibited the growth of *Salmonella* by producing a 2.3 log reduction, for a bacterial concentration of $10^6$ CFU/mL, after 3 days of incubation at 4 °C. Therefore, it can be used as a preservative to prevent food contamination. The amount of bark essential oils in cinnamon is high and the cinnamon bark essential oils are made of up to 65% of cinnamaldehyde (Burt, 2004; Du et al, 2009). Cinnamon leaf consists of eugenol and it is the major component of cinnamon leaf essential oils. Eugenol was also reported to act as an antimicrobial agent. A study conducted by Todd et al (2013) on cinnamon leaf essential oils, at
a concentration of 0.5 % (v/v), showed that it had the ability to reduce the growth of a 
*Salmonella* strain (6 log CFUs/ mL), with no significant differences between the storage 
temperatures of 4°C and 8°C.

2.1.8 Sensory evaluation

Sensory characteristics are important in selecting food products. Optimum food quality is 
important to consumers as it promotes safety and high nutrition (Peri, 2006). The consumption of 
high quality food is increasing. Since quality is significant, several methods have been used to 
evaluate the products and to determine if the product reach industry and consumer requirements 
(Sampedro *et al*, 2009). Greater quality of the food is, the higher the consumption. Therefore, 
food companies compete in enhancing their product attributes (Wei *et al*, 2012; Shahidi *et al*, 
2004).

Color is a major aspect in food products and fresh produce. Color is essential in food 
industries, and it is one of the most significant characteristics to determine consumer acceptance 
(Crisosto *et al*, 2003). Quality of product is based on consumers satisfactory of color (Leon *et al*, 
2006). Color can be measured by vision or by using color instruments such as the CIE-Lab 
system. The CIE-Lab system is a dependable method for measuring color (Tijskens *et al*, 2001). 
Commonly, color is measured by L*a*b* system (Leon *et al*, 2006). In colorimeter, L* value 
indicates the whiteness and blackness, a* value is for determining the redness and greenness, b* 
and measures the yellowness and blueness (HunterLab, 2008). A study was conducted of red 
Grapefruit Juice to determine its color. The color was measured for 90 red grapefruit Juices using 
the CIE L*a*b* values. The study found that a* value is the appropriate color indicator to 
measure pigments of beverages. From this study it can be concluded that the CIE-Lab system is a 
useful method to determine color (Lee, 2000).
Like color, flavor is one of most significant attributes in food products. The marketing of selected food product is depending on the consumer point of view on flavor (Barrett et al, 2010). In milk industries, consumers usually prefer flavored milk beverages to the bland taste of milk (Shahidi et al, 2004). Flavored milk beverages such as orange, chocolate and strawberry have been known in the United States for decades. However, the flavor decreases after a while. A study was conducted to evaluate conventional orange juice and orange juice with functional ingredients. Although the functional ingredients have several benefits for health such as vitamins and minerals, consumers have preferred the conventional orange juice (Luckow and Delahunty, 2004). This indicates that antimicrobial agents such as essential oils might be effective in reducing the microbial growth. However, they can also affect the color and flavor of flavored milk beverages. Therefore, other additives such as masking agents should be considered due to their ability to enhance color and flavor.

2.1.9 Shelf life

The long distance, and to decrease the time spoilage of food, has resulted in the importance of shelf life study. According to Goff et al (2006) one of the major concerns of dairy industries is shelf life. Several studies have examined the shelf life of beverages (Van et al 2014, Gironés et al, 2014, Sampedro et al, 2009). The quality of the product and its ability to stay at good condition for a longer time without any change is important to consumer, (Caplan and Barbano, 2013). Several methods have been used to extend the shelf life of milk beverages such as thermal processing, UV light, and packaging (Walkling et al, 2011; Walkling et al 2009; Kim et al, 2013; Orlowska et al, 2013). One of the common methods on extending the shelf life of beverages is pasteurization. For centuries, pasteurization has been used to kill most bacteria in milk pasteurization is processed at 71°C for 15 minutes (Henderson, 1971). Pasteurization is
considered a mild heat that is used to process milk. In industries, after removing the milk from
the cow, thermal technique is commonly used during processing of flavored milk. According to
Henderson (1971) more than 90% of microorganisms are destroyed after pasteurization.

Although temperature can results in extending shelf life of dairy products, it might result
in the alteration in the flavor of the product. A study conducted by Oshima et al (2014) used
nisin as a natural preservative in dairy products and found that the addition of nisin can assist in
reducing the heat treatment and it can also preserve the shelf life of the product. The popularity
of strawberry shakes makes extending their shelf life important. Although using antimicrobial
agents such as essential oils is significant in milk beverages after processing to ensure long shelf
life, it is also important to maintain specifically the quality of strawberry shakes, after applying
the essential oils treatments.
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CHAPTER 3

Objective 1: Investigate the antimicrobial effects of cinnamon leaf and bark essential oils against *Salmonella Typhimurium* (S.T.), and *Listeria monocytogenes* (L.m.) in broth culture and determine their Minimum Inhibitory Concentration (MIC).

3.1 INTRODUCTION

Foodborne illnesses are a significant cause of death (Buzby *et al.*, 2001; Scallan *et al.*, 2011). Contaminated food is estimated to cause 128,000 hospitalizations, and 3,000 deaths per year (CDC 2014). Although the U.S government attempts to prevent the contamination of food products by ensuring its safety, food pathogens are considered a primary concern in the United States (Kim, *et al.*, 2000; Buzby *et al.*, 2001). In California, an outbreak of *Listeria* led to the infection of two people and one death (CDC, 2016a). Unlike *Listeria*, a multistate outbreak in 2016 related to a strain of *Salmonella* has resulted in the hospitalizations of 204 people (CDC, 2016b).

Several methods have been used to determine the inhibitory activity of antimicrobial agents (Andrews, 2001). Current methods in food processing and safety are being promoted to inhibit or to kill foodborne pathogens in food products (Labbe *et al.*, 2001). However, methods are often insufficient, and inconvenient. Therefore, choosing the appropriate method as well as antimicrobial agents is important. Minimum inhibitory concentration is one of the most common methods used to examine the ability of antimicrobial agents to inhibit foodborne pathogens. According to Andrews (2001) minimum inhibitory concentration can be defined, “as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.” The MIC method assists in determining the ability of used antimicrobial to inhibit the growth of microorganisms overnight (Mann *et al.*, 1998; Andrews, 2001).
Essential oils have been identified as antimicrobial agents. Essential oils are a promising natural alternative of chemicals to inhibit microbial growth. The antimicrobial composition of essential oils depends on the type of oil (Smith-Palmer and Fyfe, 1998; Burt, 2004). Cinnamon oils consist of (65%) trans-cinnamaldehyde, the major antibacterial components. Cinnamon bark essential oil has shown its ability to inhibit pathogens such as *Listeria* (Burt, 2004). The objective of the study was to investigate the effectiveness of Cinnamon oils on Salmonella Typhimurium, and *Listeria monocytogenes* in broth culture.

### 3.2 MATERIAL AND METHODS

**Materials**

In this study, the two different types of bacterial pathogens were used namely, *Listeria monocytogenes* (*L.m.*)(strain V7 serotype 1/2a) and *Salmonella Typhimurium* (*S.T.*). The strains of *L.m.* were provided by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR and the *S.T.* strain was obtained from the Center for Food Safety, at the University of Arkansas, AR, U.S.A. Media used for initial culturing of bacteria was brain heart infusion (BHI) broth (EMD Chemicals Inc., Darmstadt, Germany). The agar media used for *S.T.* and *L.m.* enumeration were the Difco xylose, lysine, tergitol-4 (XLT4) agar (Becton Dickinson and Co., Sparks, MD, U.S.A) and Oxford Listeria agar (EMD Chemicals Inc., Darmstadt, Germany) respectively. The cinnamon essential oils (CEOs) from leaf and bark were provided by EOAS Organics Pvt. Ltd., (Ratmalana, Sri Lanka, supply to the US through Bulk Apothecary 125 Lena Drive Aurora, Ohio 44202).

**Culture preparation**

Frozen stock cultures of *L.m.* and *S.T.* stored at -70°C were thawed to ambient temperature after which 10μl of each culture was passed into 10 ml of BHI broth and incubated at 37°C for 24 h.
in a New Brunswick Scientific shaker (200-rpm). After the first passage, 10µl of each culture was again passed into 10 ml of BHI broth and incubated under the same conditions as above. The cultures from the second passage after incubation were used for the experiments to determine the antimicrobial effects of the cinnamon essential oils in broth system, strawberry shake, and celery.

**Antimicrobial activity of CEOs from bark and leaf in broth system**

Different concentrations of CEOs from leaf and bark were prepared in Phosphate Buffered Saline (PBS, 20 mM, pH 7.0) on the day previous to the study. Stock emulsions of 1% (v/v) were prepared by diluting 200 µl of the leaf / bark CEOs in 19.8 ml of PBS in sterile culture tubes, mixed well by vortexing for 30 seconds, after which they were diluted further to obtain 0.1%, 0.25%, 0.5% (v/v). The different concentrations of CEOs prepared (0.1%, 0.25%, 0.5% and 1% v/v) were dispensed into 96-well microtiter plates with lid (100 µl / well) along with 50 µl of 2 different concentrations of bacterial culture (10^4 and 10^9 CFU/ml). Control treatments used were mixtures of 100 µl of PBS and 50 µl of respective bacterial concentration. All the individual treatments were replicated in 3 wells and the microtiter plates were incubated at ambient temperature. Samples were taken at 0, 8, 12, 24, 48, and 72 hours from each well, serially diluted and plated on to agar plates for enumeration of bacterial colonies. For serial dilution, 20 µl of sample from each well were added to 180 µl of PBS, serially diluted and 100 µl from each dilution plated onto differential agar plates with selective supplements in triplicate for the two bacteria studied.

**Statistical analysis**

The data was analyzed by one-way analysis of variance (ANOVA) student t test using JMP® Pro 10.0.1 2012 software.
3.3 RESULTS AND DISCUSSION

**Antimicrobial activity of CEOs from bark and leaf in broth system**

Cinnamon essential oils of leaf and bark at various concentrations (0.1%, 0.25%, 0.5% and 1%) were added to broth cultures of bacteria to determine their antimicrobial activity against *Salmonella Typhimurium* (*S. T.*) and *Listeria monocytogenes* (*L. m.*). The two bacteria were found to be totally inhibited by 0.5% and 1% of CEOs of leaf and bark (Tables 1 – 4) and this inhibition was with the lower (10^4) and higher (10^9) bacterial concentrations. The leaf CEO at 0.25% and 0.1% was found to completely inhibit *S. T.* after 24 h and 48 h respectively with the bacterial concentration of 10^4 CFU/mL but was not able to produce complete inhibition with the higher bacterial concentration even after 72 h (Table 1). The bark CEO at 0.25% was found to completely inhibit *S. T.* after 12 h and 72 h respectively for the bacterial concentrations of 10^4 CFU/mL and 10^9 CFU/mL. However, 0.1% bark CEO was able to inhibit Salmonella completely only at the lower concentration of 10^4 CFU/mL after 48 h (Table 2). The leaf and bark CEO at 0.25% was found to completely inhibit *L. m.* after 72 h at a concentration of 10^4 CFU/mL, whereas with 0.1% CEOs growth was still present (Table 3 – 4). However, with a higher bacterial concentration of *L. m.* both leaf and bark CEOs at 0.1% and 0.25 % were unable to produce complete inhibition.

From our experiments it was found that the MIC for *S. T.* was 0.25% bark CEO and 0.5% leaf CEO, while the MIC for *L. m.* was 0.5 % of both bark and leaf CEOs. Minimum inhibitory concentration is one of the most common methods used to examine the ability of antimicrobial agents to inhibit foodborne pathogens. According to Andrews (2001) minimum inhibitory concentration can be defined “as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation”. Cinnamon leaf and bark have
been used as a spice in cooking for centuries and is also a component in several traditional and modern medicines (Rao et al, 2014). Many studies have shown that CEOs derived from various parts of the plant such as leaves, bark and buds have the ability to reduce growth of microbial organisms (Senhaji et al, 2007; Oussalah et al, 2007; Goni et al, 2009; Becerril and Gómez-Lus 2012; Souza et al, 2013).

Antimicrobial activity of both cinnamon bark and leaf essential oils as well as volatile components of the leaf and bark essential oils have been studied (Singh et al, 2007; Wang et al., 2008; El-Baroty et al, 2010). The study conducted by Singh et al (2007) compared the chemical composition and antimicrobial activity of leaf and bark volatile oils against several bacteria. They found that bark essential oils displayed greater zone of inhibition than the leaf essential oils against *E. coli* and *Salmonella Typhimurium*, which was in accordance with our study. Another study by Hill et al (2013) also found that the cinnamon bark extracts were more effective against *S. Typhimurium* LT2 and *Listeria innocua*. Unlu et al (2010) also found that cinnamon bark essential oils were able to produce strong inhibitory action against the growth of several gram positive and negative bacteria.

Cinnamon leaf and bark oils consist of terpenes such as β-caryophyllene, terpenoids such as linalool and phenylpropenes such as cinnamaldehyde and eugenol (Hyldgaard et al, 2012). The concentrations of each component vary depending on the cinnamon oil type and the plant part from which it is extracted. It has been well documented that the different biological properties of essential oils depend on their major components (Bakkali et al, 2008). The main component in bark and leaf CEOs are cinnamaldehyde and eugenol respectively which are very effective as antimicrobials compared to other essential oils (Prabuseenivasan et al, 2006; Matan et al, 2006; Goni et al, 2009; Vangalapati et al, 2012; Hyldgaard et al, 2012; Rao et al, 2014).
Cinnamon essential oils of bark might be showing more inhibition capability due to the presence of cinnamaldehyde, a compound that is found in higher concentrations in the bark of the plant, whereas in leaves the major component is eugenol (Burt, 2004; Vangalapati et al., 2012). Studies have found that cinnamaldehyde has more antibacterial efficacy than eugenol against bacteria such as Salmonella and Listeria (Ravishankar et al, 2010; Sanla-Ead et al., 2012), which are in accordance with the results of our study. Cinnamaldehyde have the ability to inhibit cell division and perturbs the cell membranes, while eugenol works as antimicrobial agent by permeabilizing the cytoplasmic membrane (Hyldgaard et al., 2012). This dual action of cinnamaldehyde might be the reason for its higher antimicrobial activity than eugenol.

3.4 CONCLUSION

Cinnamon essential oils (CEOs) derived from leaf and bark have shown their ability to inhibit the growth of bacteria such as Salmonella Typhimurium and Listeria monocytogenes in broth system suggesting that these can be used as a potential, natural antimicrobial agents in the food industry and improve the microbial safety of foods. Overall, CEOs from bark showed better performance with respect to antimicrobial activity and sensory acceptability than those from leaf, which might be due to the effect of cinnamaldehyde which is the main component of bark essential oil, whereas the leaf essential oils contain more of eugenol.
References


Table 1: The log reductions of *S*. Typhimurium (from $10^4$ and $10^9$ CFU/mL) when incubated with cinnamon leaf essential oils for different time periods

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concentration of cinnamon essential oils</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^4$ CFU/ml</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^9$ CFU/ml</th>
<th>P-value</th>
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Values are the mean and standard deviation.
Mean ratings with different small letters within each column represent a significant difference at *P* < 0.05.
Mean ratings with different capital letters within each row represent a significant difference at *P* < 0.05.
Table 2: The log reductions of *S. Typhimurium* (from $10^4$ and $10^9$ CFU/mL) when incubated with cinnamon bark essential oils for different time periods

<table>
<thead>
<tr>
<th>Time (h)</th>
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*Log reduction of Salmonella from initial concentration of $10^4$ CFU/ml*

<table>
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<th>Time (h)</th>
<th>Concentration of cinnamon essential oils</th>
<th>Log reduction of Salmonella from initial concentration of $10^9$ CFU/ml</th>
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$P$-values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 
Table 3: The log reductions of *Listeria* (from $10^4$ and $10^9$ CFU/mL) when incubated with cinnamon leaf essential oils for different time periods

<table>
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<tr>
<th>Time (h)</th>
<th>Concentration of cinnamon essential oils</th>
<th>P-value</th>
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<td>Log reduction of <em>Listeria</em> from initial concentration of $10^4$ CFU/ml</td>
<td>Log reduction of <em>Listeria</em> from initial concentration of $10^9$ CFU/ml</td>
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</tr>
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</tr>
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<td>48</td>
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<td>P-value</td>
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Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 


Table 4: The log reductions of *Listeria* (from $10^4$ and $10^9$ CFU/mL) when incubated with cinnamon bark essential oils for different time periods

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concentration of cinnamon essential oils</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^4$ CFU/ml</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^9$ CFU/ml</th>
<th>P-value</th>
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<td>72</td>
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<td>P-value</td>
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</table>

Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$.
CHAPTER 4

Objective 2: Investigate the effects of cinnamon leaf and bark essential oils on *Salmonella Typhimurium* (S.T.), and *Listeria monocytogenes* (L.m.) in strawberry shake

4.1 INTRODUCTION

Essential oils are used as preservatives and flavoring agents in different types of foods as they are natural products well-liked by consumers all over the world. Essential oils are also called as volatile or ethereal oils and are obtained from different plant materials such as flowers, buds, fruits, seeds, leaves, bark, wood and roots (Burt, 2004; Hyldgaard *et al*, 2012; Solórzano-Santos and Miranda-Novales, 2012). These aromatic plant essences are oily in consistency and often consists of terpenes, terpenoids, and other aromatic and aliphatic compounds which can vary with the source from which they are extracted (Bakkali *et al*, 2008; Jayasena and Jo, 2013). Essential oils can be obtained by various processes such as expression, extraction, fermentation and distillation and are known to possess antimicrobial properties against various bacteria, yeasts and mold from long back (Bassole and Juliani, 2012). Hence they are identified as a promising natural alternative for synthetic and chemical preservatives in food for inhibiting microbial growth. The antimicrobial effects of essential oils depend on various factors such as the composition, structure and functional groups present (Celikel and Kavas, 2008; Omidbeygi *et al*, 2007; Palmer, 1998; Burt, 2004).

Food industry recently is giving more importance to ensure the microbiological safety through the use of natural products. Essential oils are gaining popularity as natural antimicrobials as they are generally recognized as safe (GRAS). *Cinnamomum zeylanicum* commonly known as cinnamon was one of the first trade spices of ancient world and is known popularly for its fragrance and flavor. Cinnamon consists of various resinous compounds along with numerous essential oils such as eugenol, cinnamaldehyde, camphor, b-caryophyllene, linalool and other
Cinnamon Essential Oil (CEO) is responsible for the characteristic odor and flavor but the components vary according to the part of the plant from where it is extracted. The main component in bark CEO is trans-cinnamaldehyde (65-80%), whereas in leaf CEO it is eugenol (70-95%). CEOS have been shown to possess antimicrobial properties against bacteria, yeast and mold (Prabuseenivasan et al., 2006; Matan et al., 2006; Goni et al., 2009). Food and drug administration lists cinnamon as a GRAS substance (FDA, 2015) and it is used worldwide as a natural food additive and flavoring agent.

Foodborne bacteria such as *Salmonella Typhimurium* (S.T.), and *Listeria monocytogenes* (L.m.) are known to cause contamination of milk (CDC, 2016a; CDC, 2015; Cava et al., 2007; Mungai et al., 2015). Food borne outbreaks due to raw milk has increased from 2% in 2007-2009 to 5% in 2010-2012 (Mungai et al., 2015). A recall was reported of flavored shakes in the US (CDC, 2016b). Although pasteurization or heat treatment of milk, has gained popularity as a decontamination technique, outbreaks associated with pasteurized milk occur quite often (Ackers et al., 2000; Olsen et al., 2004; Cava et al., 2007; FDA, 2015). The objective of the study was to investigate the effectiveness of Cinnamon oils at their MIC effective combinations against *Salmonella Typhimurium*, and *Listeria monocytogenes* in strawberry shakes.

### 4.2 MATERIAL AND METHODS

**Materials**

*Listeria monocytogenes* (L.m.) (strain V7 serotype 1/2a) and *Salmonella Typhimurium* (S.T.). The strain of L.m. was provided by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR and the S.T. strain was obtained from Center for Food Safety, University of Arkansas, AR, U.S.A.). Media used for initial culturing bacteria were brain heart infusion (BHI) broth (EMD Chemicals Inc., Darmstadt, Germany). The agar media used for S.T. and L.m.
enumeration were Difco xylose, lysine, tergitol-4 (XLT4) (Becton Dickinson and Co., ® Sparks, MD, U.S.A.) agar and Oxford Listeria agar (EMD Chemicals Inc., ® Darmstadt, Germany) respectively. Strawberry shake was obtained from a local market (Atkins Nutritionals, Inc., Denver, CO). The cinnamon essential oils (CEOs) from leaf and bark were provided by EOAS Organics Pvt. Ltd., (Ratmalana, Sri Lanka, supply to the US through Bulk Apothecary (125 Lena Drive Aurora, Ohio 44202).

**Antimicrobial activity of CEOs from leaf and bark in strawberry shake**

To investigate the effectiveness of CEOs from leaf and bark against *S*. *T*. and *L*. *m*. in strawberry shakes, only 2 different concentrations were used (MIC of 0.5% and a lower concentration of 0.1%). These low concentrations of CEOs were chosen because, besides evaluating its ability to reduce the growth of *Salmonella* and *Listeria*, it should also not affect the consumer acceptance and sensory value of the final food product. Minimum inhibitory concentration was calculated to determine the highest microbial reduction achieved by the CEOs over 72 h, at 37 °C and it was determined to be 0.5% (v/v) for both *Salmonella* and *Listeria* (10⁴ and 10⁹ loadings). Strawberry shake (Atkins Nutritionals, Inc., Denver, CO) was purchased from the local market on the day of the study and pasteurized at 73°C for 15 seconds. The two different concentrations (0.1% and 0.5%) of CEOs from leaf and bark were prepared by adding 20 µl and 100 µl of the essential oils respectively into 20 ml of pasteurized and cooled strawberry shake in sterile tubes. Bacterial culture from the second passage was added to the mixture of strawberry shake and CEOs at the rate of 200 µl / tube, mixed well by vortexing and all the tubes were kept in the refrigerator for 8 days. Samples were taken, serially diluted and plated for bacterial enumeration on 0, 2, 4, and 8 days. For serial dilution, 30µl of sample from each well were added to 270 µl of PBS, serially diluted and 100 µl from each dilution plated
onto differential agar plates with selective supplements in triplicate for the two bacteria studied. The agar media used for S.T. and L.m. enumeration were the same as that used for MIC determination.

**Statistical analysis**

The data was analyzed by one-way analysis of variance (ANOVA) student t test using JMP® Pro 10.0.1 2012 software.

**4.3 RESULTS AND DISCUSSIONS**

Cinnamon essential oils (CEOs) derived from leaf and bark were used in strawberry shake at 4°C to evaluate their antimicrobial activity against *Salmonella Typhimurium* (S.T.) and *Listeria monocytogenes* (L.m.). The log reductions of the two bacteria in the strawberry shakes with CEOs of leaf and bark were found to be different from the results obtained in the broth system. The leaf and bark CEOs at 0.1% and 0.5% were found to produce similar but significant log reductions in S.T. counts (Tables 5-6) at lower and higher concentrations (10⁴ and 10⁹ CFU/mL), with 0.5% CEOs producing complete inhibition of the organism after 8 days. Similarly, both leaf and bark CEOs were able to produce significant log reductions in L.m (Tables 7-8).

However, with L.m. the leaf CEO at 0.5% was not able to completely inhibit the organism even after 8 days of storage at 4°C (Table 8) and bark CEO was found to be more effective. The results of our study indicate that the CEOs of leaf and bark in strawberry shakes at 4°C were effective after a longer time period. This is in contrast with the research of Cava *et al*., 2007 where they found that the MIC of CEOs of leaf and bark in partial skimmed milk against *Listeria monocytogenes* Scott A were lower at 7°C when compared to that at 35°C. This might be due to some antagonistic activity produced by the ingredients of strawberry shakes used in our study. In our previous study with the CEOs against bacteria in broth system, both leaf and bark essential
oils resulted in a high log reduction of $L.m$ and S.T. at 0.1% (v/v) concentration and complete inhibition of these bacteria at 0.5% (v/v) concentration. A study using nutmeg and oregano essential oils, against several foodborne pathogens, showed similar log reduction of the pathogenic bacteria when it was used in broth culture systems as compared to food system (Firouzi et al., 2007).

Cinnamon essential oils of leaf consists of several compounds such as eugenol (87.3%), β-carryophyllene (1.9%) and α-phellanderene (1.9%) and the CEO of bark consists of trans-cinnamaldehyde (97.7%), α-copaene (0.8%) and α-amorphene (0.5%) (Singh et al., 2007). Although CEOs from leaf and bark contains several components they can work as antimicrobial agents either alone or synergistically. Food systems are much more complex than the broth systems and the activity of antimicrobial agents is bound to vary depending on the food product composition. Factors such as fat and pH of the food system can also affect the antimicrobial activity of the CEOs. According to Burt (2004), the pH level of the food can affect the ability of essential oils to inhibit microbial growth with higher antimicrobial activity found at lower pH. A study conducted by Roller and Seedhar (2002), reported that the antimicrobial effect of carvacrol and cinnamic acid was higher in kiwi fruits than in honeydew melons, because the kiwi fruits had a lower pH of 3.2 – 3.6. The strawberry shake in our study had a pH around 6, and hence a higher concentration of the CEOs [0.5% (v/v)] was required to inhibit the growth of *Listeria* and *Salmonella*. For the same reason, a longer incubation period of 8 days or more was required to produce quantifiable results when using the strawberry shake. In broth cultures, on the other hand, a total inhibition of the microbes was achieved at both low and high bacterial levels against both S.T. and $L.m$. 

Other than pH, the fat content of a food product can also greatly affect the ability of essential oils to act as antimicrobial agents. The fat content of strawberry shake used in our study might also have contributed to the delayed antimicrobial activity of the CEO’s. Cinnamon essential oils were found to produce great inhibitory action against *Listeria monocytogenes* Scott A in partial skin milk which might be due to the low fat content of the milk (Cava *et al.*, 2007). Another study by Smith-Palmer *et al* (2001) found that CEOs were having greater inhibitory action against *Salmonella enteritidis* in soft cheese with low fat content when compared to cheese with full fat.

**4.4 CONCLUSION**

Cinnamon essential oils (CEOs) from leaf and bark of the plant have exhibited the ability to inhibit the growth of gram-positive and gram-negative bacteria, when applied to a food system. Although the effectivity of the CEOs was limited because of the complex nature of the food system, application of the CEOs still produced the desired antimicrobial effect. It demonstrated that cinnamon leaf and bark essential oils can be used as a potential, natural antimicrobial agents in food.
References


CDC (2015); Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update) [Internet]. : United States Centers for Disease Control and Prevention; Available from:https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html


FDA, (2015); The Dangers of Raw Milk: Unpasteurized Milk Can Pose a Serious Health Risk http://www.fda.gov/Food/ResourcesForYou/Consumers/ucm079516.htm


Table 5: The log reductions of *S. Typhimurium* (from $10^4$ and $10^9$ CFU/mL) on Strawberry shake after treatment with 0.1% and 0.5% CEOs leaf Concentration at 4°C

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^4$ CFU/ml</th>
<th>P-value</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^9$ CFU/ml</th>
<th>P-value</th>
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<td></td>
<td>Concentration of oil (%)</td>
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Values are the mean and standard deviation.
Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.
Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 

Table 6: The log reductions of *S. Typhimurium* (from $10^4$ and $10^9$ CFU/mL) on Strawberry shake after treatment with 0.1% and 0.5% CEOs bark Concentration at 4°C

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^4$ CFU/ml</th>
<th>P-value</th>
<th>Log reduction of <em>Salmonella</em> from initial concentration of $10^9$ CFU/ml</th>
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<td>&gt;0 001</td>
<td>0.3±0.1$^{cB}$ $^{a}$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>0.6±0.3$^{cB}$ $^{a}$</td>
<td>&gt;0 001</td>
<td>2.8±0.1$^{bb}$ $^{a}$</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>1.6±0.2$^{bb}$ $^{a}$</td>
<td>&gt;0 001</td>
<td>2.8±0.0$^{cB}$ $^{a}$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.4±0.4$^{bb}$ $^{a}$</td>
<td>&gt;0 001</td>
<td>4.0±0.2$^{cB}$ $^{a}$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 
Table 7: The log reductions of *Listeria* (from $10^4$ and $10^9$ CFU/mL) on Strawberry shake after treatment with 0.1% and 0.5% CEOs leaf Concentration at 4°C

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^4$ CFU/ml</th>
<th>P-value</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^9$ CFU/ml</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of oil (%)</td>
<td></td>
<td>Concentration of oil (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>0</td>
<td>0.2±0.2$_{dB}$</td>
<td>0.6±0.8$_{dA}$</td>
<td>0.010</td>
<td>0.4±0.15$_{dB}$</td>
</tr>
<tr>
<td>2</td>
<td>1.6±0.4$_{cB}$</td>
<td>1.9±0.1$_{cA}$</td>
<td>0.014</td>
<td>1.8±0.05$_{cB}$</td>
</tr>
<tr>
<td>4</td>
<td>1.9±0.1$_{bB}$</td>
<td>2.6±0.2$_{bA}$</td>
<td>0.006</td>
<td>3.0±0.07$_{bB}$</td>
</tr>
<tr>
<td>8</td>
<td>2.5±0.3$_{aB}$</td>
<td>4.0±0.0$_{aA}$</td>
<td>0.001</td>
<td>3.7±0.21$_{aB}$</td>
</tr>
</tbody>
</table>

Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 

< 0.001
Table 8: The log reductions of *Listeria* (from $10^4$ and $10^9$ CFU/mL) on Strawberry shake after treatment with 0.1% and 0.5% CEOs bark Concentration at 4°C

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^4$ CFU/ml</th>
<th>P-value</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^9$ CFU/ml</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of oil (%)</td>
<td></td>
<td>Concentration of oil (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>0</td>
<td>0.2±0.2&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>0.8±0.1&lt;sup&gt;dA&lt;/sup&gt;</td>
<td>0.4±0.1&lt;sup&gt;dB&lt;/sup&gt;</td>
<td>2.2±0.1&lt;sup&gt;dA&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1.7±0.1&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>2.0±0.1&lt;sup&gt;cA&lt;/sup&gt;</td>
<td>2.0±0.3&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>4.7±0.1&lt;sup&gt;cA&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>1.9±0.1&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>2.8±0.2&lt;sup&gt;bA&lt;/sup&gt;</td>
<td>3.2±0.2&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>5.6±0.1&lt;sup&gt;bA&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>2.5±0.2&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>4.0±0.0&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>4.0±0.2&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>9.0±0.0&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*P*-value

Values are the mean and standard deviation. Mean ratings with different small letters within each column represent a significant difference at *P* < 0.05. Mean ratings with different capital letters within each row represent a significant difference at *P* < 0.05.
CHAPTER 5:

Objective 5: Investigate the shelf life stability of cinnamon leaf and bark essential oils in strawberry shake and conduct sensory evaluation and consumer acceptability of the product.

5.1 INTRODUCTION

Quality attributes is important to consumers (Font and Guerrero 2014; Loutfi et al, 2015). The sensory characteristics of a product such as color, flavor, and aroma effects consumer satisfaction (Zellner et al, 2014; Van Loo et al, 2015). In addition, the consumption of high quality food is increasing. Two of the most significant attributes of foods quality are color and flavor. Color is significant in determining consumer’s acceptance of food products (Shahidi, 2004). Consumers show their satisfactory based on the surface color of products. The marketing of selected food product depends on the consumer preferring. Milk has a bland-taste which results in consumer preferring flavors in milk beverages such as, chocolate vanilla, and strawberry (Huang, 2002; Shahidi, 2004).

Like color, flavor is important in sensory characteristics (Barrett et al, 2010). The addition of flavors such as strawberry to milk products can impact increasing sells of milk products (Shahidi et al, 2004). High heat thermal is an effective method that can be used to ensure the safety of milk beverages. However, high heat thermal can result in undesirable changes in quality of beverages such as changes in color, or affecting the flavor (Chugh et al, 2014). In food safety, natural antimicrobial agents such as cinnamon oils can be used as flavor substance in addition to their effective ability to reduce the microbial growth and increase the shelf life of food products. Cinnamaldehyde is the main component in cinnamon oil that can be effective against foodborne pathogens (Burt, 2004). Cinnamaldehyde consist of aldehyde group, which contributes in the taste of cinnamon.
The objective of the study will investigate the effectiveness of cinnamon oils on the color of strawberry shake and consumer overall acceptance.

5.2 MATERIAL AND METHODS

Materials

Strawberry shake was tested using different techniques such as pH meter (Orion 210A, Orion Research Inc., Boston, MA, USA), refractometer (Atago Inc., Osaka, Japan) and colorimeter CR-300 (Minolta Camera Co., Ltd., Osaka, Japan). Trypticase Soy Agar (TSA) plates (EMD Chemicals Inc., Darmstadt, Germany), PDA plates (EMD Chemicals Inc., Darmstadt, Germany), unsalted crackers (Nabisco Premium Unsalted Tops Saltine Crackers, Mondelēz Global LLC, East Hanover, NJ) and spring water (Clear Mountain Spring Water, Taylor Distributing, Heber Springs, AR) were presented for palate cleansing. Strawberry shake was obtained from a local market (Atkins Nutritionals, Inc., Denver, CO). Cinnamon essential oils (CEOs), from leaf and bark, were provided by EOAS Organics Pvt. Ltd (Ratmalana, Sri Lanka, supply to the US through Bulk Apothecary 125 Lena Drive Aurora, Ohio 44202).

Analysis of strawberry shake at two different temperatures

Samples of strawberry shake were analyzed for color, pH, total soluble solids (TSS) and microbial growth over a period of 8 weeks at two different temperatures, ambient temperature and 4°C. Strawberry shake was purchased from the local market on the day of the study and was autoclaved at 121°C for 15 seconds and cooled to use for the experiments. For each temperature, five samples of strawberry shake were analyzed which were: 1) Strawberry shake (10 ml) + 10 µl of CEO from leaf (0.1%), 2) Strawberry shake (10 ml) + 10 µl of CEO from leaf (0.1%) + 100 µl of masking agent (1%), 3) Strawberry shake (10 ml) + 10 µl of CEO from bark (0.1%), 4) Strawberry shake (10 ml) + 10 µl of CEO from bark (0.1%) + 100 µl of masking agent (1%), and
5) Strawberry shake (10 ml) with no treatments added (control). The samples were all mixed in sterile glass tubes, stored for 8 weeks and evaluated for color, pH, total soluble solids (TSS) and microbial growth at 0, 2, 4, 6, and 8 weeks.

**pH:** The pH of strawberry shake was measured by using pH meter (Orion 210A, Orion Research Inc., Boston, MA, USA). The pH of all five samples for each of the two temperatures studied were recorded.

**Color:** Color is one of the important attributes of beverages. The color of the strawberry shake samples at two different temperatures was determined by using colorimeter CR-300 (Minolta Camera Co., Ltd., Osaka, Japan). Color measurements were taken over a period of 8 weeks to evaluate the changes over time. The L*a*b* color system was used for this experiment, where L*value indicates if the product color is light or dark, a*value measures the green (-a) or red (+a) color and the b* value indicates the blueness (-b) or the yellowness (+b) of the food product. The differences of color was calculated using the following equations:

\[
\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}
\]

**Total soluble solids:** Brix was used to measure the total soluble solids (TSS) in strawberry shake samples at two different temperatures by using the refractometer (Atago Inc., Osaka, Japan). The TSS was measured for all the five samples treated with and without CEOs as described for the shelf life study.

**Microbial growth:** All the five strawberry shake samples at ambient temperature and 4°C were also analyzed for microbial spoilage during the storage period. Samples were taken on 0, 2, 4, 6, and 8 weeks, serially diluted and plated to determine the log CFU/mL. The Total Plate Count (TPC) was determined using Trypticase Soy Agar (TSA) plates (EMD Chemicals Inc., Darmstadt, Germany) which were incubated at 30°C for 48 h. The growth of yeast and mold was
determined using Potato dextrose agar (PDA) plates (EMD Chemicals Inc., Darmstadt, Germany) which were incubated at 22 °C for 48h.

Sensory analysis

Sensory analysis of strawberry shakes with CEOs added as antimicrobials, was conducted in the University of Arkansas Sensory Service Center (Fayetteville, AR). A total of 75 participants (41 females and 34 males) ranging in age from 21 to 65 years (mean ± standard deviation = 36 ± 14 years) participated in the sensory analysis. Participants were recruited through the consumer profile database of the University of Arkansas Sensory Service Center, which contains profiles of more than 6,200 Northwest Arkansas residents. All participants reported no history of major disease and food allergy. There were five strawberry shake samples, i.e., four treatment samples with CEOs and a control sample with no additives. The four treatment samples contained 0.1% leaf oil, 0.1% bark oil, 0.1% leaf oil with 1% masking agent, and 0.1% bark oil with 1% masking agent, respectively. A strawberry shake sample without additives was used as a control. For each participant, approximately 30-ml of each sample was presented in a 60-ml soufflé cup identified with a three-digit code. The five strawberry shake samples were presented in a sequential monadic fashion based on the Williams Latin Square design (Williams, 1949). Participants were asked to rate their hedonic impression with respect to surface color, aroma, flavor, and texture on 9-point hedonic scales ranging from 1 (“dislike extremely”) to 9 (“like extremely”), respectively. Overall impression of each sample was also rated on the 9-point hedonic scale. Cinnamon flavor of each sample was evaluated on a 5-point “Just-About-Right” (JAR) scale (1 = “much too little”, 3 = “JAR”, and 5 = “much too much”). In addition, participants were asked to rate their willingness to purchase each sample on a 5-point category scale (1 = “definitely would not buy”, 3 = “may or may not buy”, and 5 = “definitely
will buy”). A short break (for 25 seconds) was allowed between sample presentations. During the break, unsalted crackers (Nabisco Premium Unsalted Tops Saltine Crackers, Mondelēz Global LLC, East Hanover, NJ) and spring water (Clear Mountain Spring Water, Taylor Distributing, Heber Springs, AR) were presented for palate cleansing.

**Statistical analysis**

Data analysis was performed using JMP® Pro (version 12.2, SAS Institute Inc., Cary, NC). Data obtained from antimicrobial test and physicochemical analysis were analyzed by a one-way analysis of variance (ANOVA) treating “sample” as a fixed effect. Data obtained from sensory analysis was analyzed by a two-way ANOVA treating “sample” as a fixed effect and “consumer panel” as a random effect. If a significant difference of means was determined by ANOVA, post hoc comparison between test samples were conducted using Student’s t-tests. A statistically significant difference was defined as $P < 0.05$.

5.3 **RESULTS AND DISCUSSIONS**

**pH**: pH measurements determine whether a product is acidic or basic and this is important to ensure the stability of the product during storage. The pH of strawberry shakes with CEOs of leaf and bark added was measured at 0, 2, 4, 6, and 8 weeks to determine the stability of the product during this period. The pH of strawberry shake with the addition of CEOs of leaf and bark did not change significantly from the initial pH (6.6) and was ranging from 6.5-6.6 and 6.4-6.6 respectively with leaf and bark oils. The pH also did not show any significant changes with the two temperatures studied (ambient temperature and 4°C). The addition of masking agents together with the CEOs of leaf and bark also did not alter the pH of the strawberry shake from the initial pH. Overall the pH range for the strawberry shakes with CEOs was found to be stable over a storage period of 8 weeks and was found to be on the acidic side. pH is one among the
different factors that can influence the antimicrobial effects of essential oils in food. With a lower pH of the food product antimicrobial effects of essential oils was found to be high (Burt, 2004). Roller and Seedhar (2002) found that the antimicrobial effect of carvacrol and cinnamic acid was more in kiwi fruits than in honeydew melons as kiwi fruits had a lower pH of 3.2 – 3.6.

**Color:** Color is a major aspect in food products and fresh produce and it is essential in food industries. The difference in color of strawberry shake with cinnamon leaf and bark essential oils along with masking agents over storage period at various temperatures was measured using $\Delta E^*$ equation (Figure 1-2). The results were classified as “not noticeable” ($0 < \Delta E^* < 0.5$), “slightly noticeable” ($0.5 < \Delta E^* < 1.5$), “noticeable” ($1.5 < \Delta E^* < 3.0$), “well visible” ($3.0 < \Delta E^* < 6.0$), and “great” ($6.0 < \Delta E^* < 12.0$) (Cserhalmi *et al.*, 2006). Strawberry shake with leaf CEOs at 4°C showed noticeable change in color after 2 weeks of storage which became more prominent over time, but at ambient temperature the color did not change significantly over storage time and became slightly noticeable after 8 weeks. For strawberry shakes with leaf CEOs and masking agents the color change was significantly different over time at 4°C but a noticeable change was observed only after 6 weeks of storage period (Table 9). However, for the shakes with leaf CEOs and masking agents at ambient temperatures the noticeable color change after 2 weeks became slightly noticeable overtime (Table 10). The addition of masking agent with leaf CEOs was not able to produce any noticeable change in color after 8 weeks of storage at both the temperatures. Strawberry shakes with bark CEOs alone or with bark CEOs and masking agent produced noticeable and well visible changes in color both at 4°C and ambient temperature (Tables 9 and 10). The change in color of strawberry shakes could be due to the application of high temperature (autoclaving) or the difference in components of the CEOs of leaf and bark. Even though application of high temperature helps in
the control of microbial spoilage of a product extending the shelf life of a product, it can also alter the color of the product. High heat thermal processing can result in the change of color of the beverages over storage time (Chugh et al., 2014). The color changes also could be due to the difference in major components of leaf and bark oils.

**Brix:** Brix is used to measure the total soluble solid content of a product. The initial total soluble solids of the control sample (strawberry shake with no CEOs or masking agents added) was measured to be 5.9 Brix. The addition of CEOs of leaf and bark to strawberry shakes have increased the total soluble solid content compared to the control. However, addition of CEOs of bark with and without masking agents to strawberry shakes had the same range of total soluble solid content (6.1 and 6.4). Strawberry shakes were found to have a higher total soluble solid content between (6.9 and 6.3) when CEOs of leaf were added to the shakes. Here also as observed with the bark CEOs, addition of 1% masking agent did not change the value of total soluble solid content of the shakes. Also the values of total soluble solid contents of shakes at the two different temperatures (ambient temperature and 4°C) did not change significantly with the addition of CEOs of leaf and bark.

**Microbial growth:** The stability of strawberry shakes was also measured by determining the microbial survivors during the storage period of 8 weeks. The total aerobic plates as well as the plates for yeast and mold determination did not show any growth for all the five strawberry shake samples during the entire storage period for both the temperatures studied.

**Sensory analysis**

Sensory attributes of beverages, such as color, flavor, aroma, taste, and texture, play an important role in modulating consumer acceptance, which may in turn affect willingness to purchase. It has been well known that antimicrobials, whether chemical or natural, can affect
sensory impacts of products at elevated concentrations (Meredith et al., 2013; Samant et al., 2015). Sensory analysis was designed to determine whether and how cinnamon essential oils (CEOs) can affect sensory acceptability of strawberry shakes as a function of parts of cinnamon plants, i.e., between the CEOs derived from bark versus leaf.

Table 11 shows mean ratings of consumer acceptability and willingness to purchase for the five strawberry shake samples. Overall, participants liked surface colors of the five strawberry shake samples slightly to moderately (ranging from 6.38 to 6.69 on a 9-point hedonic scale). Color liking did not significantly differ among the five strawberry shake samples ($P = 0.76$). Aroma liking significantly differed among the five shake samples ($P < 0.001$). Strawberry shake samples with CEOs derived from leaf or bark showed significantly lower ratings of aroma liking than did the shake without CEOs (i.e., control). Among strawberry shake samples with CEOs, the shake sample with both masking agent and CEOs from bark (mean ± SD = 5.92 ± 1.77) showed significantly higher ratings of aroma liking than than did those with CEOs from leaf (without flavor masking agent: 5.03 ± 1.90; with flavor masking agent: 5.28 ± 1.92). The five strawberry shake samples significantly differed with respect to flavor liking ($P < 0.001$) with the greatest liking of control sample. The strawberry shake samples with CEOs from bark, regardless of masking agent addition, showed significantly higher ratings of flavor liking than did those with CEOs from leaf. In addition, participants rated strawberry shake samples with either bark or leaf CEOs as being more intense (ranging from 3.75 to 4.09) than their just-about-right (3.00), so further research is needed to reduce cinnamon flavor intensity of strawberry shakes including CEOs. There were no significant differences with respect to JAR ratings of cinnanon flavor intensity as a function of part of cinnamon (bark versus leaf) and addition of flavor masking agent (with versus without flavor masking agent) ($P > 0.05$). Texture liking
significantly differed among the five strawberry shake samples \((P < 0.001)\) with the greatest liking of control sample. Strawberry shake samples with CEOs from bark had significantly higher ratings of texture liking than did those with CEOs from leaf. Similarly, the five strawberry shake samples significantly differed with respect to overall impression \((P < 0.001)\) and willingness to purchase \((P < 0.001)\) showing the great rating of overall impression in the control sample.

Sensory analysis shows that an addition of CEOs decreased both ratings of overall impression and willingness to purchase, which might be due to the high concentration of CEOs. Similarly, previous studies have shown variations in consumer acceptability of foods in relation to the concentration of antimicrobials \((\text{Kim and Marshall, 1999; Mytle et al, 2006})\). For example, Mytle et al (2006) showed sensory impact of clove oil on chicken frankfurters are dependent on the addition level of clove oil. Moreover, in the present study addition of flavor masking agent (1\%) was not effective in lessening the negative impact of the high concentration of CEOs on overall impression and willingness to purchase. A study conducted by Luckow et al (2006) showed that addition of 1.5\% probiotic cultures to orange juice was well accepted by consumers when high concentration (10\% v/v) of flavor masking agent was used. Thus, further research is needed to determine whether increasing the concentration of masking agents can result in higher acceptability of strawberry shakes including CEOs. Furthermore, it is worth noting that strawberry shake samples with CEOs from bark had significantly higher ratings of overall impression and willingness to purchase than did those with CEOs from leaf, respectively. In other words, consumer participants liked and wanted to purchase strawberry shakes with CEOs from bark more than those with CEOs from leaf. CEOs of bark contain more of cinnamaldehyde whereas CEOs of leaf contain more of eugenol. Taste of cinnamaldehyde might be more
acceptable to consumer participants since bark of cinnamon is mainly used in cooking than the leaves and the taste is more familiar to them.

5.4 CONCLUSION:

The demand of natural antimicrobial agents by consumers have led us to investigate cinnamon oils’ ability to reduce the microbial growth of foodborne pathogens. However, it was important to evaluate the shelf life of strawberry shake and their overall acceptance by consumers. In our study, we found that cinnamon leaf and bark essential oils their stored for 8 weeks at different temperatures had no significant change except for color. When the strawberry shake was evaluated by consumers, cinnamon bark oils was preferred compared to leaf oil. Moreover, 1% of masking agent was low. Higher masking agent may result in better acceptance of beverages with cinnamon oils.
References:


Table 9: Total color difference $\Delta E^*$ of strawberry shake at $4^\circ$C

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Leaf CEO</th>
<th>Leaf with M CEO</th>
<th>Bark CEO</th>
<th>Bark with M CEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.46±1.64a</td>
<td>9.19±1.15a</td>
<td>6.36±0.30a</td>
<td>2.2±0.28a</td>
<td>11.3±0.85a</td>
</tr>
<tr>
<td>4</td>
<td>3.38±2.71a</td>
<td>1.08±0.39b</td>
<td>5.09±0.60a</td>
<td>5.7±0.52a</td>
<td>1.46±0.91b</td>
</tr>
<tr>
<td>6</td>
<td>5.41±3.26a</td>
<td>1.79±0.41b</td>
<td>4.98±2.50a</td>
<td>6.2±0.06a</td>
<td>8.15±2.87a</td>
</tr>
<tr>
<td>8</td>
<td>4.05±2.36a</td>
<td>1.26±0.45b</td>
<td>8.64±3.71a</td>
<td>3.2±0.41a</td>
<td>8.35±2.07a</td>
</tr>
</tbody>
</table>

Control: strawberry shake without cinnamon essential oils and flavor masking agent (M).
Values are the mean and standard deviation.
Mean ratings with different letters within each row represent a significant difference at $P < 0.05$. 
<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Leaf CEO</th>
<th>Leaf with M CEO</th>
<th>Bark CEO</th>
<th>Bark with M CEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.54±0.71ab</td>
<td>2.46±0.20b</td>
<td>1.74±0.91b</td>
<td>4.26±0.64ab</td>
<td>5.12±1.61a</td>
</tr>
<tr>
<td>4</td>
<td>2.12±0.47a</td>
<td>4.07±0.16a</td>
<td>5.34±0.52a</td>
<td>3.31±0.78bc</td>
<td>5.11±0.23a</td>
</tr>
<tr>
<td>6</td>
<td>1.36±0.20ab</td>
<td>4.43±0.65a</td>
<td>1.73±0.68b</td>
<td>5.24±0.56a</td>
<td>0.87±0.11b</td>
</tr>
<tr>
<td>8</td>
<td>0.97±0.18b</td>
<td>2.21±1.06b</td>
<td>4.81±2.19a</td>
<td>2.16±1.36c</td>
<td>2.95±2.33ab</td>
</tr>
</tbody>
</table>

Control: strawberry shake without cinnamon essential oils and flavor masking agent (M). Values are the mean and standard deviation. Mean ratings with different letters within each row represent a significant difference at $P < 0.05$. 
Table 11: Mean ratings of the five strawberry shake samples with respect to sensory acceptance and willingness to purchase.

<table>
<thead>
<tr>
<th></th>
<th>Strawberry shake samples with/without cinnamon essential oils (CEOs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Color liking</td>
<td>6.69 ± 1.42&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aroma liking</td>
<td>7.09 ± 1.22&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavor liking</td>
<td>6.76 ± 1.68&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Texture liking</td>
<td>6.71 ± 1.57&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Overall impression</td>
<td>6.69 ± 1.67&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cinnamon flavor JAR</td>
<td>N/A</td>
</tr>
<tr>
<td>Willingness to purchase</td>
<td>3.47 ± 1.08&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Control: strawberry shake without cinnamon essential oils and flavor masking agent (M). Values are the mean and standard deviation. N/A: Not applicable because control did not include cinnamon essential oils. Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 

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CHAPTER 6

Objective 4: Evaluate the inhibitory effects cinnamon leaf and bark essential oils *Salmonella Typhimurium* (S.T.), and *Listeria monocytogenes* (*L.m.*) in fresh celery.

6.1 INTRODUCTION

Introduction

Foodborne illnesses in the United States is a major public health concern with one in every 6 Americans getting sick through consumption of contaminated food (Mangen *et al*., 2015; Scallan *et al*., 2011; Nou and Luo, 2010). According to the CDC, illnesses caused by *Listeria* have resulted in approximately 1600 illnesses and 260 deaths every year (CDC, 2014). Similar to *Listeria, Salmonella* has caused several hospitalizations resulting in 19,000 illnesses and 380 deaths annually (CDC, 2016). Outbreaks associated with consumption of fresh produce have increased during the last decade (Callejón *et al*., 2015; Pielaat *et al*., 2014; Ziuzina *et al*., 2014). Both *Salmonella* and *Listeria* have been related to different foodborne outbreaks in fresh produce such as fruits and vegetables as well as in seafood and dairy products. Vegetable consumption is increasing annually in the United States due to its health benefits such as lowering risk of several diseases like cardiovascular diseases, cancer, and diabetes. It contains essential vitamins and minerals as well as are rich in proteins and fiber (Rahal *et al*., 2014). However, vegetables are vulnerable to contamination as they are easily perishable and minimally processed (Callejón *et al*., 2015; Birmpa and Vantarakis, 2013; Schnabel *et al*., 2015).

Normally, foods produced are processed to minimize the risk of contamination by foodborne pathogens. However, minimally processed foods have an increased chance of contamination by various foodborne pathogens (Birmpa and Vantarakis, 2013). Although many chemical and artificial compounds used as antimicrobial agents have shown high ability to inhibit microbial
growth, consumer preference towards natural preservatives and antimicrobials in foods has resulted in the need to investigate use of different natural antimicrobial agents (Gyawali and Ibrahim, 2014; Tajkarimi and Cliver, 2010). This has led to the investigation of antimicrobial properties of plant extracts such as cilantro, coriander, dill, and cinnamon (Soylu and Evrendilek, 2009; Seow et al, 2014; Perdones et al, 2014). Several studies have been conducted to evaluate the antimicrobial activity of plant extracts against many foodborne pathogens (Ahn and Mustapha, 2007; Doddanna et al, 2013; Al-Mariri and Safi 2014; Gupta et al, 2013). Plant extracts contain essential oils that are usually extracted from plants by different methods such as expression and distillation (Olmedo et al., 2014; Bassole and Juliani, 2012). Essential oils have been shown to possess several biological properties such as antioxidative, antibacterial, and antifungal properties (Teixeira et al., 2013; Ojeda et al, 2013; Amorati et al, 2013; Bajpai et al, 2013; Diao et al, 2014; Nuzhat and Vidyasagar, 2014). For thousands of years, plant oils have been used for different purposes in food such as their flavor and attractive aroma until the antimicrobial properties of some essential oils were realized (Burt, 2004). Many investigations were carried out to evaluate the antimicrobial activity of essential oils extracted from different plants such as oregano, thyme, cinnamon, basil, and garlic (Seydim and Sarikus 2006, Hussain et al, 2008, Emiroğlu et al, 2010, Abdollahzadeh et al, 2014; Clemente et al, 2016).

Cinnamon essential oils (CEOs) can be extracted from different parts of the plant such as leaf, bark, roots and buds. They are commonly used for several reasons such as natural preservatives, enhancement of flavor, and as medicine (Matan et al, 2006, Wang et al, 2009). The main component of CEOs derived from bark and leaves are trans-cinnamaldehyde and eugenol (Matan et al, 2006; Singh et al, 2007). Cinnamon essential oils have been studied for their antimicrobial properties against several foodborne pathogens (Goni et al 2009, El-Baroty et
The aim of this study was to investigate the antimicrobial effects of cinnamon essential oils obtained from leaf and bark of the plant, towards foodborne pathogens such as *Salmonella* Typhimurium and *Listeria monocytogenes* on fresh celery.

**6.2 MATERIAL AND METHODS**

**Materials**

*Listeria monocytogenes* (*L.m.*) (strain V7 serotype 1/2a) and *Salmonella* Typhimurium (S.T.). The strain of *L.m.* was provided by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR and S.T. strain was obtained from Center for Food Safety research laboratory at University of Arkansas, Ar., U.S.A. Media used for initial culturing of bacteria was brain heart infusion (BHI) broth (EMD Chemicals Inc., ® Darmstadt, Germany). The agar media used for *S.T.* and *L.m.* enumeration were Difco xylose, lysine tergitol-4 (XLT4) (Becton Dickinson and Co., ® Sparks, MD, U.S.A.) agar and Oxford Listeria agar (EMD Chemicals Inc., ® Darmstadt, Germany) respectively. The Cinnamon essential oils (CEOs), from leaf and bark, were provided by EOAS Organics Pvt. Ltd., (Ratmalana, Sri Lanka supply to the US through Bulk Apothecary (125 Lena Drive Aurora, Ohio 44202).

**Antimicrobial effects of CEOs from leaf and bark on contaminated celery**

Fresh celery procured from the local market was washed thoroughly to remove the dirt and debris. It was then placed in a bag to sanitize with bleach (NaOCl) for 5 to 7 minutes which was followed by a rinse with deionized water. The celery was then taken out, dried for 5-10 min and cut into pieces approximately 2cm long and having a weight of 10 g. The 10 g pieces of celery were dipped in bacterial cultures with concentrations of $10^9$ and $10^4$ CFU/mL for 2 minutes. The celery pieces thus contaminated were left under a biosafety (BSL-2) hood to dry
for 30 minutes. Cinnamon leaf and bark essential oils were prepared in PBS, a day before the study to obtain 0.5% (v/v) concentrations of CEOs of leaf and bark. A garden sprayer was used as a conventional mechanism to spray the CEOs of leaf and bark on the contaminated celery samples. The celery samples were sprayed 5 times (2sec/spray), dried under BSL-2 for 30 min, again dipped in bacterial cultures as described above and dried for 30 minutes. The samples were then bagged and stored at 4°C for 7 days. Sampling was done on 0, 1, 3, and 7 days where the samples were homogenized in PBS using a stomacher, serially diluted and plated onto selective agar plates containing selective supplements. The log reductions for the different treatment groups receiving CEOs derived from leaf and bark, were observed by counting the colonies and comparing to the control group, which received no treatments.

**Statistical analysis**

The data was analyzed using one-way analysis of variance (ANOVA) & Student’s t-test with the JMP® Pro 10.0.1 software (Cary, NC).

6.3 RESULTS AND DISCUSSION

Cinnamon leaf and bark essential oils, at 0.5% (v/v) concentration was used in this study against *Listeria monocytogenes* and *Salmonella* Typhimurium on celery. At lower concentration of S.T (Table 12), both leaf and bark CEOs produced log reductions of 0.8 and 1.2 when incubated for 7 days at 4°C. With *L.m*. these log reductions were 0.8 and 1.1 logs (Table 13).

The leaf and bark CEOs were able to reduce S.T by 2.8 and 3.5 logs (Table 12) respectively, while with *L.m.* these were found to be 3.9 and 3.8 logs (Table 13) after 7 days of storage at the same temperature as above.

The concentrations of the components in CEOs vary depending on the CEO type with CEOs from bark consisting mainly of *trans*-cinnamaldehyde (65-80%) while CEO from leaf
contains mainly eugenol (70-95%). *Trans*-cinnamaldehyde and eugenol have shown antimicrobial abilities against bacteria, yeast and mold (Prabuseenivasan *et al*., 2006; Matan *et al*, 2006; Goni *et al*, 2009). A study conducted by Ravishankar *et al* (2010) on cinnamaldehyde and carvacrol as antimicrobial agents against a strain of *Salmonella* on celery, has proved the ability of cinnamaldehyde to inhibit the growth of the bacteria by producing a 2.3 log reduction at a bacterial concentration of $10^6$ CFU/mL after 3 days of incubation at 4°C. These results are in accordance with our study where bark CEOs were found to produce higher reduction of *Salmonella* Typhimurium on celery than the leaf CEOs.

The antimicrobial agents applied on fresh produce surface such as the celery, were less susceptible to factors such as pH, fat, and proteins unlike the strawberry shake system. But the retention of CEOs on surface of celery by conventional spraying is doubtful as the retention of antimicrobial agents on fresh produce may vary depending on the method used to apply the agent. A study using organic acids and plant extracts against *Salmonella* in fresh produce has reported that, using traditional spray to apply antimicrobial agents was not sufficient due to the uneven surface of the produce, and that it might not ensure the safety of the produce (Ganesh *et al*, 2010). In our study, the celery samples were sprayed five times to ensure that the CEOs from leaf and bark were completely covering the celery, before it was left under the hood to dry. Another study conducted using the dipping method of fresh leaf lettuce and radish sprouts, with several essential oils such as oregano and cinnamon essential oils, at varying concentrations showed that a significant log reduction of 0.96 and 0.32 CFU/g were achieved for oregano and cinnamon essential oils, respectively, after seven days of storage at 4 °C (Hyun *et al*, 2015). Compared to these results, our study found that the CEOs from leaf when applied at 0.5% (v/v) concentration on celery sticks resulted in a higher log reduction of both *L.m.* (1.0 and 3.9
CFU/mL) and S.T (0.88 and 2.85 CFU/mL), at low and high bacterial concentrations. Also for celery samples applied with 0.5 % (v/v) of CEO from bark, the reductions were 1.1 and 3.8 CFU/mL for L.m and 1.2 and 3.5 CFU/mL for S.T, at low and high bacterial concentrations, respectively (Table 12-13).

Several studies have examined the antimicrobial activity of cinnamon leaf and bark essential oils (Wang et al., 2009; Hill et al, 2013). According to Todd et al. (2013), cinnamon leaf essential oil at a high concentration of 0.5% and a longer storage time had the ability to reduce the growth of a Salmonella strain (6 logs/mL), with no significant differences observed between the storage temperatures of 4°C and 8°C. In our study also, the leaf essential oils have been shown to reduce the growth of both Listeria and Salmonella, when incubated at 4°C with higher log reductions observed with longer storage time.

6.4 CONCLUSION

Natural extracts of cinnamon, i.e. leaf and bark essential oils have the ability to reduce the growth of foodborne pathogens at different microbial concentrations. The results of our study were promising for the use of cinnamon essential oils as a stand-alone antimicrobial agent for minimally processed fresh produce, in order to ensure the safety of vegetables. The inhibition of the two bacteria used in our study by application of CEOs on fresh celery however was not as high as the inhibitory effects of the CEOs in strawberry shake. This might be due to the fact that the CEOs were added to strawberry shake in closed containers and had more surface contact with the bacteria. Hence a better application technique on fresh produce such as celery may produce even higher inhibitory effects against bacteria.
References:


Table 12: The log reduction of *Salmonella* at 0.5% cinnamon leaf and bark essential oils at 4 °C on celery

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Salmonella</em> concentration of 10^4 CFU/mL</th>
<th>P value</th>
<th>Log reduction of <em>Salmonella</em> concentration of 10^9 CFU/mL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type of oil</td>
<td></td>
<td>Type of oil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>Bark</td>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td>0</td>
<td>0.0±0.1_dB</td>
<td>0.9±0.2_dA</td>
<td>&lt;0.0001</td>
<td>0.0±0.2_cB</td>
</tr>
<tr>
<td>1</td>
<td>0.9±0.2_dB</td>
<td>1.1±0.2_cA</td>
<td>&lt;0.0001</td>
<td>2.6±0.0_bB</td>
</tr>
<tr>
<td>3</td>
<td>0.7±0.3_dB</td>
<td>1.4±0.7_aA</td>
<td>&lt;0.0001</td>
<td>2.8±0.0_aB</td>
</tr>
<tr>
<td>7</td>
<td>0.8±0.1_bbB</td>
<td>1.2±0.2_bA</td>
<td>&lt;0.0001</td>
<td>2.8±0.2_aB</td>
</tr>
</tbody>
</table>

Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 

94
Table 13: The log reduction of *Listeria* at 0.5% cinnamon leaf and bark essential oils at 4°C on celery

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^4$ CFU/mL</th>
<th>P value</th>
<th>Log reduction of <em>Listeria</em> from initial concentration of $10^9$ CFU/mL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of oil (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>Bark</td>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td>0</td>
<td>0.9±0.1&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>0.4±0.2&lt;sup&gt;CA&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>0.0±0.1&lt;sup&gt;dB&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>0.8±0.2&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>0.9±0.2&lt;sup&gt;bA&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>2.6±0.1&lt;sup&gt;cB&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>0.8±0.3&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>1.0±0.8&lt;sup&gt;bA&lt;/sup&gt;</td>
<td>0.005</td>
<td>3.0±0.0&lt;sup&gt;BB&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>1.0±0.4&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>1.1±0.4&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>0.007</td>
<td>3.9±0.2&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are the mean and standard deviation.

Mean ratings with different small letters within each column represent a significant difference at $P < 0.05$.

Mean ratings with different capital letters within each row represent a significant difference at $P < 0.05$. 
CHAPTER 7

7.1 OVERALL CONCLUSION

Cinnamon essential oils (CEOs) derived from leaf and bark have shown their ability to inhibit the growth of bacteria such as *Salmonella Typhimurium* and *Listeria monocytogenes* both in broth system and strawberry shakes, suggesting that these can be used as a potential natural antimicrobial in food industry to improve the microbial safety of foods. Overall, CEOs from bark showed better performance with respect to antimicrobial activity than did those from leaf. CEOs of leaf and bark also have shown significant differences with respect to overall impression and likings of color, aroma, flavor and texture, but not in color liking. Consumer panelists liked and wanted to purchase strawberry shakes with CEOs of bark more than those with CEOs of leaf. Since 1% flavor masking agent was not effective in reducing strong flavor of CEOs in strawberry shakes, further research is needed to find an optimum concentration of flavor masking agent that can lessen the negative impact of CEOs on consumer acceptability of strawberry shakes. In conclusion, our findings demonstrate that CEOs derived from bark are better than those derived from leaf with respect to antimicrobial activity, as well as sensory aspect in strawberry shakes.
Appendix 1: Color of strawberry shake with cinnamon oils at 0, 2, 6, 4, and 8 weeks at 4C

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatments</th>
<th>L</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>76.2±0.64</td>
<td>2.40±0.27</td>
<td>5.14±0.50</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>78.5±0.98</td>
<td>2.78±0.58</td>
<td>5.87±0.66</td>
</tr>
<tr>
<td></td>
<td>Leaf/M</td>
<td>79.8±0.84</td>
<td>3.59±0.11</td>
<td>6.92±0.11</td>
</tr>
<tr>
<td></td>
<td>Bark</td>
<td>70.9±0.88</td>
<td>1.25±0.57</td>
<td>7.01±1.00</td>
</tr>
<tr>
<td></td>
<td>Bark/M</td>
<td>78.8±0.79</td>
<td>2.47±0.71</td>
<td>8.51±0.77</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>69.9±2.00</td>
<td>1.31±0.28</td>
<td>3.98±0.51</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>71.7±0.99</td>
<td>1.72±0.79</td>
<td>4.70±1.20</td>
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<tr>
<td></td>
<td>Leaf/M</td>
<td>74.3±0.51</td>
<td>1.36±0.16</td>
<td>4.51±3.68</td>
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<tr>
<td></td>
<td>Bark</td>
<td>69.5±0.34</td>
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<tr>
<td></td>
<td>Bark/M</td>
<td>68.4±0.80</td>
<td>0.34±0.22</td>
<td>4.41±0.91</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>73.1±2.54</td>
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<td>Leaf</td>
<td>77.6±0.73</td>
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<tr>
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<td>Leaf/M</td>
<td>75.0±0.53</td>
<td>2.13±0.25</td>
<td>6.02±0.47</td>
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<tr>
<td></td>
<td>Bark</td>
<td>76.6±0.60</td>
<td>0.85±0.56</td>
<td>6.30±0.44</td>
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<td>78.5±0.21</td>
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<td>6</td>
<td>Control</td>
<td>68.9±0.44</td>
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<td>3.80±0.47</td>
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<td>1.66±0.55</td>
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<td>71.2±0.21</td>
<td>0.86±0.77</td>
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<tr>
<td>8</td>
<td>Control</td>
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<td>1.19±0.14</td>
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<td>71.7±0.22</td>
<td>0.79±0.28</td>
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</tbody>
</table>

Control (no oils treatment)
Values are the mean of triplicate measuring of L, a, b values ± standard deviation
Means following the same letters in the column within the same day are not significantly different (P > 0.05).
**Appendix 2: Color of strawberry shake with cinnamon oils at 0, 2, 6, 4, and 8 weeks at ambient temperature**

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatments</th>
<th>L</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>76.2±0.64</td>
<td>2.40±0.27</td>
<td>5.14±0.50</td>
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<td></td>
<td>Leaf</td>
<td>78.5±0.98</td>
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<td>5.87±0.66</td>
</tr>
<tr>
<td></td>
<td>Leaf/M</td>
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<td>3.59±0.11</td>
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<td>0.73±0.9</td>
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</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>76.9±0.2c</td>
<td>2.96±0.5a</td>
<td>6.23±0.5a</td>
</tr>
<tr>
<td></td>
<td>Leaf/M</td>
<td>75.3±0.5</td>
<td>2.5±0.9</td>
<td>5.90±1.0</td>
</tr>
<tr>
<td></td>
<td>Bark</td>
<td>75.3±0.1</td>
<td>2.50±0.7</td>
<td>9.35±0.2</td>
</tr>
<tr>
<td></td>
<td>Bark/M</td>
<td>72.6±0.1</td>
<td>1.26±0.1</td>
<td>7.90±0.2</td>
</tr>
</tbody>
</table>

Control (no oils treatment)

Values are the mean of triplicate measuring of L, a, b values ± standard deviation

Means following the same letters in the column within the same day are not significantly different (P > 0.05).
Appendix 2: Sensory study approval form by University Institutional Review Board & consent forms.

MEMORANDUM

TO: Navam Hettiarachchy
    Han-Seok Seo
    Wafaa Birnawi

FROM: Ro Windwalker
      IRB Coordinator

RE: New Protocol Approval

IRB Protocol #: 16-05-780

Protocol Title: The Effects of Using Cinnamon Leaf and Bark Essential Oils in Strawberry Shakes on their Sensory Attributes and Shelf Life

Review Type: ☑ EXEMPT ☐ EXPEDITED ☐ FULL IRB

Approved Project Period: Start Date: 06/01/2016 Expiration Date: 05/31/2017

Your protocol has been approved by the IRB. Protocols are approved for a maximum period of one year. If you wish to continue the project past the approved project period (see above), you must submit a request, using the form Continuing Review for IRB Approved Projects, prior to the expiration date. This form is available from the IRB Coordinator or on the Research Compliance website (https://vpred.uark.edu/unit/irb/index.php). As a courtesy, you will be sent a reminder two months in advance of that date. However, failure to receive a reminder does not negate your obligation to make the request in sufficient time for review and approval. Federal regulations prohibit retroactive approval of continuation. Failure to receive approval to continue the project prior to the expiration date will result in Termination of the protocol approval. The IRB Coordinator can give you guidance on submission times.

This protocol has been approved for 75 participants. If you wish to make any modifications in the approved protocol, including enrolling more than this number, you must seek approval prior to implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

If you have questions or need anything else, please contact me at 109 MLKG Building, 5-2208, or irb@uark.edu.

109 MLKG • 1 University of Arkansas • Fayetteville, AR 72701-1201 • (479) 575-2208 • Fax (479) 575-6527 • Email irb@uark.edu
The University of Arkansas is an equal opportunity affirmative action institution.
The Effect of Using Cinnamon Leaf and Bark Essential Oils in strawberry shake on their sensory attributes and shelf life

Consent to Participate in a Research Study
Principal Researcher: Navam S. Hettiarachchy
Co-Researcher: Han-Seok Seo
Co-Researcher: Wafaa Brawi

INVITATION TO PARTICIPATE
You are invited to participate in a research study about strawberry shake drink incorporated with cinnamon leaf and bark essential oil as natural antimicrobial agents against foodborne pathogens. You are being asked to participate in this study because you are interested in pathogenically safer strawberry shake drink due to the use of cinnamon essential oil as a natural antimicrobial ingredient in the beverage and are not allergic to cinnamon products.

WHAT YOU SHOULD KNOW ABOUT THE RESEARCH STUDY

Who is the Principal Researcher?
Navam S. Hettiarachchy, Ph.D., Faculty Department of Food Science
Email: nshettar@unlck.edu
Campus phone: 479-575-4779

What is the purpose of this research study?
The purpose of this study is to incorporate essential oils (cinnamon leaf and bark oils) as natural antimicrobial against foodborne pathogens into strawberry shake beverage targeting for all consumers. Commercial strawberry shake will be used as the base beverage. Pure cinnamon leaf and bark oils will be added into the base beverage. Masking agents will be added to minimize the flavor and taste of the essential oils. These beverages will be evaluated for consumer acceptability.

Who will participate in this study?
Approximately seventy five (75) random untrained participants will be selected. All participants must be over 18 with no allergies to cinnamon products, and interested in evaluating strawberry shake beverage incorporated with cinnamon essential oil as natural antimicrobial agent for safer ready to drink beverages.

What am I being asked to do?
Your participation will require the following:
Each panelist will have 2 samples to evaluate. All panelists will be instructed to start the test with visual observation, smell, taste, and evaluate one by one sample. The participants will be administered a paper ballots accompanied with all sample cups to express their evaluation on samples’ sensory attributes including appearance, aroma, flavor, sweetness, mouthfeel, aftertaste, and overall acceptability.

What are the possible risks or discomforts?
These products contain cinnamon leaf and bark essential oils which may cause food allergies. No individuals who are allergic to with cinnamon products should participate in this study.

What are the possible benefits of this study?
Outcome of this research will be helpful to understand the consumer acceptability to pathogenically safer beverages containing cinnamon essential oil. This will be the first time that cinnamon essential oils are being used as natural antimicrobial ingredient in beverages. We expect that the results from this study will potentially provide commercial interest in utilizing cinnamon essential oils in safer food products which can prevent of minimize contamination of foodborne pathogen bacteria in ready to drink beverages.

IRB #16-05-780
Approved: 06/01/2016
Expires: 05/31/2017
How long will the study last?
The study will last for 15 minutes for each panelist.

Will I receive compensation for my time and inconvenience if I choose to participate in this study?
Participants will receive a Wal-Mart gift card worth $10.00. There will be several time slots available for the participants to select as per their convenience.

Will I have to pay for anything?
No, there will be no cost associated with your participation.

What are the options if I do not want to be in the study?
Your participation in this research is completely voluntary and you may withdraw from this study anytime during the study, if you are selected to be a panelist. Your job, your grade, your relationship with the University, etc. will not be affected if you refuse to participate.

How will my confidentiality be protected?
All information will be kept confidential to the extent allowed by applicable State and Federal law. In addition, you will be assigned a code number and all information will be recorded anonymously. Results from the research will be reported as aggregate data.

Will I know the results of the study?
At the conclusion of the study you will have the right to request feedback about the results. You may contact the Principal Researcher, Navam S. Hettiarachchy. You can reach her through her email nheitia@gmail.com or her phone number (479)-575-4779. You will receive a copy of this form for your files.

What do I do if I have questions about the research study?
You have the right to contact the Principal Researcher or Faculty Advisor as listed below for any concerns that you may have.

Principal Researcher: Navam S. Hettiarachchy    Food Science    nheitia@gmail.com    479-575-4779
Co-Researcher: Han-Seok Seo    Food Science    hanseo@gmail.com    479-575-4778
Co-Researcher: Wafa Baniawi    Food Science    wbanawi@email.arizona.edu    479-575-0846

You may also contact the University of Arkansas Research Compliance office listed below if you have questions about your rights as a participant, or to discuss any concerns about, or problems with the research.

Ro Windwalker, CIP
Institutional Review Board Coordinator
Research Compliance
University of Arkansas
109 MLKG Building
Fayetteville, AR 72701-1201
479-575-2308
irb@uark.edu

I have read the above statement and have been able to ask questions and express concerns, which have been satisfactorily responded to by the investigator. I understand the purpose of the study as well as the potential benefits and risks that are involved. I understand that participation is voluntary. I understand that significant new findings developed during this research will be shared with the participant. I understand that no rights have been waived by signing the consent form. I have been given a copy of the consent form.

IRB #16-06-790
Approved: 06/01/2016
Expires: 05/31/2017
Appendix 4: Sensory ballot.

Please just observe this sample. Please look at the sample closely and concentrating on only the **color** of the sample, which statement best describes your **impression** of the **color** of this product?

Color

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Neither</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extremely</td>
<td>Very Much</td>
<td>Moderately</td>
<td>Slightly</td>
<td>Not</td>
<td>Like</td>
<td>Moderately</td>
<td>Very Much</td>
<td>Extremely</td>
<td>Dislike</td>
</tr>
</tbody>
</table>

**Question # 2 - Sample ______**

Please just observe this sample. Considering the **aroma** of the sample, which of the statements below best describes your **impression** of the **aroma**?

Aroma

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Neither</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extremely</td>
<td>Very Much</td>
<td>Moderately</td>
<td>Slightly</td>
<td>Not</td>
<td>Like</td>
<td>Moderately</td>
<td>Very Much</td>
<td>Extremely</td>
<td>Dislike</td>
</tr>
</tbody>
</table>

**Question # 3 - Sample ______**

**After tasting the sample**: Considering only the **flavor** of the sample, which of the statements below best describes your **impression** of the **flavor**?

Flavor

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Dislike</th>
<th>Neither</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
<th>Like</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extremely</td>
<td>Very Much</td>
<td>Moderately</td>
<td>Slightly</td>
<td>Not</td>
<td>Like</td>
<td>Moderately</td>
<td>Very Much</td>
<td>Extremely</td>
<td>Dislike</td>
</tr>
</tbody>
</table>

**Question # 4 - Sample ______**
Please taste the sample and considering only the **flavor** of the **sample**, which of the statements below best describes your **perception** of the **cinnamon flavor intensity**?

Cinnamon Flavor JAR

Please taste the sample and considering only the **flavor** of the **sample**, which of the statements below best describes your **perception** of the **cinnamon flavor intensity**?

- Much too Little
- Too Little
- Just About Right
- Too Much
- Much too Much

**Question # 5 - Sample _____**

Please taste the sample and considering only the **texture (mouth-feel)** of the **sample**, which of the statements below best describes your **impression** of the **texture (mouth-feel)**?

**Texture**

- Dislike Extremely
- Dislike Very Much
- Dislike Moderately
- Dislike Slightly
- Neither Like nor Dislike
- Like Slightly
- Like Moderately
- Like Very Much
- Like Extremely

**Question # 6 - Sample _____**

All things considered, which statement best describes your **overall impression** of this product?

**Overall Impression**

- Dislike Extremely
- Dislike Very Much
- Dislike Moderately
- Dislike Slightly
- Neither Like nor Dislike
- Like Slightly
- Like Moderately
- Like Very Much
- Like Extremely

**Question # 7 - Sample _____**

All things considered, which of the statements below best describes your **willingness to buy** this product?
Willingness to Buy

<table>
<thead>
<tr>
<th>Definitely Would Buy</th>
<th>Probably Would Buy</th>
<th>May or May Not Buy</th>
<th>Probably Would Not Buy</th>
<th>Definitely Would Not Buy</th>
</tr>
</thead>
</table>

Please have a bite of cracker and a sip of water before passing your tray through the metal door to get your next sample.

**Question # 1 - Sample _____**

Please just observe this sample. Please look at the sample closely and concentrating on only the **color** of the sample, which statement best describes your **impression** of the **color** of this product?

**Color**

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike Very Much</th>
<th>Dislike Moderately</th>
<th>Dislike Slightly</th>
<th>Neither Like nor Dislike</th>
<th>Like Slightly</th>
<th>Like Moderately</th>
<th>Like Much</th>
<th>Like Extremely</th>
</tr>
</thead>
</table>

**Question # 2 - Sample _____**

Please just observe this sample. Considering the **aroma** of the sample, which of the statements below best describes your **impression** of the **aroma**?

**Aroma**

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike Very Much</th>
<th>Dislike Moderately</th>
<th>Dislike Slightly</th>
<th>Neither Like nor Dislike</th>
<th>Like Slightly</th>
<th>Like Moderately</th>
<th>Like Much</th>
<th>Like Extremely</th>
</tr>
</thead>
</table>

**Question # 3 - Sample _____**

**After tasting the sample:** Considering only the **flavor** of the sample, which of the statements below best describes your **impression** of the **flavor**?

**Flavor**
Dislike Extremely Dislike Very Much Dislike Moderately Dislike Slightly Neither Like nor Dislike Like Slightly Like Moderately Like Very Much Like Extremely

Question # 4 - Sample ______

Please taste the sample and considering only the flavor of the sample, which of the statements below best describes your perception of the cinnamon flavor intensity?

Cinnamon Flavor JAR

Much too Little Too Little Just About Right Too Much Much too Much

Question # 5 - Sample ______

Please taste the sample and considering only the texture (mouth-feel) of the sample, which of the statements below best describes your impression of the texture (mouth-feel)?

Texture

Dislike Extremely Dislike Very Much Dislike Moderately Dislike Slightly Neither Like nor Dislike Like Slightly Like Moderately Like Very Much Like Extremely

Question # 6 - Sample ______

All things considered, which statement best describes your overall impression of this product?

Overall Impression

Dislike Extremely Dislike Very Much Dislike Moderately Dislike Slightly Neither Like nor Dislike Like Slightly Like Moderately Like Very Much Like Extremely
**Question # 7 - Sample ______**

All things considered, which of the statements below best describes your *willingness to buy* this product?

<table>
<thead>
<tr>
<th>Definitely Would Buy</th>
<th>Probably Would Buy</th>
<th>May Not Buy</th>
<th>Probably Would Not Buy</th>
<th>Definitely Would Not Buy</th>
</tr>
</thead>
</table>

Please have a bite of cracker and a sip of water before passing your tray through the metal door to get your next sample.

**Question # 1 - Sample ______**

Please just observe this sample. Please look at the sample closely and concentrating on only the *color* of the sample, which statement best describes your *impression* of the *color* of this product?

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike Very Much</th>
<th>Dislike Moderately</th>
<th>Dislike Slightly</th>
<th>Neither Like nor Dislike</th>
<th>Like Slightly</th>
<th>Like Moderately</th>
<th>Like Very Much</th>
<th>Like Extremely</th>
</tr>
</thead>
</table>

**Question # 2 - Sample ______**

Please just observe this sample. Considering the *aroma* of the *sample*, which of the statements below best describes your *impression* of the *aroma*?

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Dislike Very Much</th>
<th>Dislike Moderately</th>
<th>Dislike Slightly</th>
<th>Neither Like nor Slightly</th>
<th>Like Slightly</th>
<th>Like Moderately</th>
<th>Like Very Much</th>
<th>Like Extremely</th>
</tr>
</thead>
</table>
Question # 3 - Sample ______

After tasting the sample: Considering only the flavor of the sample, which of the statements below best describes your impression of the flavor?

Flavor

Dislike  Dislike  Dislike  Dislike  Neither  Like  Like  Like
Extremely  Very  Moderately  Slightly  Like  Slightly  Moderately  Very
Much  Much  Much  Much  moderately  much  extremely  much

Neither  Like  Like  Like  Like  Like  Like  Like
nor  Slightly  Slightly  Slightly  Slightly  Slightly  Slightly  Slightly
Dislike  Dislike  Dislike  Dislike  Neither  Like  Like  Like
Slightly  Slightly  Slightly  Slightly  Slightly  Slightly  Slightly  Slightly