Investigation of West African plant extracts as alternative, natural sources of antimicrobials against foodborne pathogens and spoilage organisms

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 6, 2018

Keywords: Antimicrobial, Foodborne, Plant Extracts, Microorganisms, Illness, Spoilage

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Abstract

Consumers demand clean-label food products, necessitating the search for new, natural antimicrobials to meet demand while ensuring food safety. The purpose of this project was to evaluate the antimicrobial properties of crude methanolic and ethanolic extracts from plants [*Alchornea cordifolia, Senna alata, Psidium guajava, Cryptolepis sanguinolenta, Solanum torvum, Piper guineense,* and *Aframomum melegueta*] from West Africa against foodborne microorganisms [*Listeria monocytogenes, Salmonella, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Vibrio parahaemolyticus, Bacillus subtilis, Lactobacillus fermentum,* and *Saccharomyces cerevisiae*] and determine which have the best potential for further development. A well diffusion assay was used to screen antimicrobial activity of extracts against strains of common foodborne pathogens and plant extracts with the highest antimicrobial activity [ethanolic extracts of *P. guajava*, *C. sanguinolenta* and *S. alata*] were selected and used to further evaluate their antimicrobial effects on the growth of various microorganisms over time.

When screening the antimicrobial activity of the selected plant extracts on bacterial growth, *C. sanguinolenta* exhibited the strongest antimicrobial activity, decreasing levels of *V. parahaemolyticus* after 2 h, and *S. aureus* and *L. fermentum* after 4 h to below detectable limits of 1.00 log colony forming units (CFU)/mL. *P. guajava* required 2, 4 and 8 h to decrease *V. parahaemolyticus*, *L. fermentum* and *S. aureus*, respectively, to below detection limits. *S. alata* did not perform as well, requiring 24 h to reduce *E. coli* and *Salmonella* to below detection limits. Growth of *B. subtilis* was inhibited by all extracts, but levels were not decreased to below detectable limits. The minimum inhibitory concentrations (MIC) were then determined for *P.*

guajava and *C. sanguinolenta* against *E. coli* ATCC 2196 and *Salmonella* Typhimurium and found to be 0.50 and 1.00% (w/v), respectively. Concentrations of 0.75% (w/v) *P. guajava* and 0.25% (w/v) *C. sanguinolenta* were then added to tryptic soy broth (TSB) inoculated with *Salmonella* or *E. coli* with an adjusted pH 6, 5, or 4.5. Adjusting pH had no effect on the antimicrobial activity of *P. guajava* against *Salmonella*, whereas, at a pH of 6 and 5, effectiveness of *C. sanguinolenta* was decreased, with cell counts of *Salmonella* rising over 2.00 log CFU/mL greater than samples with unaltered pH after 24 h. The inhibitory effect of both plant extracts against *E. coli* were decreased by the lowered pH; at an unaltered pH, it was inhibited to below the detection limits of 1.00 log CFU/mL after 24 h, however, at a pH of 6, cell numbers rose to over 8.00 log CFU/mL and 6.00 log CFU/mL when *P. guajava* and *C. sanguinolenta* were applied, respectively. Lastly, antimicrobial activity of *P. guajava* at 1.00% (w/v) and *C. sanguinolenta* at 0.50% (w/v) against *E. coli* and *Salmonella* were evaluated in milk at room temperature for 72 h. *P. guajava* exhibited bacteriostatic antimicrobial activity against *E. coli* and continued to keep levels of *Salmonella* less than that of the control from 8 - 72 h at 22 °C. The antimicrobial activity of *C. sanguinolenta* remained significantly less than the control from 8- 72 h at 22 °C against both microorganism, but was not as effective as *P. guajava* at the level tested. The extracts were overall less effective in reducing bacterial populations when tested in a model food system, rather than the ideal conditions in microbiological media.

To summarize this research, extracts were more effective against Gram-negative bacteria when compared to Gram-positive bacteria and yeast. This study suggests that *C. sanguinolenta* and *P. guajava* may be useful as alternative antimicrobials against select pathogens causing foodborne illness; however, antimicrobial efficacy may be lowered when applied in a food system.

Acknowledgments

Thank you is not enough to express my gratitude for the love and support my parents have provided for me, specifically in the last two years. The constant encouragement and unconditional love has provided me with the motivation to keep working towards my goals. The past two years haven't been easy, but your kind words make everything a little easier. Also, a thank you to my extended family for caring so much for me.

To my friends, I have made in Auburn, Brittany, Alex, and Meredith, you are all so special to me and have encouraged me more than you'll ever know. Also, thank you Dr. Berry for giving me my sweet girl, Pumpkin. Having a dog has changed my life for the better and provided me with a new outlook and perspective. To Michelle Hayden, you have helped make Auburn a home away from home for me. Your guidance, advise, and conversations have meant so much to me and helped me though the last two years to achieve my goals.

Lastly, thank you to Dr. Monu for taking me as a graduate student and providing me with the tools and guidance for success and thank you to all the Food Science faculty for your encouragement and guidance. Your success and drive is a constant encouragement to achieve my goals and look forward to a successful food science career. To all my lab mates, Adam, Janet, Sayma, Alejandra, Clayton and Zoila, thank you for all your assistance with my research and your friendships.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
DNA	Deoxyribose Nucleic Acids
DMSO	Dimethyl Sulfoxide
EHEC	Enterohemorrhagic *Escherichia coli*
FDA	Food and Drug Administration
MIC	Minimum Inhibitory Concentration
MHB	Mueller-Hinton Broth
MRS	De Man, Rogosa, and Sharpe
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SAS	Statistical Analysis Software
SNK	Student-Newman-Keuls
SPP	Species
STEC	Shigatoxin Producing *Escherichia coli*
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

UHT	Ultra-High Temperature
US	United States
WHO	World Health Organization
YPD	Yeast Extract Peptone Dextrose

Chapter 1 Introduction

The existence of foodborne illness, food spoilage, food waste, the resulting negative economic impact of these issues, and consumer interests have all pushed the food industry to find alternative, safe, and natural antimicrobials to use in foods and beverages. The number of people who fall ill every year in the United States due to foodborne illness is a major concern, as is the degree of waste that is happening due to food spoilage. Consumers have also influenced the demand for novel antimicrobials due to the perceived association of current synthetic preservatives with diseases and negative effects on children. They also have a desire for clean label products. These combined concerns have led to research that investigates plant extracts as potential sources of antimicrobials. West Africa is one region of great biodiversity and most plants found in this region are only investigated for their potential medicinal applications, with an overall lack research on their potential application as antimicrobials in food and beverage products.

It is estimated by the Centers for Disease Control and Prevention (CDC) (2018) that each year in the United States over 48 million people fall ill from foodborne disease, resulting in approximately 130,000 hospitalizations and 3,000 deaths. In the most recent annual surveillance report published by the CDC (2015), it was stated that the bacterial pathogens responsible for the most outbreak-related illnesses and hospitalizations were *Salmonella* and Shiga Toxin Producing *Escherichia coli.* Foodborne illness is not the only negative effect of microbial growth in food. Food spoilage is also an important issue because bacterial and fungal microorganisms can cause spoilage by degrading nutrients and/or altering desired organoleptic characteristics (Negi, 2012).

Current alternative methods of controlling foodborne illness and spoilage consist of acidification, pasteurization, ultrahigh temperature (UHT) treatments, fermentation, storage temperature control, and addition of various natural and synthetic antimicrobials. However, these current methods of control do not completely control all microorganisms, which has been proven by continued spoilage issues and foodborne disease outbreaks (Davidson, 2001; Negi, 2012).

The consumer demand for more natural foods has led to research using more naturally derived means of food preservation. In the US, there is also a growing popularity for fast, ready-to-eat foods, which are more susceptible to microbial contamination and growth (Ahn, Grun, & Mustapha, 2007). Therefore, controlling both contamination and spoilage of these products through natural antimicrobials is increasingly important. Consumers have questioned the effects of food and diet on behavior and health since as early as 1922 (Shannon, 1922). Commonly controversial additives include nitrates and nitrites, benzoates, sorbates, and sulfites (Sharma, 2015), each being linked to a variety of dangerous and serious conditions, such as, migraines, stomach cancer, asthma, and attention deficit disorders (Rosati & Saba, 2004). Although these additives are considered safe by the Food and Drug Administration (FDA), consumers are worried about the long-term effects of accumulation in the body and the sensitivity of children (Rosati & Saba, 2004).

Plants used for medicinal purposes date back centuries, all over the world. It is estimated by the World Health Organization (WHO) that about 80% of the global population utilizes herbal medicine as part of their primary sources of health care (Adeshina, Onaolapo, Ehunmidu, & Odama, 2010). Various plant structures, such as the roots, leaves, stems, and fruit have been found to possess potent compounds which enhance their antimicrobial properties. Some of the compounds that are shown to provide the greatest antimicrobial characteristics are tannins,

flavonoids, alkaloids, glycosides, and more. These secondary metabolites work both alone and synergistically to inhibit growth of microorganisms (Adeshina et al., 2010). There are many plants indigenous to Western Africa that contain these sought after antimicrobial properties and can be found in the forests of the Eastern Region of Ghana. The Centre for Plant Medicine Research, Mampong Akuapem, Ghana collects, identifies and studies such plants (Ameyaw, Duker, & Mills-Robertson, 2005). These medicinal plants represent a vast source for obtaining lesser known and potent, natural antimicrobial agents (Adeshina et al., 2010).

The purpose of this study is to investigate a variety of plant extracts from Western Africa as possible natural antimicrobials against a variety of foodborne microorganisms. To achieve this goal, several specific objectives are listed: general screening of extracts against foodborne pathogens, descriptive screening of selected extracts against a variety of foodborne pathogens and spoilage microorganisms, determination of the minimum inhibitory concentration (MIC) for select extracts against select pathogens, and lastly, assessing antimicrobial efficacy against pathogens in a food system.

The food industry is in search of new sources of natural antimicrobials that have potential to preserve foods and aid in reducing foodborne disease in a variety of products, including produce, deli meats, and ready-to-eat products. Plants from West Africa have been shown to possess many antimicrobial properties; therefore, evaluating these plant extracts as a natural source of antimicrobials may lead to a new preventative control method to reduce foodborne illness and spoilage, while also meeting consumer needs.

Chapter 2 Literature Review

2.1 West African plant extracts

Active secondary metabolites present in commonly used medicinal plants harvested in the tropical forests of Western Africa show antimicrobial potential to combat foodborne pathogens. Different parts of the plants were investigated for their potential antimicrobial properties, such as the roots, leaves, stems, and bark (Ebi, 2001). However, most of these studies have focused on infectious disease rather than foodborne disease. The phytochemicals that are produced as secondary metabolites exhibit chemical actions, altering the microorganisms on a chemical, physical and/or biological level. Such actions involve disruption of the membrane, inhibiting cells uptake of necessary nutrients, and eventually cell lysis and death (Otake, Makimura, Kuroki, Nishihara, & Hirasawa, 1991; Ikigai, Nakae, Hara, & Shimamura, 1993). Commonly identified phytochemicals in plants consist of polyphenols, flavonoids, carotenoids, and more (Geissman, 1963; Cowan, 1999; Edeoga, Okwu, & Mbaebie, 2005; Negi, 2012). Plants that contain high concentrations of tannins as secondary metabolites are known to have astringent properties, making them antibacterial by nature (Adeshina et al., 2010). Previous studies show that tannins can rid the cell of the bacterial proteins and nutrients needed for growth and survival (Gatsing, Moudjii, Kuiate, Nkah, & Fodouop, 2008). Another potent phytochemical of importance, triterpene, possesses lipophilic characteristics that inhibit the strength of the bacterial membrane (Cowan, 1999). Specifically, there is an effect on the non-mevalonate pathways that are essential for the survival and reproduction of harmful microorganisms due to

carbon availability, synthesis of components that make up the cell membrane, and availability of essential proteins (Nayak et al., 2010).

The potency of the compounds extracted from the plant matter can be dependent on the type of organic solvent used for extraction (Cowan, 1999). The polarity of the extraction solvent will extract metabolites with the same polar structure. Many crude plant extracts use ethanol or methanol as extraction solvents, which result in extracts with antimicrobial properties against a broad spectrum of microorganisms (Okeke, Ogundaini, Ogungbamial, & Lamikanra, 1999). Potency of the plant extract can also vary by the time of year or region of plant cultivation and the process used for extraction (Othman et al., 2011).

Alchornea cordifolia

Alchornea cordifolia is a plant commonly found in the forests of West Africa, in both Nigeria and Ghana, where it is most traditionally used as medicine. Crude extracts from the leaves of this plant have been shown to exhibit antimicrobial characteristics (Hassan, Umar, Lawal, Bilbis, & Muhammad, 2006; Kubmarawa, Ajoku, Enwerem, & Okorie, 2007; Adeshina et al., 2010). The leaves have been found to contain secondary metabolites, that when extracted by various solvents, show antimicrobial activity to both Gram-positive and Gram-negative bacteria (Gatsing, Nkeugouapi, Nji-Nkah, Kuiate, & Tchouanguep, 2010). The components found to be the most abundant in the extract were tannins, flavonoids, glycosides, resins, and carbohydrates. The components that make the crude leaf extract from *A. cordifolia* most active against Gram-positive microorganisms are the tannins and flavonoids, when they are present together (Adeshina et al., 2010).

Overall, *A. cordifolia* is generally known to have broad spectrum antimicrobial properties (Adeshina et al., 2010). Common foodborne pathogens that this plant has been shown to have bacteriostatic or bactericidal effects upon are *E. coli, Bacillus subtilis, Pseudomonas,* and *S. aureus,* as well as yeast, specifically, *Candida* spp. (Ogunlana & Ramastad, 1975; Ebi, 2001).

Senna alata

Senna alata is a small shrub that commonly grows in Western Africa, specifically utilized in Nigeria, where it is often used to treat digestive issues, dermatological issues, inflammation, infections and diabetes symptoms (Hennebelle, Weniger, Joseph, Sahpaz, & Bailleul, 2009). The antimicrobial properties of this plant are attributed to the chemical components found when extracted with an organic solvent, such as methanol, ethanol, or petroleum ether (Owoyale, Olatunji, & Oguntoye, 2005). In a study done by Owoyale and others (2005) testing the antimicrobial and antifungal properties of the ethanolic extract of *S. alata* leaves, the chemical component found to be the most potent and effective against the growth of microorganisms was a flavonoid glycoside. Other active compounds found to be present in the extract were steroids, saposins, phenols, volatile oils, and tannins (Adedayo et al., 2001; Doughari & Okafor, 2007).

Crude extracts made from the leaves or roots of this plant were most commonly studied (Adedayo et al., 2001; Doughari & Okafor, 2007). Common microorganisms this plant is active against, consisting of both Gram-positive and Gram-negative bacteria, are *S. aureus, Salmonella, E. coli., Streptococcus faecalis,* and *B. subtilis* (Doughari & Okafor, 2007).

Psidium guajava

Psidium guajava, commonly known in the United States as guava, is a flavorful fruit. However, other components of this plant, apart from its fruit, have useful characteristics. Antimicrobial potential has been most detected in the roots, bark, fruit, and leaves of the plant (Arima & Danno, 2002; Gutierrez, Mitchell, & Solis, 2008). In Africa, this plant has traditionally been used as a preservative, due to the fruit's acidic nature, and as a treatment for scurvy (Jaiarj et al., 1999). Other applications of this plant extract's activity have consisted of being anti-diarrheal, antimicrobial, antioxidant, antimutagenic, antimalarial, and anti-inflammatory treatments. The components that make this plant so versatile and potent consist of phenolic compounds, carotenoids, and cytokinins (Gutierrez, Barry-Ryan, & Bourke, 2008), as well as flavonoids such as naringenin, flavone, and flavonol (Arima & Danno, 2002). Each of these compounds that are identified contribute to this plant's antimicrobial potential.

Scientific literature states methanolic extracts of this plant exhibit antimicrobial effectiveness against both Gram-positive and Gram-negative microorganisms, such as, *E. coli, B. subtilis, S. aureus,* and *Salmonella enteritidis* (Arima & Danno, 2002; Nair & Chanda, 2007). *Vibrio* spp. has also been found to be susceptible to the antimicrobial activity of this plant (Lutterodt, Ismail, Basher, & Baherudin, 1999).

Cryptolepis sanguinolenta

Cryptolepis sanguinolenta is a plant that grows in tropical areas of Africa and is most commonly cultivated and used in Ghana (Mills-Robertson, Tay, Duker-Eshun, Walana, & Badu, 2012). It has been used as folk medicine traditionally for its antifungal, antimalarial and antibacterial properties (Paulo et al., 1994; Cimanga et al., 1996). The root of this plant is most

commonly utilized due to high concentrations of active compounds, such as the alkaloid cryptolepine (Dwuma-Badu et al., 1978; Tackie et al., 1991). Cryptolepine has been found in the crude extract of this plant (Addy, 2003) and has high antimicrobial effects (Bierer at al., 1998). Ethanol, as the organic extraction solvent, resulted in the presence of high concentrations of phyto-constituents, reducing sugars, polyuronides, alkaloids, and anthocyanides (Mills-Robertson et al., 2012).

This extract has shown more inhibition of Gram-positive bacteria, most likely due to the difference in cell structure compared to Gram-negative microorganisms. Gram-positive bacteria have a single, thick cell wall of peptidoglycan, whereas Gram-negative bacteria have a thinner layer of peptidoglycan with a lipopolysaccharide outermost membrane (Mills-Robertson, Aboagye, Duker-Eshun, Kaminta, & Agbeve, 2009; Mills-Roberts et al., 2012). Common microorganisms this extract has been found to be effective against are *S. aureus, B. subtilis,* and *Pseudomonas* spp. (Boakye-Yiadom & Heman-Ackah, 1979; Cimanga et al., 1991).

Solanum torvum

Solanum torvum is a plant that has been reported as a powerful antibacterial and antifungal agent that is used in traditional medicine in Bangladesh and Nigeria to treat pain and abscesses (Chah, Muko, & Oboegbulem, 2000; Bari, Islam, Khan, & Mandal, 2010). Secondary metabolites present in the crude extracts of the leaves, stems and roots of this plant that make it a powerful and potential antimicrobial source are the steroidal alkaloids, saposins, flavonoids, glucosides and tannins (Bari et al., 2010; Sivapriya, Dinesha, Harsha, Gowda, & Srinivas, 2011).

When testing the zones of inhibition in a disc diffusion assay, the methanolic extract showed greater inhibition of the Gram-positive microorganism, *S. aureus,* and the fungi,

Aspergillus and *Candida,* when compared to the Gram-negative microorganisms, *Salmonella* and *E. coli.* However, when tested for the minimum inhibitory concentration (MIC), antimicrobial activity was not detected against the Gram-negative microorganisms, making this selective as an antimicrobial agent for Gram-positive microorganisms and an antifungal agent (Chah et al., 2000).

Piper Guineense

Piper guineense is commonly utilized in Nigeria due to its potent and effective medicinal properties. It has been used to treat a range of illnesses such as dysentery to bronchitis. Both bactericidal and bacteriostatic properties have been reported for this plant, which are traced back to the high concentrations of powerful secondary metabolites in the leaves and seeds (Okigbo and Igwe, 2007; Nwinyi, Chinedu, Ajani, Ikpo, & Ogunniran, 2009). The secondary metabolites that are most important in this extract are alkaloids, reducing sugars, tannins, and saposins (Konning, Agyare, & Ennison, 2004). Ethanolic and methanolic extractions of this crude plant extract have better antimicrobial activity when compared to an aqueous plant extract, due to the solvents' abilities to extract more phytochemicals from the plant matter (Nwinyi et al., 2009; Konning et al., 2004).

When tested against Gram-positive microorganisms [*S. aureus* and *B. subtilis*], Gram-negative microorganisms [*E. coli*], and fungi [*Pseudomonas, Candida,* and *Aspergillus*], antimicrobial activity was observed, demonstrating that this plant has broad-spectrum antimicrobial activity against bacteria and fungi (Nwinyi et al., 2009; Konning et al., 2004).

Aframomum melegueta

Aframomum melegueta is a plant commonly found in rainforests near Nigeria and is utilized for its medicinal and nutritious properties, specifically being used to treat various infectious diseases such as measles and leprosy, and is also used as a remedy for excessive lactation (Iwu, Duncan, & Okunji, 1999). The seeds, fruits, and leaves of this plant have been utilized for the potent phytochemicals they are known to possess, such as alkaloids, saposins, and triterpenes (Doherty, Olaniran, & Kanife, 2010; Voukeng et al., 2012).

When an essential oil is derived from this plant, it has been found to be more active against Gram-positive bacteria than Gram-negative bacteria (Simon et al., 2007). However, when this plant was tested as an ethanol extract, it showed to be an effective antimicrobial agent against Enterobacteriaceae such as *E. coli, Salmonella* and *Shigella* at a concentration of 50 mg/ mL (Doherty et al., 2010). These results demonstrate that the essential oil and crude extract of this plant most likely contain different active secondary metabolites, thus exhibiting different antimicrobial activity.

2.2 Common foodborne microorganisms

Nine different microorganisms were used in this project to screen the antimicrobial activity of methanolic and ethanolic crude extracts from seven plants found in forests in the Eastern Region of Ghana. The microorganisms used were *Listeria monocytogenes, Escherichia coli, Salmonella* spp.*, Staphylococcus aureus, Enterococcus faecalis*, *Bacillus subtilis, Lactobacillus fermentum, Vibrio parahaemolyticus, and Saccharomyces cerevisiae.* Strains of each microorganism were selected based on their association with foodborne illness outbreaks or food spoilage.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive microorganism that is ubiquitous in nature, being found in sewage, water, and soil. It is often present where lactic acid bacteria(LAB) are active, which gives cause of the high number of *Listeria* outbreaks in dairy products. When it is ingested orally, it grows in the intestinal tract where it can penetrate the tissue and circulate in the blood stream. This bacterial pathogen is particularly dangerous for pregnant women, due to its ability to cross into the placenta and cause abortion or stillbirth. It can withstand a wide variety of temperatures (1- 49 °C) and pH (4-9) (Jay, Loessner, & Golden, 2005a). The United States, in comparison with other countries such as the United Kingdom and Austria, has the strictest policies when it comes to the presence of this microorganism in food. They have no allowable limits and any *L. monocytogenes* detected in foods is considered as an adulterant, subjecting the product in question to recalls and public warning (Jay et al., 2005a). A recall was announced on March 10, 2017 by Vulto Creamery for all its raw milk cheeses due to a *Listeria* outbreak that affected four different states, leading to a total of 8 people being hospitalized and two deaths. The CDC investigation of this outbreak confirmed that Vulto was in fact the source of the outbreak (2017b).

Escherichia coli

E. coli was first established as a Gram-negative foodborne pathogen in 1971, in the US, having over 200 known serotypes. The main serotypes that are known to be the most dangerous are *E. coli* O157:H7 and the non-O157 STEC strains. Due to the prevalence of the non-O157 strains in foodborne outbreaks, the Food Safety and Inspection Service (FSIS) named the dangerous non-O157 strains the ‘Big 6’ (USDA, FSIS, 2010). Enterohemorrhagic *E. coli*

O157:H7 (EHEC) is classified as one of the most dangerous strains of *E. coli* because it produces Shiga-like toxins that lead to serious toxic infections. It can survive in foods at a low pH for an extended period and is most commonly found in beef more than any other food source; however, it is still of concern in other meat, poultry, seafood and fresh crops. *E. coli* is also one of the leading causes of traveler's diarrhea, acute watery diarrhea, and is common for travelers to encounter when first entering a foreign country (Jay, Loessner, & Golden, 2005b). In 2017, an outbreak of *E. coli* STEC O157:H7, the Shiga-toxin producing strain, was identified in Healthy Brand Soynut Butter and was isolated from unopened containers during laboratory testing. There have been 32 reported incidences in 12 different states associated with this outbreak while it was tracked from January to April 2017. There was a major recall of this product; however, due to its potential for such a long shelf-life, others may still be affected in the future if they were not made aware of the recall. It was found that 81% of the total people affected were under the age of 18 (CDC, 2017a).

Salmonella

Another foodborne pathogen of great concern is *Salmonella*. Like *E. coli,* it is Gram-negative with similar growth properties. Over 2,000 serovars of *Salmonella enterica* have been identified. *Salmonella* most often colonizes in mammals and birds (McClelland et al., 2001). To be infected by this pathogen from foods, a significant number of cells must be ingested. Improper handling and preparation of foods contribute to the concern of this pathogen in homes and in the food service industry (Jay, Loessner, & Golden, 2005c). According to the foodborne illness outbreak surveillance program, FoodNet, in 2016 *Salmonella* was one of two pathogens

reported causing the highest incidence of foodborne illnesses, matching the data reported in 2015 (CDC, 2016b).

Staphylococcus aureus

S. aureus is the most common strain of *Staphylococcus* involved in foodborne outbreaks. It is a Gram-positive bacterium that requires specific organic compounds for growth, such as amino acids for nitrogen sources and B vitamins. Gastroenteritis from this pathogen is caused by ingesting foods that contain enterotoxins produced by the microorganism. The known hosts for *S. aureus* are humans and other domestic animals. In general, low numbers have been found in almost all food products that have been handled by humans unless a heat processing step has been applied during production. *S. aureus* cells, in comparison to enterotoxins, are much more sensitive to heat. The foods most commonly associated with an outbreak of this microorganism are meat and poultry dishes (Bennet, Walsh, & Gould, 2013). The outbreaks for this microorganism are most commonly associated with mishandled foods and improper refrigeration of foods after being prepared (Jay, Loessner, & Golden, 2005d).

Enterococcus faecalis

Enterococcus faecalis is a microorganism commonly found in fecal matter due to its presence in natural gut microflora. It is of great significance regarding sanitation practices because of its growing resistance to antimicrobials. Although it was once used as a fecal indicator for water due to its similarities with coliforms, it is generally less numerous. It is a Gram-positive bacterium that requires specific organic material for growth, specifically B vitamins and certain amino acids. It can grow at a much wider range of pH than any other

foodborne pathogens. It can also grow under very harsh environments such as low oxidation-reduction (Eh) potential, thus, leading to its microaerophilic classification (Jay, Loessner, & Golden, 2005e). Most enterococci are not generally regarded as foodborne pathogens; however, they are known to be acquired through consumption of foods. They also cause food intoxication by producing biogenic amines that are consumed by humans (Opera & Zervos, 2007). Enterococci are also linked to food spoilage. High levels of these microorganisms in cheese often lead to deterioration of sensory characteristics in cheese products. Due to this microorganism's thermal resistance, it can survive pasteurization and persist into the next stage of cheese making (Giraffa, 2003).

Bacillus subtilis

Bacillus is a spore forming, Gram-positive microorganism with varying degrees of harmfulness, based on the strain. Some strains of *B. subtilis* are often used in fermented foods such as tofu and as a bacterial component in probiotics (Inatsu et al., 2006; Hong et al., 2008; Patel, Ahire, Pawar, Chaudhari, & Chincholkar, 2009). Another species of this microorganism, *Bacillus cereus*, is highly associated with foodborne illness due to the production of enterotoxins (Hong et al., 2008). There was an outbreak of *B. cereus* in 1993 at an elementary school where 14 people were affected by symptoms, with no deaths (CDC, 1994). In the United States, the leading food that is found to contain *B. cereus* is fried rice, due to this microorganism's presence in uncooked rice. The spores produced by this microorganism are typically heat resistant and will be passed onto foods (Terranova & Blake, 1978; Bean & Griffin, 1990; CDC, 1994). *B. subtilis* is very well characterized and has a similar genome to most other Gram-positive

microorganisms, making it an excellent model bacterium for microbiological testing (Borriss et al., 2018).

Lactobacillus fermentum

L. fermentum is an anaerobic, Gram-positive lactic acid producing microorganism. It is often linked to its potential use in probiotics and other food supplements (Ramos, Thorsen, Schwan, & Jespersen, 2013). The characteristics that make it applicable as a probiotic are acid tolerance, adherence to epithelial cells and tissues, and potential to influence the activity of bacterial adhesion (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; Ramos et al., 2013). *Lactobacillus* spp. are of great importance for fermentation, specifically in wine making. They are responsible for the step called malolactic fermentation (MLF) and if not controlled properly, can lead to unwanted metabolic activity. Once this step is complete, sulfate is typically added to halt fermentation, as over fermentation will lead to undesired organoleptic characteristics (Garcia-Ruiz et al., 2012). Another way strains of LAB are utilized in the food industry is for preservation. LAB can produce lactic acid, creating undesirable conditions for spoilage microorganisms to grow and reproduce (Gerez, Torres, Font de Valdez, & Rollan, 2013). The functional components of LAB that make it a strong antimicrobial are flavoproteins and peroxidases, that while in the presence of oxygen, produce hydrogen peroxide, resulting in strong oxidation potential and destruction of cellular function (Davidson, Post, Branen, & McCurdy, 1983; Condon, 1987; Gerez et al., 2013).

Vibrio parahaemolyticus

V. paraheamolyticus is a Gram-negative, halophilic microorganism that has a much shorter generation time than the afore mentioned microorganisms (Beuchat, 1974), requiring high amounts of salt to survive. It is known to contaminates shellfish in coastal ocean waters (Baross & Liston, 1970; Bartley & Slanetz, 1971; Kaneko & Colwell, 1973). The average generation time of *V. paraheamolyticus* is 12-14 minutes as compared to *E. coli* that has an average generation time of 16 to 17 minutes at the optimal growth temperature of 37 °C (Mason, 1935; Ulitzur, 1974). Seafood such as oysters, clams, crab and other similar crustaceans are the leading cause of outbreaks and the main carrier of the pathogenic forms consumed by humans; cross-contamination is the second leading cause (Beuchat, 1982). The foodborne outbreaks caused by this microorganism are reported worldwide; however, it remains a significant issue in Asian countries such as Japan and Taiwan (Lin & Scharwtz, 2003). Of the 118 foodborne outbreaks reported in Taiwan in 2000, 84 were linked to *V. paraheamolyticus* (Anoymous, 2001). In the U.S. there was an outbreak in 2013 associated with this microorganism due to consumption of shellfish from various Atlantic harvesting zones that affected over 100 people, while 6 were hospitalized (CDC, 2013).

Saccharomyces cerevisiae

This specific strain of yeast is often referred to as bakers' or brewers' yeast due to its strong fermentation abilities and acid tolerance; it is most commonly used in bread, beer, and wine production, as well as other various fermented foods (Jay, Loessner, & Golden, 2005f). The main functions during fermentation are production of alcohol, formation of aroma, simulating LAB, addition of nutrients, and inhibiting mold growth (Jespersen, 2003). It is rarely responsible

for spoilage in a wide variety of foods (Jay et al., 2005f); however, it has been linked to spoilage of fruit juices and can lead to over fermentation of beverages (Parish, 1991). It has been shown that contamination of the juice products can be linked to yeast present on the outside of the fruits during processing (Iqbal et al., 2016).

2.3 Application of plant extracts in the food industry

There is currently very little use of plant extracts in the food industry as a means of food safety, but there is use of them as a source of added antioxidants (Basaga, Tekkaya, & Acikel, 1997; Campo, Amiot, & Nguyen-The, 2000). For example, rosemary, a common household spice, contains a great deal of powerful phenolic compounds. The antioxidant properties of this extract have been linked to food preservation and have been investigated as an antimicrobial source in foods (Campo et al., 2000; Moreno, Scheyer, Romano, & Vojnov, 2006)

Advantages and disadvantages

The medicinal and therapeutic properties of plants against major diseases have been established for centuries, thus indicating the potential uses for various plants as sources of alternative and natural antimicrobials. Botanicals in food products are a continuously rising trend in the food industry. By adding botanicals to foods as preservatives, they may not only preserve the food but also have beneficial health effects for consumers (Negi, 2012).

The efficacy of plant extracts as antimicrobials is heavily influenced by common food system factors such as pH, temperature, protein content, lipid concentration, and other naturally occurring components (Ahn, Grun, & Mustapha, 2007). It has been reported that the overall

antimicrobial capability decreases when the plant extract is added to a food system (Stechini, Giavedoni, Sarais, & Lerici, 1993; Shelef, 1984; Pandit & Shelef, 1994; Ahn et al., 2007).

There is great interest in the further investigation of indigenous plants in various regions of Africa; however, the means and resources are very limited for them to do so. There are several key challenges they are facing, such as, lack of infrastructure to support the researchers and farmers, as well as the way the plant parts are sold in local markets. There is very little support which is leading to a major lack of quality and standardization of these plants (Simon et al., 2007). If proper means and resources were provided, further research into the antimicrobial potential of indigenous plants could provide an opportunity for the rest of the world to benefit from the biologically diverse continent and for communities to engage in global trade.

Future Research

There is a need for standardized methods for determination of MICs of plant extracts (Klancnik, Piskernik, Jersek, & Mozina, 2010). When these standard methods are established, they need to be rapid, accurate, reproducible and inexpensive to best replicate results for many different types of extracts and microorganisms of interest (Hostettuman, Wolfender, & Rodroguez, 1997; Klancnik et al., 2010). It must be noted that colorimetric methods are not effective for testing MIC of crude plant extracts that are highly pigmented, thus, other means of identification must be considered. It is very important for toxicity studies to be conducted after the MIC is determined to investigate whether the plant extract is toxic to humans or animals, if such has not already been determined (Gatsing et al., 2010). It has also been stated in the literature that identifying, isolating, and extracting the key bioactive ingredients would greatly increase the efficacy of the extract (Doherty et al., 2010). There is very limited data and research

of the application of plant extracts as antimicrobials in food systems, thus continued research is necessary (Klancnik et al., 2010), specifically in precooked meats (Ahn et al., 2007). By further investigating plant extracts as natural antimicrobials, there is potential to further develop them into applicable antimicrobials to help reduce foodborne illness, spoilage, and meet consumer needs.

Chapter 3 Materials and Methods

3.1 Plant material and preparation of extracts

The plants used were: *Alchornea cordifolia, Senna alata, Psidium guajava, Cryptolepis sanguinolenta, Solanum torvum, Piper guineense,* and *Aframomum melegueta*. The extracts were obtained from the Centre for Scientific Research into Plant Medicine in Mampong, Ghana. Plants were collected from forests in the Eastern Region of Ghana and identification of the plants was performed by the curator of the herbarium of Centre for Plant Medicine Research, Mampong Akuapem, Ghana.

Preparation of ethanolic (E) and methanolic (M) extracts

The plant was washed thoroughly, chopped into pieces, air-dried, and milled into a coarse powder. The powdered plant matter was macerated in 70% v/v ethanol or 100% v/v methanol with periodic stirring, decanted after 72 hours, and filtered. The ethanol was evaporated on a rotary evaporator and the aqueous concentrated extract freeze-dried to obtain a powdered extract. This was performed by the Centre for Scientific Research into Plant Medicine. Next, they were shipped to Auburn University and stored at -20 °C until used.

Extract Preparation

Stock solutions were prepared by combining the dry extract with dimethyl sulfoxide (DMSO; Macron Find Chemicals, Center Valley, PA) to achieve a final concentration of 20% (w/v). Stock solutions were made as needed for each plant extract and stored at 4 °C.

3.2 Bacterial and fungal strains and inoculum preparation

The microorganisms used were obtained from frozen stock at the Department of Poultry Science food safety labs at Auburn University, which were collected in premade vials containing glycerol and beads and kept at -80 °C. The microorganisms used for microbial assays were strains of *Listeria monocytogenes, Escherichia coli, Salmonella* spp.*, Staphylococcus aureus, Enterococcus faecalis*, *Vibrio parahaemolyticus*, *Lactobacillus fermentum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. All cultures were grown in 9.0 mL of their specified growth media (Table 3.1) and incubated at their specific time and temperature. Cultures were grown in broth and plated on their specified media to determine the initial concentration of each bacterial strain before use in experiments. This protocol was performed three times and the average population of inoculated broth was estimated.

Table 3.1 Organism identification and growth parameters

Microorganism	Strain Identification	Growth Medium	Time (h)	Temperature (°C)
Listeria monocytogenes	ATCC 19115 ATCC 7644	Tryptic Soy Broth (TSB) Tryptic Soy Agar (TSA)	18-24	32
Escherichia coli	ATCC BAA 2196 ATCC BAA 2210 ATCC 5053 O157:H7 AU2 301	TSB, TSA	18-24	37
Salmonella	Heidelberg Typhimurium	TSB, TSA	18-24	37
Staphylococcus aureus	ATCC 27664	TSB, TSA	18-24	37
Enterococcus faecalis	ATCC 29212	TSB, TSA	18-24	37
Vibrio parahaemolyticus	VP12	Blood Heart Infusion (BHI) broth + 3% NaCl Marine Agar	18	37
Lactobacillus fermentum	ATCC 14932	De Man, Rogosa, and Sharpe (MRS) broth MRS+ agar powder	24	37 (anaerobically)
Bacillus subtilis	ATCC 6633	Muller-Hinton (MH) broth MH + agar powder	24	37
Saccharomyces cerevisiae		yeast extract peptone dextrose (YPD) broth YPD + agar powder	48	32

3.3 Inhibition zone assay

Using an inhibition zone assay, all methanolic and ethanolic plant extracts were evaluated against the microorganisms *L. monocytogenes, E. coli,* and *Salmonella* spp. Each bacterial strain was grown by using a loop to streak an isolated colony from a stock tryptic soy agar (TSA; Hardy Diagnostics, Santa Monica, CA) plate and added to tryptic soy broth (TSB; Neogen, Lansing, MI). The cultures were incubated at 37 °C for 24 h, except for *Listeria monocytogenes* that was incubated at 32 °C for 24 h. The inocula were serially diluted in 0.1% peptone water (Neogen) to achieve approximately 10^6 CFU/mL and were spread onto 18 mL TSA plates, creating a bacterial lawn, then dried, and 6.0 mm diameter wells were made in agar using a sterile cork borer. A volume of 50 μL was added to a well for each of extracts at concentrations of 20%, 10% and 5% (w/v). There were two antibiotic controls, nalidixic acid and trimethoprim, and one solvent control sample, DMSO. Plates were incubated at 32 °C for *L. monocytogenes* and 37 °C for *E. coli* and *Salmonella* for 24 h. The diameter of the inhibition zone was measured in mm. This protocol was performed three times total.

3.4 Effect of plant extracts on microbial growth

The descriptive screening method, an inhibition curve test, was used to determine the antimicrobial activity of ethanolic plant extracts [*P. guajava*, *C. sanguinolenta,* and *S. alata*], which were selected based on their antimicrobial activity in the previous screening assay. Inoculated broths of *E. coli*, *Salmonella* Typhimurium, *S. aureus, E. faecalis*, *V. parahaemolyticus, L. fermentum, B. subtilis,* and *S. cerevisiae* were serially diluted in broth to achieve an approximant population 10^5 CFU/mL, which was then combined with each individual extract at a concentration of 2.5% (w/v) to make a 2-mL final working volume sample. A control

sample contained the inoculum and 10% or 5% DMSO in broth. The concentration of DMSO used for the control samples was determined based on the sensitivity of each microorganism to the dissolving agent. All inoculated samples were incubated for 18 - 24 h at 37 °C [48 h at 32 °C for *S. cerevisiae*]. Samples were taken at 0, 4, 8, and 24 h [*E. coli*, *Salmonella* Typhimurium, *S. aureus, E. faecalis, L. fermentum, B.* subtilis], 0, 2, 4, 6, and 18 h [*V. parahaemolyticus*], and 0, 6, 12, 18, 24, 36, and 48 h [*S. cerevisiae*] and were serially diluted in 0.1% peptone water, spread on specified agar plates, incubated for the specified time and temperature (Table 3.1), and log colony forming units (CFU) per mL was determined. This protocol was performed three times total.

3.5 Minimum inhibitory concentration (MIC) assay

The MIC of *P. guajava* and *C. sanguinolenta* were determined using a macrodilutions assay which tracked inhibition over time. Various concentrations of the extracts, ranging from 0.025% to 2.0% (w/v), were tested against *E. coli* ATCC BAA 2196 and *Salmonella* Typhimurium inocula at an initial population of approximately 10^5 CFU/mL. Samples were made by combing each inoculum with the various plant extract concentrations to achieve a final working volume of 2-mL and incubated at 37 °C for 24 h. DMSO control samples (10% (w/v)) without plant extracts were used to observe uninhibited bacterial growth. Samples were taken at 0, 8 and, 24 h, diluted in 0.1% peptone water, spread plated onto TSA, incubated for 24 h, and log CFU/mL was determined. The MIC against each microorganism was determined based on the concentration at which the microorganism exhibited <1.0 log CFU/mL growth over 24 h. This protocol was performed in duplicate three times total.

3.6 Effect of pH on antimicrobial activity of plant extracts

The antimicrobial effects of concentrations of 0.75% (w/v) *P. guajava* and 0.25% (w/v) *C. sanguinolenta* against *E. coli* ATCC BAA 2196 and *Salmonella* Typhimurium were evaluated in TSB at an adjusted pH of 4.5, 5, and 6, using 1 N HCl (EDM Millipore Coa., Billerica, MA). Unaltered pH of TSB was approximately 7.80. Concentrations of extracts were selected as less than the MIC to observe the efficacy of plant extracts against observable populations of microorganisms. Inoculated cultures were serially diluted in TSB and pH altered TSB to achieve the approximate population of 10^5 CFU/mL. The plant extracts were added to the inoculated broth for a final working volume of 2-mL and incubated at 37 °C for 24 h. Controls for each pH contained 20% DMSO and no plant extract. Samples were evaluated at 0 and 24 h, serially diluted in 0.1% peptone water, spread plated on TSA plates, incubated at 37 °C for 24 h, and log CFU/mL was calculated. This protocol was performed in duplicate three times total.

3.7 Antimicrobial efficacy of plant extracts in milk

The antimicrobial effects of 1.00% (w/v) *P. guajava* and 0.50% (w/v) *C. sanguinolenta* against *E. coli* ATCC BAA 2196 and *Salmonella* Typhimurium were evaluated in milk (UHT 2% reduced fat milk). Concentrations of plant extract were chosen at the MIC values to determine if the food system would affect antimicrobial activity of the extracts. Dilutions were performed in milk to achieve the approximate population of 10^5 CFU/mL. The extracts were added to the inoculated milk for a final working volume of 4-mL and incubated at 22 °C for 24 h. Inoculated milk without any extract and un-inoculated milk (to determine background microflora levels) were used as control samples. Samples were taken at 0, 4, 8, 24, 48, and 72 h,

serially diluted in 0.1% peptone water, spread plated on TSA plates incubated at 37 °C for 24 h, and log CFU/mL was calculated. This protocol was performed in duplicate three times total.

3.8 Statistical Analysis

Data was analyzed using SAS software (version 9.4, SAS Institute, Cary, NC) by using an analysis of variance (ANOVA), indicating the significance of multiple means from one large data set (Abdi & Williams, 2010). The means were analyzed both by treatment and by hour using the Student-Newman-Keuls (SNK) test which can indicate the significance of the different treatments and sampling hours, based on a 95% confidence level.

Chapter 4 Results and Discussion

4.1 Inhibition zone assay

The antibacterial properties of the selected West African plant extracts were assessed using an inhibition zone assay based on our methods previously described. Extract concentrations of 20% (200 mg/mL), 10% (100 mg/mL), and 5% (50 mg/mL) were tested against *L. monocytogenes, E. coli* and *Salmonella* spp. to determine their effectiveness as antimicrobial agents. Methanolic and ethanolic plant extracts were selected for this experiment based on their ability to properly dissolve in DMSO (Table 4.1). Based on our methods, the results of this experiment indicate that the ethanolic extracts *C. sanguinolenta*, *P. guajava*, and *S. alata* at a concentration of 20% exhibited the greatest antimicrobial potential against *Salmonella* and *E. coli*. None of the extracts were effective against the Gram-positive bacterium, *L. monocytogenes*. The results of this experiment show more antimicrobial effectiveness for ethanolic plant extracts vs. methanolic extracts which was exhibited by the inactivity of methanolic extracts of *S. alata* and *P. guajava* compared to the antimicrobial activity displayed by ethanolic extracts of the same plants (Table 4.1). Results in scientific literature have found methanolic extracts to be effective based on similar procedures to this experiment. When Doughari & Okafor (2007) tested methanolic extracts of *S. alata* leaves and Abdelrahim, Almagboul, Omer, & Elegami (2002) evaluated methanolic extracts of *P. guajava* bark, they found the extracts to be effective against E. coli and Salmonella, similar to what was found with the ethanolic extracts of these plants used in our study. The results of these experiments in

comparison to those found in scientific literature vary due to extraction solvent used (Cowan, 1999), the region and season cultivation of plants took place, and extraction methods performed (Othman et al., 2011). Antimicrobial activity of an ethanolic extract of *C. sanguinolenta* from Ghana was evaluated in a study performed by Mills-Robertson and others (2012). They found the plant extract to be active against *E. coli*; however, it did not exhibit antimicrobial activity against *Salmonella* spp., like the results found in this study.

Currently published research investigating the effect of these West African plant extracts were performed against bacterial and fungal microorganisms typically associated with medical research. There is a lack of research testing their antimicrobial properties against microorganisms associated with food, thus leading to further screening of their effectiveness against a variety of microorganisms associated with foodborne illness and spoilage in the following experiments.

Table 4.1 Zones of inhibition (mm) of growth of foodborne pathogens[a] by African plant extracts

		L. monocytogenes		*E. coli*				*Salmonella*	
Controls and Plant Extract	**Concent -ration % (w/v)**	ATCC 19115	ATCC 7644	ATCC BAA 2196	ATCC BAA 2210	ATCC BAA 5053	O157:H7 AU2 301	Heidelberg	Typhimurium
Trimethoprim	4%	36±0.00[b]	26±0.00	27±0.71	27±0.71	30±2.83	32±2.83	n.i.	31±0.71
Nalidixic acid	4%	n.i.[c]	16±0.00	21±0.71	24±0.00	n.i.	19±2.12	n.i.	n.i.
Dimethyl sulfoxide	70%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Psidium guajava (M)[d]	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	20%	12±0.00	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Senna alata (M)	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	20%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Aframomum melegueta (E)[e]	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	20%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Piper guineense (E)	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	20%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Solanum torvum (M)	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	20%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Psidium guajava (E)	5%	n.i.	n.i.	8±0.00	n.i.	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	10±1.41	n.i.	n.i.	n.i.	8±0.00	n.i.
	20%	n.i.	n.i.	12±1.41	10±0.00	10±0.00	n.i.	15±0.71	n.i.
Cryptolepis sanguinolenta (E)	5%	n.i.	n.i.	8±0.00	8±0.00	n.i.	n.i.	n.i.	n.i.
	10%	n.i.	n.i.	8±0.00	11±2.12	n.i.	8±0.00	n.i.	n.i.
	20%	n.i.	n.i.	11±0.71	10±0.00	10±0.00	12±0.00	n.i.	n.i.
Senna alata (E)	5%	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	10±0.00	n.i.
	10%	12±0.00	n.i.	8±0.00	n.i.	n.i.	n.i.	11±0.71	8±0.00
	20%	12±0.00	n.i.	9±0.71	10±0.00	n.i.	12±0.00	14±0.00	10±0.00

[a]Obtained by adding 50 μL of each dilution of antimicrobial substance to each 6 mm well.
[b]Values are mean ± standard deviation, diameter of the halo was measured.
[c]n.i.= No zone of inhibition was observed
[d]M = 100% Methanol extraction
[e]E = 70% Ethanol extraction

4.2 Effect of plant extracts on microbial growth

Based on the results of the previous experiment, the ethanolic extracts of *C. sanguinolenta*, *P. guajava*, and *S. alata* were further assessed against a variety of foodborne microorganisms. A descriptive screening method was used to evaluate the antimicrobial activity of the plant extracts over time against the selected bacteria and yeast strains. *E. coli* ATCC BAA 2196 and *Salmonella* Typhimurium from the previous experiment were used, as well as *V. parahaemolyticus, S. aureus, E. faecalis, L. fermentum, B. subtillis,* and *S. cerevisiae* (Figures 4.1-4.8). The results of this study, based on the methods performed in this experiment, show *C. sanguinolenta*, *P. guajava*, and *S. alata* inactivated *E. coli* (Figure 4.1)*, Salmonella* Typhimurium (Figure 4.2)*, V. parahaemolyticus* (Figure 4.3)*,* and *S. aureus* (Figure 4.5) to below the detection limit of <1.00 Log CFU/mL after 24 h of incubation. A study performed by Lutterodt and others (1999) showed that *V. cholera,* another strain of foodborne illness causing vibrionosis*,* is highly susceptible to the extract from *P. guajava.*

The results of this experiment show inactivation of *S. aureus* to below detection limits after 4 h by *C. sanguinolenta* (Figure 4.5). There is an alkaloid present in *C. sanguinolenta* called cryptolepine, thought to be the most potent alkaloid present in this plant extract and is the main indoquinoline alkaloid present in the extract (Mills-Robertson et al., 2009; Sawer, Berry, & Ford, 2005). Sawer and others (2005) performed an experiment testing the isolated alkaloid cryptolepine at concentrations of 10, 20, and 40 μg/mL against *S. aureus*, resulting in decreased cell levels from an initial population of 6 log CFU/mL to between 2 – 3 log CFU/mL by 5 h at 37 °C. Cryptolepine is thought to decrease the population of microorganisms by cell lysis and change of cell morphology (Mills-Robertson et al., 2009; Sawer et al., 2005). Previous studies have also found early inactivation of microorganisms by cryptolepine at concentrations of 40

μg/mL and 160 μg/mL*,* showing bactericidal and fungicidal effects on *E. coli* by 5 h and *S. cerevisiae* after 20 h, respectively (Sawer, Berry, Brown, & Ford, 1995). However, in this study, *C. sanguinolenta* did not have a fungicidal effect on *S. cerevisiae* after 48 h treatment, but was able to decrease cell populations by approximately 1 log CFU/mL by 48 h (Figure 4.4). This result could be due to the lower concentrations of cryptolepine present in this extract or that the alkaloid is more potent when extracted and separated from the other constituents. Based on the results from all eight tested microorganisms, *C. sanguinolenta* and *P. guajava* exhibited a stronger antimicrobial effect than *S. alata*, overall. The most effective extract tested was *C. sanguinolenta,* being able to inactivate four [*Salmonella, V. parahaemolyticus, S. aureus, and L. fermentum*] of the eight microorganisms tested to below detectable limits of < 1.00 log CFU/mL after 8 h treatment.

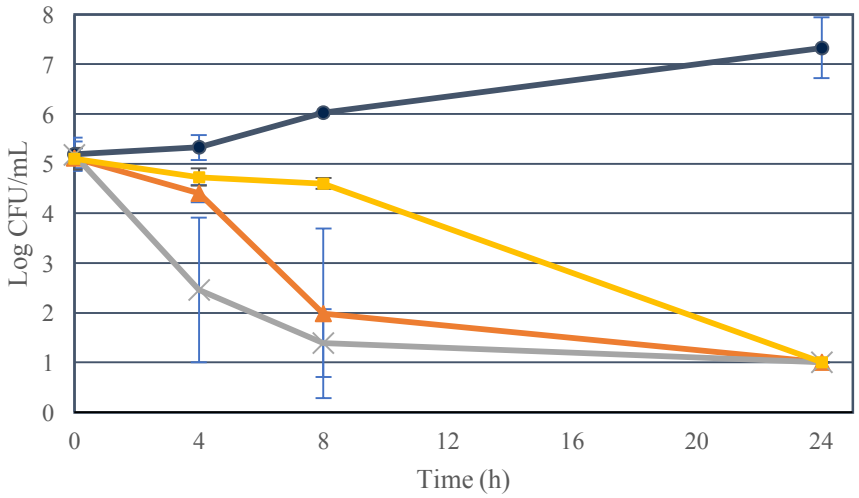

Figure 4.1 Effect of 10% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *E. coli* ATCC BAA 2196 at 37 °C for 24 h in TSB

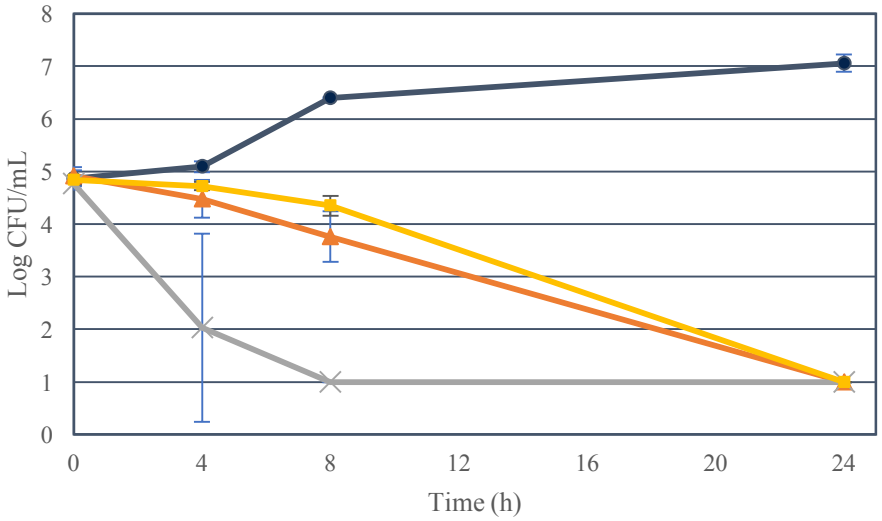

Figure 4.2 Effect of 10% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *Salmonella* Typhimurium at 37 °C for 24 h in TSB

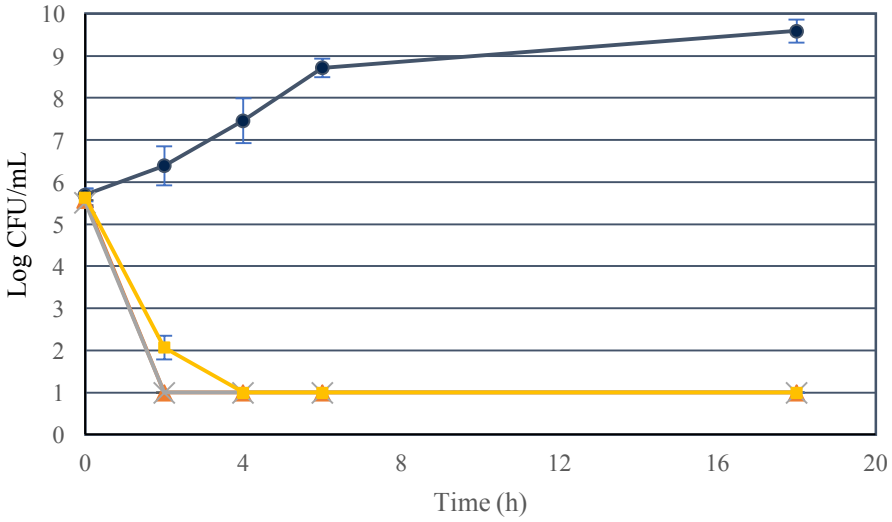

Figure 4.3 Effect of 5% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *V. parahaemolyticus* VP12 at 37 °C for 18 h in MH broth + 3% NaCl

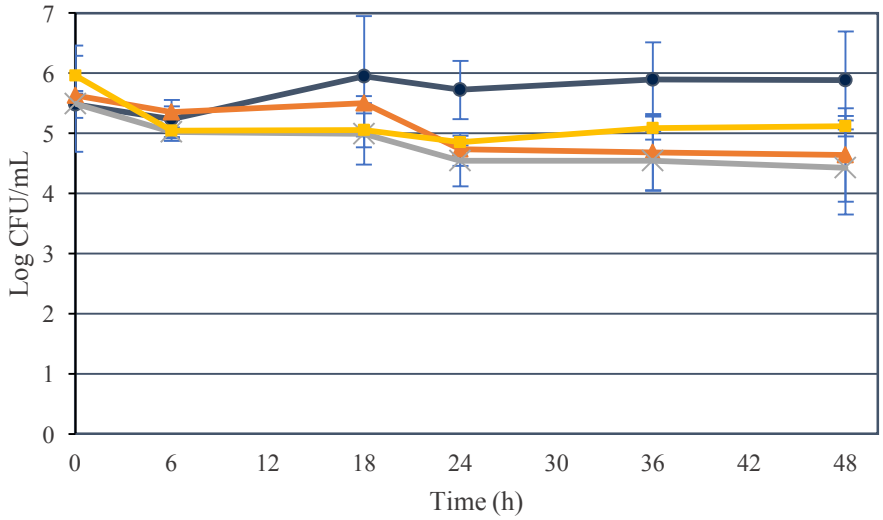

Figure 4.4 Effect of 5% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *S. cerevisiae* for 32 °C for 48 h in YPD broth

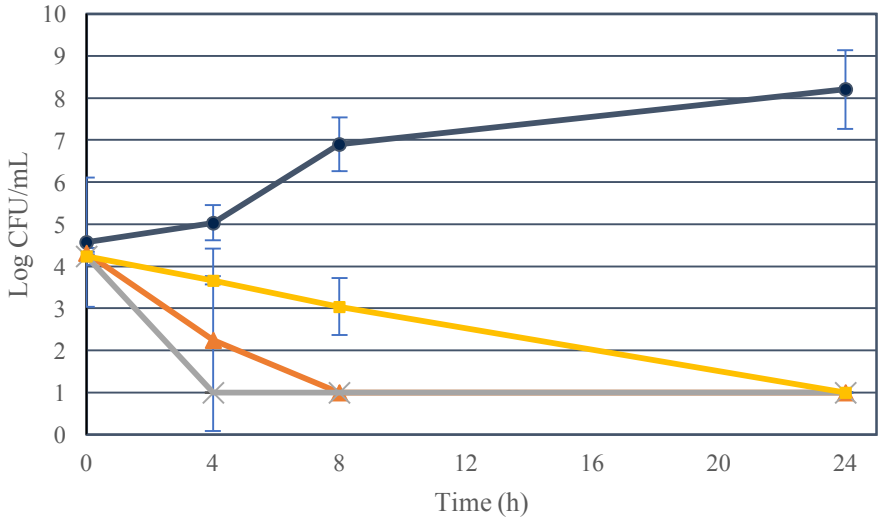

Figure 4.5 Effect of 10% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *S. aureus* ATCC 27664 at 37 °C for 24 h in TSB

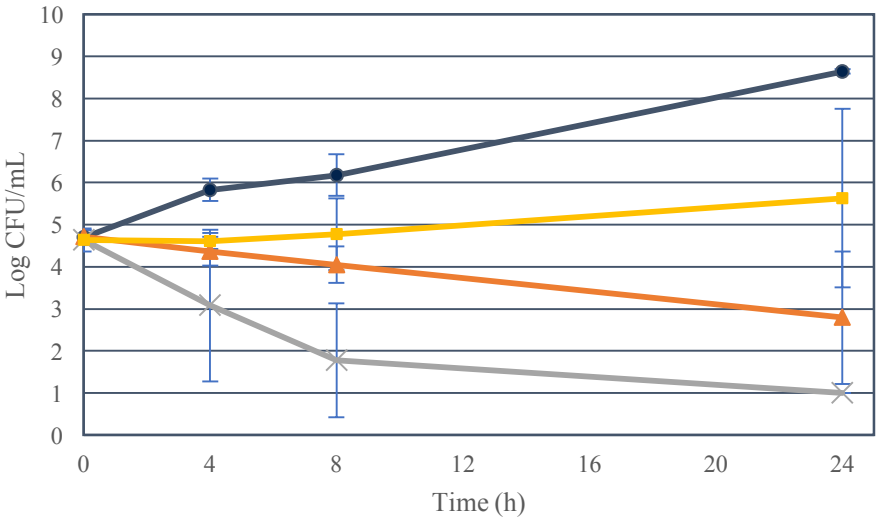

Figure 4.6 Effect of 10% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *E. faecalis* ATCC 29212 at 37 °C for 24 h in TSB

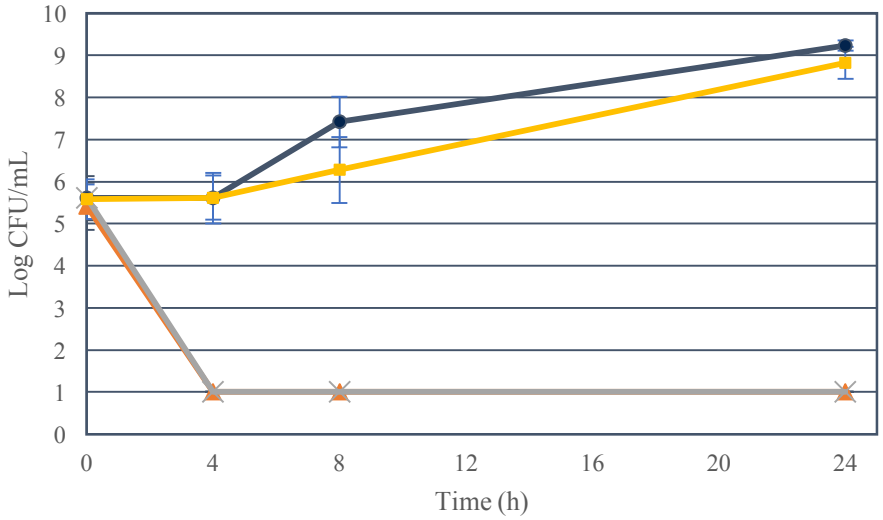

Figure 4.7 Effect of 5% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *L. fermentum* ATCC 14932 at 37 °C for 24 h in MRS broth

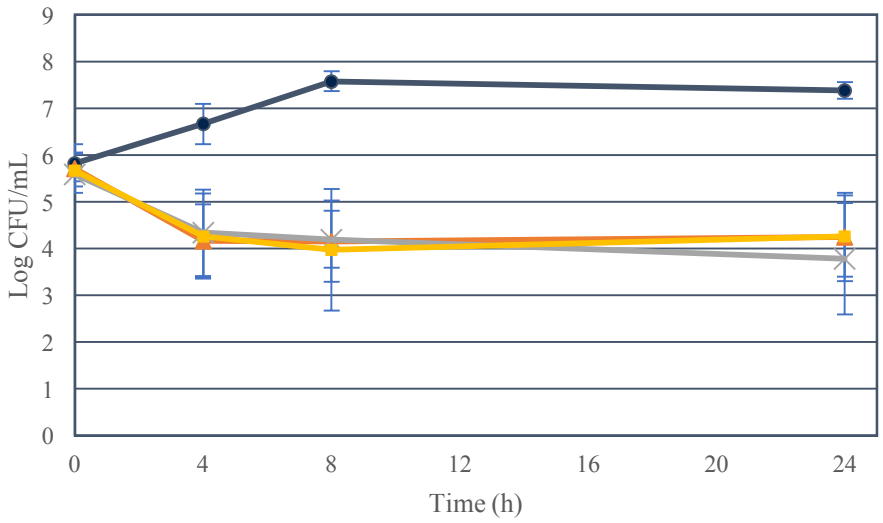

Figure 4.8 Effect of 5% DMSO (●), 2.5% (w/v) *P. guajava* (▲), 2.5% (w/v) *C. sanguinolenta* (x), and 2.5% (w/v) *S. alata* (■) on *B. subtilis* ATCC 6633 at 37 °C for 24 h in BHI broth

4.3 Minimum inhibitory concentration (MIC) assay

Before testing the antimicrobial efficacy in acidic conditions or in a model food system, the MIC under optimal growth conditions in microbiological media was determined for the plant extracts *P. guajava* and *C. sanguinolenta* against *E. coli* and *Salmonella.* The MIC was defined as the concentration of antimicrobial at which the microorganism expressed less than one log of growth after 24 h of incubation. The results (Table 4.2) show that *P. guajava* against *Salmonella* and *E. coli* had an MIC of 1.00% (w/v) [10 mg/mL] and *C. sanguinolenta* against *Salmonella* and *E. coli* had an MIC of 0.50% (w/v) [5 mg/mL], after 24 h at 37 °C using the macrodilutions technique (Figures 4.9-4.12).

A study performed by Mills-Robertson and others (2012) evaluated the MIC of *C. sanguinolenta* against *E. coli* and found it to be 32 mg/mL. The extract used in that study was an ethanolic root extract, which is the same as used in this study. Differences in the MIC are most likely due to the phytochemical composition of these extracts based on geographic location, collection and extraction methods specific to each plant extract (Othman et al., 2011). It has been indicated that the potent compound, cryptolepine, is greatly responsible for the strong antimicrobial properties of *C. sanguinolenta* by inhibiting DNA synthesis of cells (Sawer et al., 1995). Thus, different cryptolepine concentrations would affect relative antimicrobial activity.

Ethanolic extracts of leaves, stems, and roots from *P. guajava*, in a study performed by Sanches, Cortez, Schiavini, Nakamura, & Filho (2005), were found to have an MIC against *E. coli* of greater than 1 mg/mL. In contrast, in this study the MIC of the bark extract of *P. guajava* was found to be 10 mg/mL, ten times greater than the concentration indicated in the previously mentioned study. Results in the scientific literature stated that the leaf extracts of *P. guajava* collected in Bangladesh have been found to be inactive against *Salmonella* and *E. coli* strains

(Hoque, Bari, Inatsu, Juneja, & Kawamoto, 2007). Different phytochemicals are present at varying concentrations throughout the plant and are dependent upon the region and conditions it is grown in (Othman et al., 2011). The bark has been shown to possess greater antimicrobial properties against stains of *E. coli* and *S. aureus* (Abdelrahim, Almagboul, Omer, & Elegami, 2002).

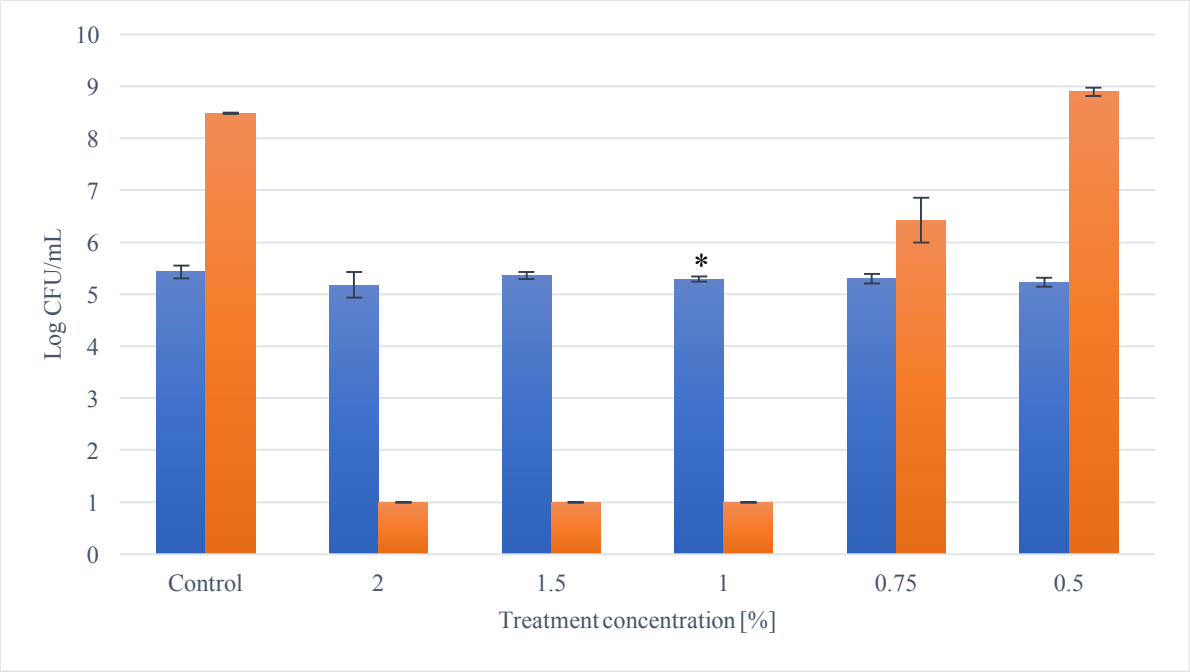

Figure 4.9 Antimicrobial activity of *P. guajava* at various concentrations against *Salmonella* Typhimurium at 37 °C in TSB for [■] 0 h and [■] 24 h to determine MIC, $n = 6$
* Indicates MIC

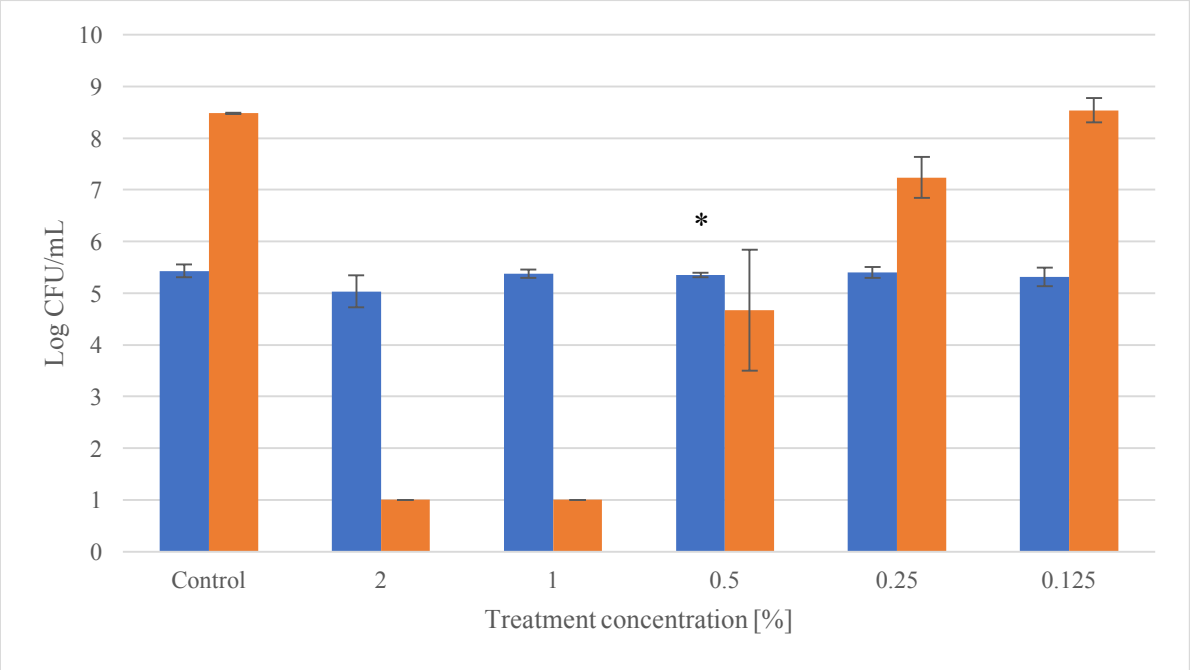

Figure 4.10 Antimicrobial activity of *C. sanguinolenta* at various concentrations against *Salmonella* Typhimurium at 37 °C in TSB for [■] 0 h and [■] 24 h to determine MIC, $n = 6$
* Indicates MIC

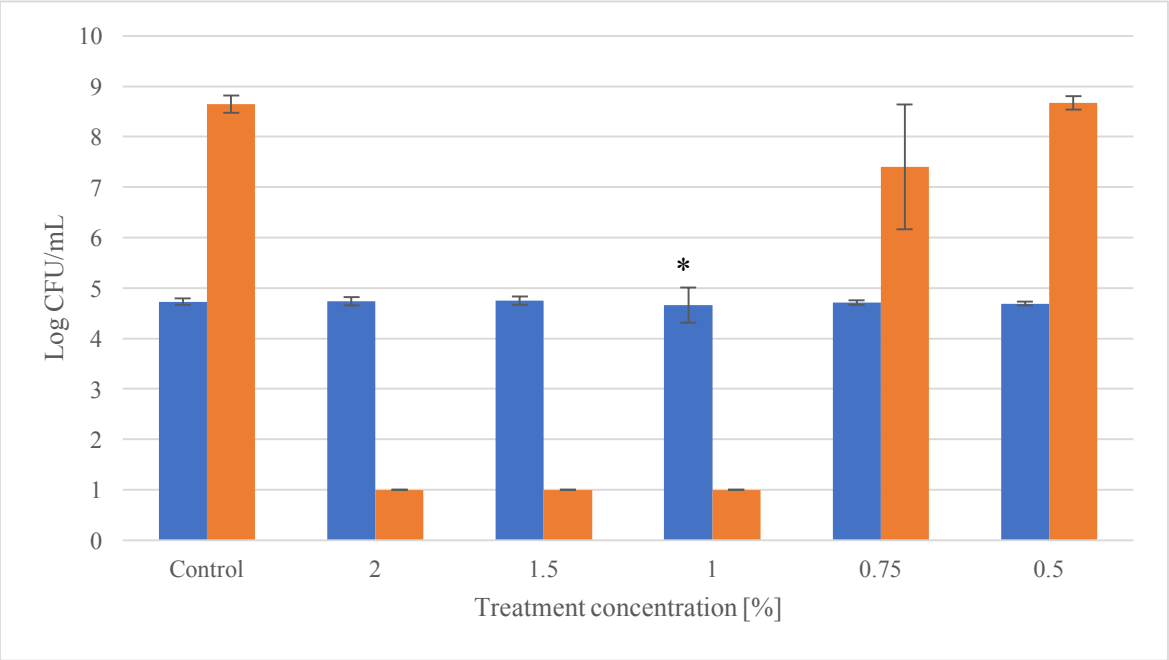

Figure 4.11 Antimicrobial activity of *P. guajava* at various concentrations against *E. coli* ATCC BAA 2196 at 37 °C in TSB for [■] 0 h and [■] 24 h to determine MIC, $n = 6$
* Indicates MIC

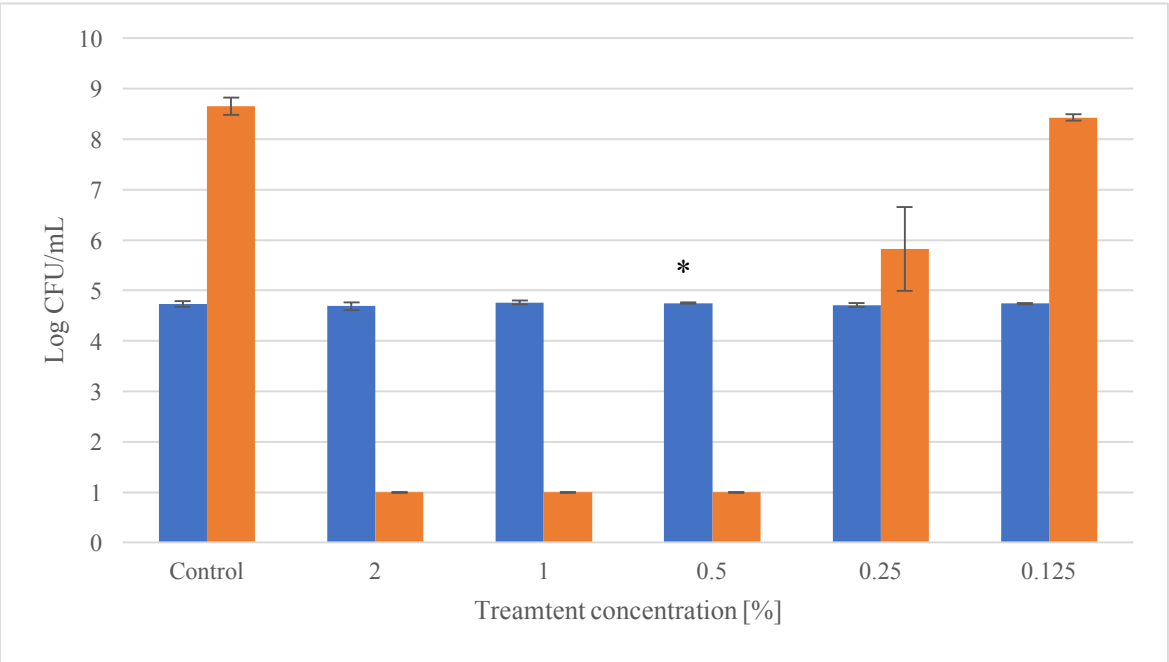

Figure 4.12 Antimicrobial activity of *C. sanguinolenta* at various concentrations against *E. coli* ATCC BAA 2196 at 37 °C in TSB for [■] 0 h and [■] 24 h to determine MIC, $n = 6$
* Indicates MIC

Table 4.2 MIC[a] values for *P. guajava* and *C. sanguinolenta* against *Salmonella* Typhimurium and *E. coli* ATCC BAA 2196

	Salmonella Typhimurium	*E. coli* ATCC BAA 2196
	% (w/v)	% (w/v)
P. guajava	1.00	1.00
C. sanguinolenta	0.50	0.50

[a] MIC was defined as the concentration of antimicrobial at which the microorganism expressed less than one log of growth after 24 h of incubation.

4.4 Effect of pH on antimicrobial activity of plant extracts

The effect of pH was measured against the known inhibitory properties at concentrations of 0.75% (w/v) *P. guajava* and 0.25% (w/v) *C. sanguinolenta* against *Salmonella* and *E. coli* (Figure 4.13-4.16). The unadjusted pH of the TSB was 7.80. Increased antimicrobial activity was exhibited by 0.75% (w/v) *P. guajava* and 0.25% (w/v) *C. sanguinolenta* against *E. coli* in this experiment than previously seen in the MIC experiment (Figure 4.9, 4.10) due to acquiring a new batch of the freeze-dried plant extract from Africa. As mentioned previously, there can be variation in activity of crude plant extracts. Adjusting the pH to 6, 5, and 4.5 had no effect on the antimicrobial activity of *P. guajava* against *Salmonella*. However, after 24 h of incubation, *C. sanguinolenta* had less of an antimicrobial effect against *Salmonella* (Figure 4.14) at all adjusted pH levels, with initial counts ca. 2 log more than the unaltered control after 24 h of incubation. The antimicrobial efficacy of *P. guajava* and *C. sanguinolenta* against *E. coli* (Figure 4.15, 4.16) was decreased, overall when pH was lowered in comparison to the unaltered samples. When *P. guajava* was applied to *E. coli* the cell population increased by about 4 log at pH 6, there was a 2 log decrease of cells at pH 5, and there was a bacteriostatic effect at pH 4.5 from 0 – 24 h of

incubation. *P. gujava* and *C. sanguinolenta* were the least effective against *E. coli* at pH 6, causing the level of cells to rise to over 8.00 and 6.00 log CFU/mL, respectively, after 24 h of incubation. In previous scientific literature, the effect of pH on the antimicrobial activity of rosemary extract was greater at acidic pH values (Del Campo, Amito, & Nguyen-The, 2000). To further elucidate the effect of pH on the antimicrobial activity, research needs to be performed to further assess the antimicrobial effects of the plant extracts on the growth rates and lag phase of microorganisms (Gutierrez et al., 2008).

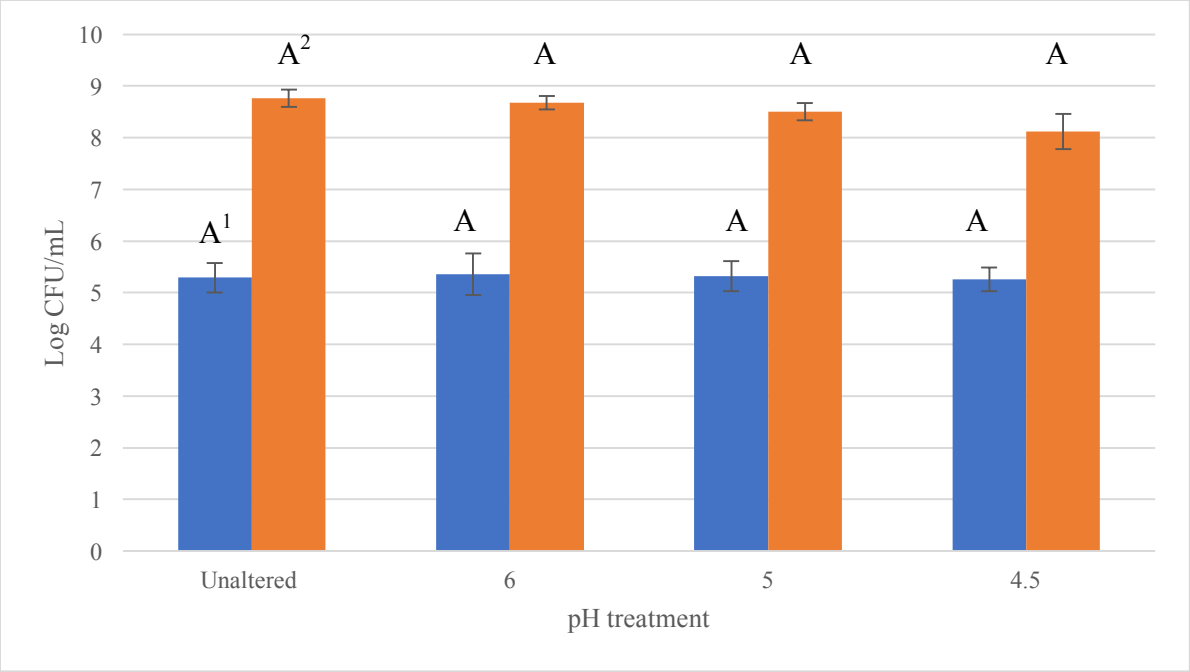

Figure 4.13 Efficacy of 0.75% (w/v) *P. guajava* against *Salmonella* Typhimurium at 37 °C for [■] 0 h and [■] 24 h in TSB at different pH, $n = 6$

[1] Student-Newman-Keuls (SNK) test, ($P \leq 0.05$) indicating significant differences between treatments at 0 h

[2]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 24 h

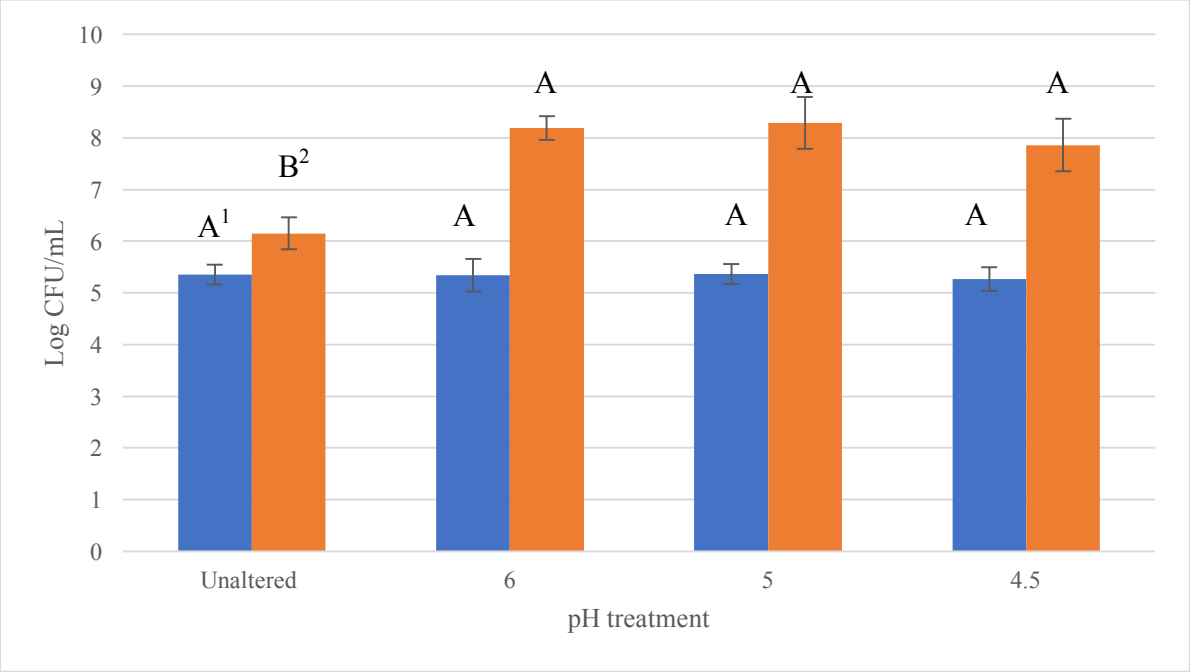

Figure 4.14 Efficacy of 0.25% (w/v) *C. sanguinolenta* against *Salmonella* Typhimurium at 37 °C for [■] 0 h and [■] 24 h in TSB at different pH, $n = 6$

[1]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 0 h

[2]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 24 h

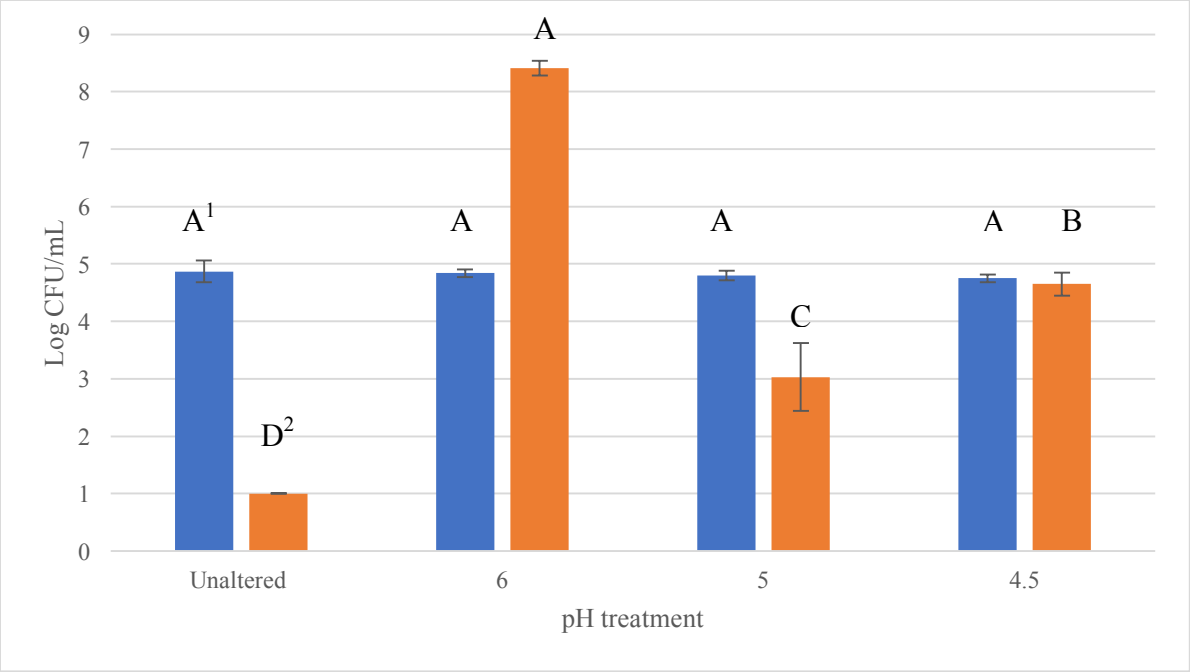

Figure 4.15 Efficacy of 0.75% (w/v) *P. guajava* against *E. coli* ATCC BAA 2196 at 37 °C for [■] 0 h and [■] 24 h in TSB at different pH, $n = 6$
[1]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 0 h
[2]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 24 h

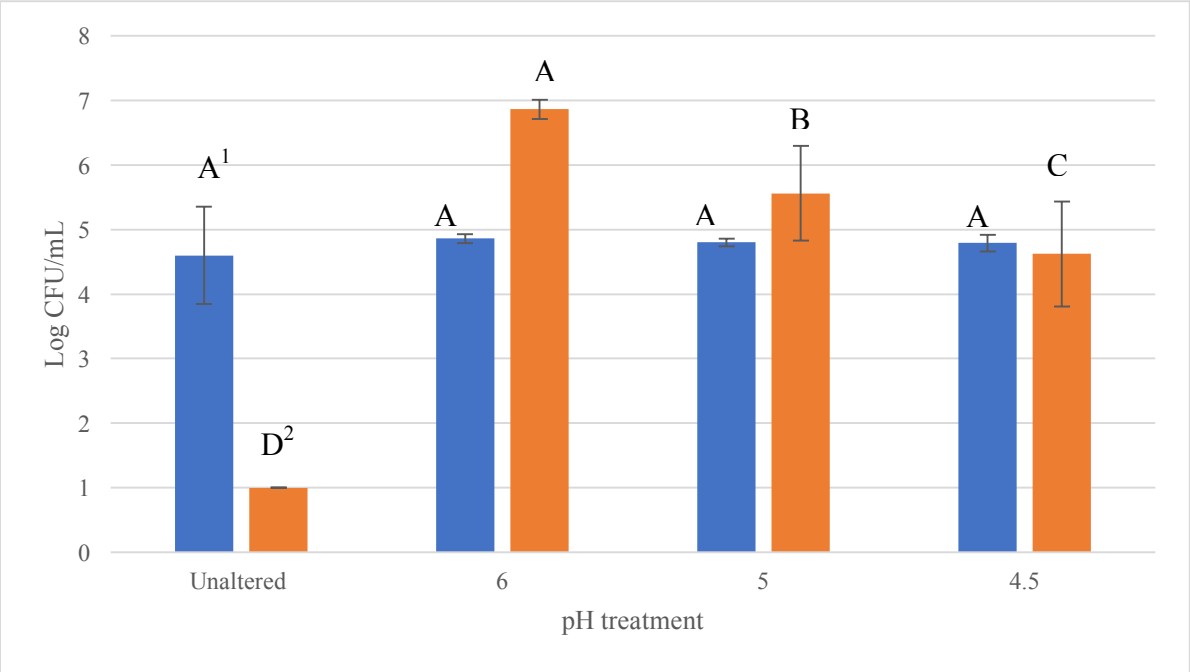

Figure 4.16 Efficacy of 0.25% (w/v) *C. sanguinolenta* against *E. coli* ATCC BAA 2196 at 37 °C for [■] 0 h and [■] 24 h in TSB at different pH, $n = 6$
[1]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 0 h
[2]SNK test, ($P \leq 0.05$) indicating significant differences between treatments at 24 h

4.5 Antimicrobial efficacy of plant extracts in milk

Milk was used as a model food system for this experiment to show the combined interaction of the extracts with the protein, fat, and carbohydrates present in milk (Figure 4.17, 4.18). There was no observed growth in the uninoculated milk control sample for these experiments after 72 h, indicating that there were not significant numbers of background microflora present in the milk (data not shown). *P. guajava* and *C. sanguinolenta* were applied at the MIC values found in microbiological media of 1.00% and 0.50% (w/v); respectively. However, it was expected that the observed antimicrobial activity in milk would be decreased, due to the body of literature that stated higher concentrations of antimicrobials are generally needed to reduce bacterial populations in a complex food system where conditions make phytochemicals less effective at injuring or killing bacterial cells (Higginbotham, Burris, Zivanovic, Davidson, & Stewart, 2014). The complex parts of the food systems such as protein, fat, and carbohydrates, may interact with antimicrobials, and lessen their effectiveness in comparison to their ability to express antimicrobial activity in ideal growth conditions such as nutrient broth. For example, fat content of a food system is often one of the main constituents linked to decreasing antimicrobial efficacy of other natural antimicrobials obtained from plants, such as essential oils (Gutierrez et al., 2008), due to its complexity. Specifically, it has been stated in the scientific literature that the antimicrobial effectiveness clove, cinnamon, and *Picea excelsa* essential oils against *L. monocytogenes* were decreased in high fat dairy samples (Canillac & Mourey, 2004; Cava, Nowak, Taboada, & Marin-Iniesta, 2007). Mint oil has been shown to have less of an antibacterial effect against *S. enteritidis* in food systems with high fat content (Tassou, Drosinos, & Nychas, 1995).

The results of this experiment in milk agree with the assertions of decreased antimicrobial activity in food systems (Figures 4.9-4.12). Compared to the MIC values for these plant extracts against *Salmonella* and *E. coli* in TSB at 37 °C, the ideal conditions for bacterial growth (in the milk at 22 °C) the plant extracts exhibited decreased antimicrobial activity. *P. guajava* exhibited bacteriostatic antimicrobial activity against *E. coli* and continued to keep levels of *Salmonella* less than that of the control from 8 - 72 h at 22 °C. The antimicrobial activity of *C. sanguinolenta* remained significantly less than the control from 8- 72 h at 22 °C against both microorganism, but was not as effective as *P. guajava* at MIC values determined in microbiological media at ideal conditions. In addition, after 48 h, the number of *Salmonella* cells was only <1.00 log CFU/mL lower than that of the control. The results of this experiment suggest that *P. guajava* may be effective for extending shelf life of products like the one tested.

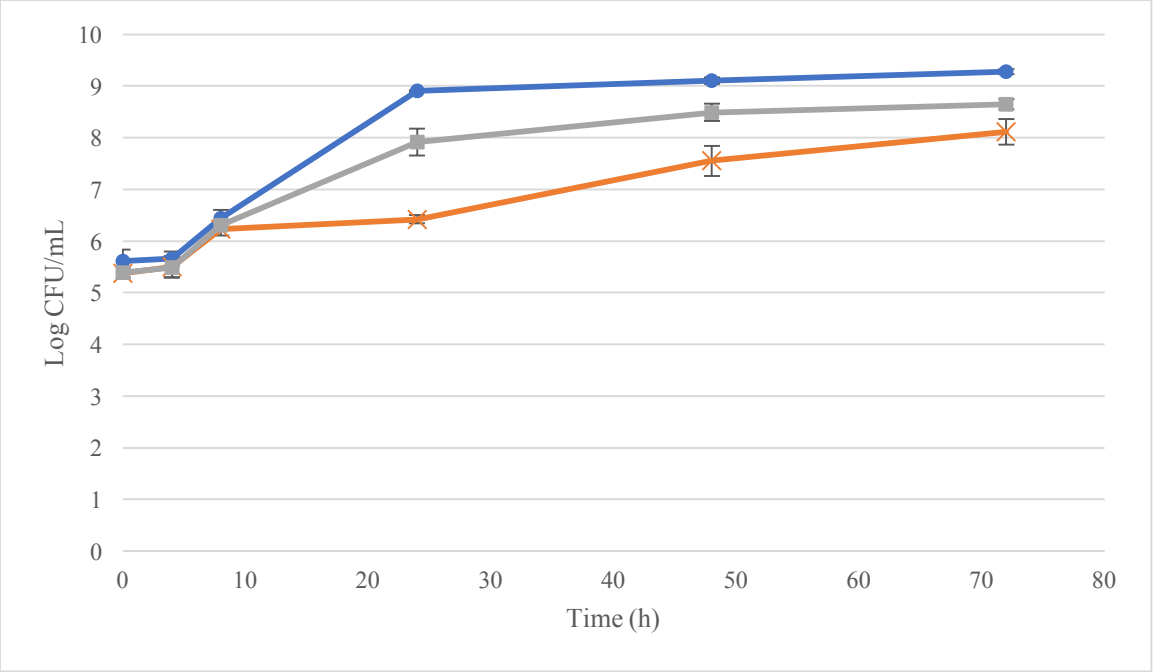

Figure 4.17 Efficacy of 1.00% (w/v) *P. guajava* (X), 0.50% (w/v) *C. sanguinolenta* (■), and Control (●) against *Salmonella* Typhimurium in milk at 22 °C for 72 h, $n = 6$

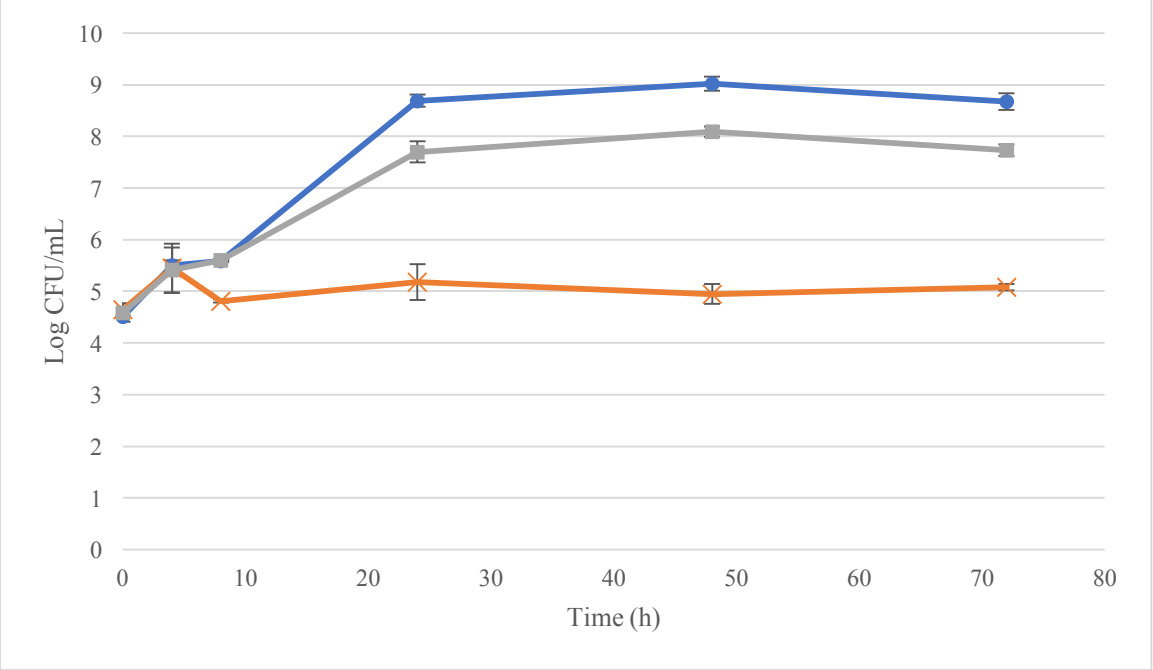

Figure 4.18 Efficacy of 1.00% (w/v) *P. guajava* (X), 0.50% (w/v) *C. sanguinolenta* (■), and Control (●) against *E. coli* ATCC BAA 2196 in milk at 22 °C for 72 h, $n = 6$

Chapter 5 Conclusions

The purpose of this study was to evaluate the antimicrobial potential of seven ethanolic and seven methanolic extracts of plants obtained from West Africa against a variety of foodborne microorganisms. After general screenings of antimicrobial potential of the plant extracts against foodborne pathogens and spoilage microorganisms, ethanolic extracts of *P. guajava* and *C. sanguinolenta* were tested for antimicrobial efficacy in acidic conditions and when applied in a model food system, milk.

After a general well diffusion screening of the original 14 extracts it was found that the most effective were the ethanolic extracts *S. alata, P. guajava,* and *C. sanguinolenta.* These showed the greatest antimicrobial potential against Gram-negative microorganisms, *E. coli* and *Salmonella*; all extracts exhibited little to no effectiveness against the Gram-positive microorganism *L. monocytogenes.* The next step was to move onto a more descriptive screening method to observe antimicrobial activity of the plant extracts against a variety of foodborne pathogens and spoilage microorganisms. These results led to the conclusion that *P. guajava* and *C. sanguinolenta* were more effective, overall, than *S. alata.* All extracts were less effective against Gram-positive microorganisms.

The previous results led to selecting two foodborne Gram-negative microorganisms (*Salmonella* Typhimurium and *E. coli* ATCC BAA 2196) to be further tested for their susceptibility to the antimicrobial properties of *P. guajava* and *C. sanguinolenta.* The MIC was determined for both plant extracts against the two microorganisms. Next, the antimicrobial efficacy of these plant extracts was evaluated in pH altered broth. The results of this study

concluded that antimicrobial activity of both extracts against *Salmonella* was decreased at all pH levels. Against *E. coli* the efficacy of the plant extracts was highly dependent on pH, specifically exhibiting the least antimicrobial activity at pH 6.

Lastly, when the extracts were applied to the same two microorganisms in the food system model, milk, results showed decreased antimicrobial activity compared to the MIC experiment performed in nutrient broth at ideal conditions. *P. guajava* still exhibited a bacteriostatic effect against *E. coli* when in the milk, suggesting it may be potentially useful for extending shelf life of similar products.

In conclusion, these studies show potential for *P. guajava* and *C. sanguinolenta* to be used as a natural antimicrobial in food products to increase food safety. However, further research needs to be conducted to study the effects of combining these extracts with other hurdles in the food industry to increase their antimicrobial effectiveness and their effect on color and flavor on food products.

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