

Association of foodborne pathogens with *Capsicum annuum* fruit
and evaluation of the fruit for antimicrobial compounds

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ABSTRACT

Hot peppers are gaining popularity in the United States as both a vegetable and a spice. In 2008, jalapeño peppers were involved in a multistate outbreak of *Salmonella* Saintpaul. This is the first outbreak implicating jalapeños as a vehicle for foodborne illness. Hot peppers contain many compounds thought to possess antimicrobial characteristics. This research was conducted to provide more information on the interactions of pathogenic bacteria and jalapeño peppers, as well as to identify properties of *Capsicum annuum* that affect bacterial survival, growth, and inhibition.

Behavior of pathogens associated with jalapeños was investigated by inoculating jalapeño fruits with a cocktail of *Listeria monocytogenes*, *Salmonella enterica*, or *Escherichia coli* O157:H7 on the intact external surface, injured external surface, or intact internal cavity and storing the jalapeños at 7°C or 12°C. Intact external jalapeño surfaces did not support the growth of the bacteria tested under storage conditions of 7°C. However, *L. monocytogenes* populations remained detectable throughout the 2 week study. At 7°C, pathogenic bacteria were able to survive but not grow on injured and internally inoculated jalapeños, but populations increased at 12°C ($p=0.05$). The most supportive growth environment for the pathogenic bacteria was the internal cavity of jalapeños held at 12°C. This study demonstrated the importance of intact uninjured produce and proper storage temperatures for food microbial safety.

Inhibitory properties of jalapeños were studied by making extracts from fresh jalapeño peppers to test for antimicrobial activity. A disk diffusion assay determined that

the extracts were capable of inhibiting the growth of the pathogenic bacteria tested. *Listeria monocytogenes* was especially sensitive to the extracts. Jalapeño extracts were fractionated using HPLC and used for inhibition assays using disk diffusion and growth curve generation. Two fractions stimulated bacterial growth ($p=0.05$), while two other fractions inhibited bacterial growth. The inhibitory fractions were separated further using HPLC and tested for antimicrobial activity. Fraction E1 suppressed the growth of *L. monocytogenes*. HPLC-MS analysis revealed that Fraction E1 contained compounds known as capsianosides. To prove that inhibition is caused by capsianoside(s) and determine minimum inhibitory concentrations, a method to isolate the pure compound should be developed.

Dedication

This is dedicated to Ian Bacon for all the sharing of ideas and honest feedback he provided. I could not have done it without him.

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CHAPTER 1

Introduction

Americans are being encouraged to increase the amount of fresh fruits and vegetables they consume to fight obesity and maintain a healthy intake of essential vitamins, minerals, and antioxidants (USDA, 2011; Blanck et al., 2008). As consumption of fresh produce increases, so does the occurrence of foodborne outbreaks related to fresh fruits and vegetables (Sivapalasingam et al., 2004). In fact, incidence of foodborne outbreaks connected with produce rose from 0.7% to 6.0% of annual outbreaks from 1973 to 1997 (Sivapalasingam et al., 2004). A more recent report by DeWaal and Bhuiya (2007) states that produce was accountable for 13% of foodborne outbreaks between the years 1990 and 2005. At present, 2 of the 9 (22%) multistate outbreaks in the year 2011 have been linked to contaminated produce (CDC, 2011). Enhancing the safety of produce intended to be consumed raw is challenging. Produce is traditionally grown outdoors, in a natural environment, which leaves it exposed to a variety of contamination sources including soil, water, manure, and animals in or near the field (Sapers et al., 2006). Adding to the challenge, produce is most often consumed with little to no processing, eliminating any chance for a kill step to reduce pathogenic bacterial loads (Gorny, 2006).

Outbreaks of foodborne illness related to produce are commonly linked to consumption of leafy greens, melons, and raw tomatoes (CDC, 2011; FDA, 2008). There have been 16 outbreaks of salmonellosis linked to tomatoes since 1999; as a result authorities prematurely identified the vehicle of a summer 2008 national salmonellosis outbreak to be tomatoes imported from Mexico. Once trace back investigations were completed, the actual carrier of the contamination was determined to be jalapeño peppers (*Capsicum annum*) (CDC, 2008). Salsas containing multiple ingredients including both fresh cut tomatoes and fresh cut jalapeños were

linked to those affected in the outbreak. Jalapeños were not considered initially, as they had not previously been identified as a vehicle for foodborne outbreak (CDC, 2008). From this outbreak, we have learned that jalapeño peppers are capable of carrying *Salmonella enterica* to a consumer and cause illness. Little else is known about the interactions of pathogenic bacteria and jalapeño peppers.

The need to understand the interaction of pathogens with fresh produce has been addressed by the United States Food and Drug Administration (FDA) (FDA, 2008). The institute has proposed a number of initiatives to reduce the presence of pathogenic organisms in these foods. For example, the FDA has developed the 2004 Action Plan to Minimize Foodborne Illness Associated with Fresh Produce Consumption. One of the specific aims of the Action Plan is to prevent the contamination of fresh produce with pathogens. In order to target this objective, the Action Plan seeks to develop and assist in the development of additional guidance relevant to the production, packing, processing, transportation, distribution, or preparation of fresh produce. The proposed research addresses this point in the Action Plan through the study of growth and survival of foodborne pathogens under different conditions which the peppers may face during post-harvest handling.

Prevention and control of hazards in food safety is also emphasized by the USDA (USDA, 2009). One focus of the USDA Specialty Crop Research Initiative is the promotion of research for the development of methods in prevention and control of food safety hazards including those associated with fresh produce. Examining the potential antimicrobial activity from natural extracts of *C. annuum* fruits addresses this need by exploring potential compounds that can combat the growth of harmful bacteria associated with fresh produce. Antimicrobial substances isolated from *Capsicum* fruit may possibly be used as a rinse agent, dip treatment, or

a component of wax for fresh produce to eliminate human pathogens or prevent their growth on fresh produce.

Consumers are becoming more skeptical of “unnatural” food ingredients, causing a resurgence of interest in natural antimicrobial compounds. Foods are typically preserved by compounds such as nitrite, sodium benzoate and sodium metabisulfite that have been tested and proven safe (Gould and Russell, 2003). However, there are occasional reports of allergic reactions to these preservatives, and even potential formation of carcinogenic by-products like nitrosamines from nitrite (Roller, 2003). Essential oils isolated from plant sources have been found to be effective antimicrobial agents, and there is ongoing research to identify more antimicrobial plant sources. Extracts from *C. annuum* fruit have been investigated to some extent, and antimicrobial properties have been reported with mixed results. Crude tissue extracts from several different *C. annuum* varieties have growth inhibiting properties against species of *Bacillus*, *Clostridium*, and *Streptococcus* (Cichewicz and Thorpe, 1996). Extracts from *C. annuum* fruits using isopropanol as a solvent inhibited the growth of *L. monocytogenes*, *S. enterica*, *Bacillus*, and *Staphylococcus* in liquid media to varying degrees (Dorantes et al., 2000). Contradictory results were found when *S. typhimurium* was introduced to both bell pepper and jalapeño fruit extract, and bacteria grew readily in each (Nutt et al., 2003). Little is known about antimicrobial properties of *C. annuum* extract when tested against various foodborne pathogens and no antimicrobial research has been conducted using HPLC-fractionated extracts of *C. annuum*.

An understanding of how foodborne bacterial pathogens interact with *C. annuum* fruits under post-harvest conditions is lacking. This research aims to address how foodborne bacterial pathogens survive and grow when introduced to *C. annuum* fruit under normal and temperature

abusive storage conditions. As an additional measure to improve food safety, this research will investigate the fruit of *Capsicum annum* L. var. jalapeño as a source of natural antimicrobial.

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CHAPTER 2

Review of the Literature

The Current State of Food Safety

Foodborne illness is caused by consuming food or beverages that are contaminated with pathogenic microorganisms. There is a wide variety of foodborne pathogens that can survive the vast range of environments within food and beverages, and these pathogens can cause a wide variety of illness. Traditionally, biological food contamination has been associated with animal products (Meng and Doyle, 2002). However, the epidemiology of foodborne disease has changed rapidly over the past two decades (Brandl, 2006) and the frequency of documented outbreaks associated with fresh fruits and vegetables has increased in the United States in recent years (Burnett and Beuchat, 2000). It was estimated that less than 1% of all foodborne outbreaks were associated with produce in the 1970's where 6% of outbreaks were tied to produce in the 1990's (Sivapalasingam et al., 2004). The reasons behind this rise in incidence are numerous and complex.

Increased Incidence of Produce Related Outbreaks

There are many explanations regarding why we are seeing such a significant increase in produce-associated foodborne illnesses. Fruits and vegetables do not grow year round in most parts of the United States, and consumer desire for a wide variety of produce during the off-season means that fruits and vegetables must travel further distances to get to the consumer (Lynch et al., 2009). This demand has caused production and handling practices to change (Beuchat, 1996). Production facilities are becoming more centralized and produce is being distributed further distances than before. The majority of fresh fruits and vegetables will see

minimal processing before consumption, meaning there will be no kill step to reduce microorganism populations (Brandl, 2006). For consumers, this means that a piece of produce contaminated by microorganisms thousands of miles away may enter their home and be consumed with little to no intervention steps to reduce or eliminate the harmful microorganisms.

Foodborne illness may also appear to be on the rise due to advanced surveillance networks, which may be catching more outbreaks than in previous years (Doyle and Erickson, 2008). One example of a successful surveillance network is the FoodNet system which was established in 1996 as a resource for investigation and surveillance of foodborne disease in the United States. FoodNet is a component of the CDC's Emerging Infections Program in collaboration with the USDA-FSIS, the FDA, and several state health departments. FoodNet conducts population-based, active surveillance for laboratory-confirmed infections from 9 pathogens commonly transmitted through food. FoodNet also determines the impact on human health from these foodborne illnesses by conducting epidemiological studies. States actively participating in FoodNet surveillance include California, Oregon, New Mexico, Colorado, Minnesota, Tennessee, Georgia, New York, Connecticut, and Maryland. The CDC has also implemented a national network known as PulseNet for public health officials and food regulatory agency laboratories. Participants in PulseNet conduct pulsed-field gel electrophoresis analysis of food-disease causing bacteria to generate a “fingerprint” for each bacteria type. The “fingerprint” can be used to distinguish different bacterial isolates, and storing this information in a databank allows investigators to link food illnesses that are caused by bacteria of the same pulse-type (CDC, 2009). The CDC also runs the National *Salmonella* Surveillance System and the National *Shigella* Surveillance System, where information is reported electronically through the Public Health Laboratory Information System (PHLIS). The National *Salmonella*

Surveillance System is based on data collected by state and territorial public health laboratories. *Salmonella* isolates are submitted to the state public health laboratory by clinical diagnostic laboratories. The state laboratory confirms the isolates as *Salmonella*, performs serotyping, and submits data through PHLIS. As systems like these continue to improve and expand, the number of foodborne illnesses reported are likely to increase as well.

In addition to increased surveillance, another explanation for the growing incidence of produce related illnesses is the changing habits and practices of the consumer over the past few decades. Consumers are eating meals outside of the home more often, and nearly eighty percent of foodborne illnesses originate from sources outside of the home (Taege, 2002). Salad bars are a staple in many restaurants. Processing of fresh and fresh cut produce, like those items found on salad bars, is one the most rapidly growing areas of food processing (Meng and Doyle, 2002). Not surprisingly, a number of produce-associated outbreaks have involved salad bars (Osterholm et al., 2007). Overall, consumption of fruits has raised 15-20% and consumption of vegetables has increased 25-30% in the past 30 to 40 years (Taege, 2002). This increase in consumption may be the result of the recognition that fruits and vegetables are very beneficial to health.

In 1991, the National Cancer Institute (NCI) partnered with the Produce for Better Health Foundation (PBH) to promote increased consumption of fruits and vegetables. The “five a day for better health” campaign was created to reduce cancer among Americans and encourage lasting health (NCI, 2003). The partnership has since expanded to include the Center for Disease Control and Prevention (CDC) and the United States Department of Health and Human Services (HHS). Since the introduction of the “five a day” campaign, a revamped campaign emerged titled “Fruits and Veggies – More Matters™” (PBH, 2009). This campaign uses factors such as age, sex, and individual activity level to recommend the amount of fruits and vegetables a person

should consume a day. There is an emphasis on not only amount consumed, but the variety and color consumed as well. A recent revamp of the food pyramid has resulted in the MyPlate icon, a simplified schematic of the amounts of food from each food group that should be eaten with each meal (USDA, 2011). The new MyPlate icon suggests that half of the food eaten each meal should consist of fruit and vegetables. Programs such as “Five a day”, “More Matters”, and MyPlate are influential in persuading American consumers to increase the proportion of fruits and vegetables in their diet. As the American diet increases in terms of fresh produce consumption, it is logical that an increased number of illnesses related to produce would follow.

Interactions of Bacterial Pathogens with Fresh Produce

One topic of major interest in food safety is the source and prevention of produce contamination, including pre-harvest and post-harvest factors. The epidemiology of foodborne disease is shifting and many pathogens that use animals as a reservoir are being spread to fresh fruits and vegetables (Brandl, 2006). It is now recognized that bacterial pathogens are able to grow and survive on, and even within, fruits and vegetables (Sapers et al., 2006). There are a number of ways in which produce can become contaminated pre-harvest. Pathogens can be transmitted to produce in the field via contaminated manure, manure compost, sewage sludge, irrigation water, runoff water from livestock operations, and wild and domesticated animals (Doyle and Erickson, 2008).

The plant environment is considered a harsh habitat for bacterial pathogens. Bacteria experience UV from sunlight, a lack of available nutrients, fluctuations in temperatures and osmotic conditions, and competition from natural bacterial plant inhabitants (Brandl, 2006). Survival of enteric organisms on a plant host varies depending on the organism’s adaptability,

the host produce item, and the environmental conditions faced in the field. It is generally believed that bacterial pathogens will survive but not grow on plant surfaces (Harris et al., 2003), likely due to the plants natural barriers like cell walls and waxes. When nutrients become available to bacterial pathogens via physical damage or by biological means (bacteria or fungi), conditions become favorable for pathogens to multiply (Harris et al., 2003). It has been noted that *S. enterica* inoculated in high concentrations onto the surface of tomato fruits can persist for weeks (Zhuang et al., 1995). In addition, leaf and root surfaces are known to have microsites dispersed throughout where nutrients and water are available to bacteria (Leveau and Lindow, 2001; Miller et al., 2001).

Microorganisms have been proven to live inside healthy, intact fruits and vegetables, as first demonstrated by Samish et al (1963). Bacteria that colonize the internal tissue of plants without causing disease are known as endophytes (Kobayashi and Palumbo, 2000). There are over 129 known species of endophytic bacteria that have been isolated from the internal tissue of healthy plants (Kobayashi and Palumbo, 2000). Common internal plant inhabitants include *Pseudomonas*, *Enterobacter*, and *Agrobacterium* (Hallman et al., 1997). The potential for enteric pathogens like *E. coli* and *S. enterica* to persist as endophytes has recently been elucidated (Bernstein et al., 2007; Jablasone et al., 2005; Guo et al., 2002; 2001). There are a number of tactics that bacteria may use to enter produce. Because most plants are covered by a protective layer of wax, bacteria have difficulty penetrating (Bartz, 2006). Natural openings found on plant surfaces such as stomata or lenticels are entry points for bacteria. Submerging produce into water that differs with the temperature of the produce can cause contaminated water to enter the produce due to pressure differentials (Eblen et al., 2004). Bacteria can also gain entrance into produce through wounds, bruises, and stem scars (Burnett et al., 2000; Janisiewica et al., 1999).

Studies have shown that bacterial pathogens are capable of internalizing a plant through the root system (Franz and van Bruggen, 2008; Kutter et al., 2006; Cooley et al., 2003; Wachtel et al., 2002). Results indicate that bacteria may enter the plant through junctions in the root, where nutrients and water are readily available (Jabalson et al., 2004). *S. enterica* and *E. coli* are of particular concern in alfalfa and bean sprouts, where they can be internalized through the roots and persist in warm and moist conditions until they reach the consumer (Taormina and Beuchat, 1999; Jaquette et al., 1996). Activities that cause injury to the root, such as transplantation or cultivation, can provide sites of bacterial entry (Hallman et al., 1997). Contaminated flowers can lead to internalization of enteric pathogens into the fruit, as demonstrated with *Salmonella* and tomato plants (Guo et al., 2001). A pathogen that has become internalized in produce will be unaffected by washing and disinfecting rinses, making this of particular food safety concern.

Bacteria may be transported through the plant vasculature structure once it has become internalized. Warriner et al. (2003) inoculated mung beans with either bioluminescent *E. coli* or *S. enterica* serovar Montevideo and allowed the beans to sprout for up to 4 days while roots and hypocotyls were tested for bacterial presence. Both *E. coli* and *S. enterica* growth were closely related to roots but not the hypocotyls. *Escherichia coli* and *S. enterica* were detected in the internal tissue of the bean sprouts, and imaging during sprouting suggested that both bacteria were internalized and then distributed through the plant during the early stages of sprouting (Warriner et al., 2003). *Salmonella enterica* was shown to internalize the roots of barley in high numbers and spread to the plant stems and leaves (Kutter et al., 2005). When plants were grown in a gnotobiotic system, *E. coli* O157:H7 became internalized in cress, lettuce, radish and spinach seedlings but were not recovered from mature plant tissue (Jabalson et al., 2005).

Risks from Farm to Fork

The chain of events bringing produce from the farm to the plate is complex, and there are a number of points where produce can be introduced to pathogens from harvest and beyond. Produce harvested from the field will encounter one of the following fates: enter the market whole with minimal processing; become fresh cut with minimal processing; or undergo a processing step and be sold canned or frozen. Most produce will be sold whole or fresh cut and therefore see very little processing before reaching the consumer. The lack of extensive processing increases a consumer's risk of encountering a pathogenic organism. Produce could be contaminated by pathogens during growth in the field, harvest, processing, or distribution. The sources of contamination are often referred to as "pre-harvest" or "post-harvest".

Pre-harvest contamination could occur due to contaminated soil, irrigation water, feces in the field, improperly-composted fertilizer, wild or domesticated animals, insects, or improper handling by humans (Lynch et al., 2009). Post-harvest sources of contamination include: humans, harvesting equipment, transport containers, wash and rinse water, animals, and improper storage temperature of the foods (Beuchat and Ryu, 1997).

The risk of physically damaging produce is always high when mechanical means are used to gather the crop. Cut surfaces on produce make nutrients more readily available to microbes and encourage the attachment of bacteria. Produce that undergoes processing like cutting, shredding, peeling, and dicing also create surfaces that microbes are more likely to attach (Doyle and Erikson, 2008).

Cross contamination may occur at any point of direct contact between contaminated and non-contaminated produce. Contaminated water may also pose a cross-contamination threat. Produce can be contaminated via cross contamination by processing equipment, as was the case

of an outbreak of *E. coli* O157:H7 in orange juice (Martinez-Gonzales et al., 2003). The surface of a cutting wheel used to shred lettuce was the point of contamination of lettuce by *S. enterica* Bovismorbificans in Australia (Stafford et al., 2002). Contaminated produce can spread bacteria to water and cross contaminate other produce, as demonstrated by Wachtel and Charkowski (2002). In this study, one dry piece of lettuce contaminated with *E. coli* O157:H7 was capable of contaminating 100% of lettuce stored in water within a refrigerator.

There are clearly a number of factors that must be considered and monitored when dealing with fresh produce. In order to prevent or reduce contamination of produce, it is important that all workers are aware of the steps they can take to reduce the likelihood of contamination. Programs such as Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs) are available to farmers and workers in order to provide training on how simple steps can be taken to avoid or reduce contamination.

Listeria monocytogenes: Basic Characteristics

The genus *Listeria* is composed of six species: *monocytogenes*, *ivanovii*, *innocua*, *seeligeri*, *welshimeri*, and *grayii*. Only two of the species are considered pathogenic: *L. monocytogenes* in humans, and *L. ivanovii* in other mammals (Donnelly, 2001). *Listeria monocytogenes* is the causative agent of listeriosis, a rare but highly fatal foodborne illness. Listeriosis is of particular concern for groups of the population that are immunocompromised, such as pregnant women, the elderly, patients effected by the human immunodeficiency virus, and those undergoing immunosuppressing therapy as treatment for cancer. Fatality rates of those with listeriosis range from 20-30% in hospitalized patients, making it the leading cause of death from a foodborne pathogen (Kathariou, 2002). *Listeria monocytogenes* is ubiquitous in the

environment and is often found in soil and water. The organism is capable of growing in temperatures ranging from -1.5°C to 50°C and within a pH range of 4.3 to 9.6 (Lou and Yousef, 1999). There is a need for a highly sensitive method of detection for *L. monocytogenes* due to the strict zero-tolerance policy set for the organism. Currently, the most reliable methods for detection are outlined by the USDA for meat and poultry products (Cook, 1999) and the FDA for dairy, fruits, vegetables, and seafood products (Hitchens, 1995). However, when Hays et al. (1992) conducted a survey of 899 foods that were positive for low levels of *L. monocytogenes*, the FDA method detected the organism on only 65% of samples, while the USDA method was successful for only 74% of foods. Alternative rapid methods such as enzyme linked immunosorbant assay, DNA probe and amplification, and lateral flow have been developed, but a lengthy enrichment method would need to be in place for these methods to be able to detect small numbers of organisms in food (Cook, 1999). Currently a protocol to detect *L. monocytogenes* with the sensitivity and accuracy needed for the food industry does not exist.

Listeria monocytogenes are facultative anaerobic, Gram-positive microorganisms that live intracellularly within its infected host. There are several key virulence factors which help *L. monocytogenes* to evade the host immune system by invading cells, replicating within, and translocating into neighboring cells. The virulence factors include the hemolysin listeriolsin O, two phospholipases, and an actin polymerizing protein known as ActA. The bacterial cells express the surface proteins internalin A and internalin B, which are recognized by host cell receptors (Braun et al., 2000; Mengaud et al., 1996). Once the bacterial cell associates with the host cell, a cascade of signals causes the host cell to internalize the *L. monocytogenes* cell into a vacuole. The bacteria are able to escape from the vacuole into the cytoplasm through the expression of listeriolsin O. Within the cytoplasm, *L. monocytogenes* replicates and uses its

ActA protein to polymerize actin provided by the host cell to produce an “actin tail”, propelling the bacterial cell to push a pseudopod through the wall of the infected host cell and into a neighboring host cell. *Listeria monocytogenes* is able to escape the now double-membraned vacuole using listeriolysin O and phospholipases to enter the cytoplasm of the new cell and restart its replication cycle (Kathariou, 2002).

Infection with *L. monocytogenes* begins with an onset of influenza-like symptoms, including fever. Gastrointestinal symptoms such as nausea, vomiting, and diarrhea may also present in more serious forms of listeriosis. In the most extreme cases of infection, symptoms can include septicemia, meningitis, encephalitis, and intrauterine and cervical infection in pregnant women which can lead to spontaneous abortion or stillbirth (FDA, 2009). Although there are only 1600 cases of listeriosis a year, 415 cases result in death (FDA, 2009).

There are several methods of controlling *L. monocytogenes*. Pasteurization of milk and dairy products will kill the bacterium. Good sanitation will prevent build-up in processing facilities. Acid development in fermented foods will prevent *L. monocytogenes* from growing. Proper cooking of meat and seafood will kill this bacterium.

Association of *L. monocytogenes* with Fresh Produce

Listeria are ubiquitous in the environment, and their potential to contaminate fresh fruits and vegetables has long been recognized. *Listeria monocytogenes* has been isolated from produce such as cucumbers, peppers, potatoes, radishes, sprouts, tomatoes, and leafy greens (Beuchat, 1996; Arumugaswamy et al., 1994). There have been two major outbreaks in produce due to contamination of *L. monocytogenes*; a 1979 outbreak due to salad items served as part of a hospital meal and a 1981 outbreak in Canada due to contaminated cabbage in coleslaw. Although

there are few outbreaks due to *L. monocytogenes* documented annually, the incidence of the pathogen associated with food is high (Harvey and Gilmour, 1992). The high infective dose of *L. monocytogenes* ($\sim 10^6$ cells) in healthy individuals may be one reason so few outbreaks are reported. It has been speculated that improper handling by food service workers is more likely a cause of *L. monocytogenes* contamination than from natural contamination of the raw product (Harvey and Gilmour, 1992). Therefore, the importance of good sanitation and handling practices is emphasized to lower the risk of contamination of produce.

Pathogenic *Escherichia coli*: Basic Characteristics

Of the known human pathogens, *E. coli* are one of the most versatile. Not only are *E. coli* the dominant commensal organism of the human gastrointestinal tract, they are also a potential pathogen that can cause a variety of diseases. *Escherichia coli* can cause up to six clinical syndromes of diarrhea with overlapping but distinct epidemiology (Montville and Matthews, 2005). It can cause a variety of illnesses ranging from urinary tract infections to wound infections.

The ability of a single species to co-exist symbiotically with its host and to cause so many distinct diseases is due to the genetic heterogeneity of the organism. The various clinical syndromes attributed to *E. coli* are caused by pathotypes that differ from each other and from commensal strains because they have acquired distinct sets of virulence genes (Park et al., 1999). These genes are carried on plasmids, on lysogenic bacteriophages, or on pathogenicity islands. The distinctive clinical syndromes recognized in patients infected with different pathotypes of *E. coli* are a direct result of the interactions with the host that are encoded by the various combinations of gene sets. These combinations of virulence genes were not selected for their

ability to cause harm to humans, but to allow the organism to occupy distinctive niches within a host.

E. coli are Gram-negative, non-sporeforming rods. They are facultative anaerobes. They may be motile with peritrichous flagella, or they may be non-motile. *Escherichia coli* grow in a temperature range of 10°C to 50°C with optimum growth at 30°C to 37°C. *Escherichia coli* can normally grow at a pH ranging from 4.3 to 9 with an optimum of 6 to 8 (Jay et al., 2005). Some strains of *E. coli* O157:H7 isolated from apple cider can survive pH as low as 2.5 (Benjamin and Datta, 1995).

E. coli are serologically differentiated based on three major surface antigens: O (somatic), H (flagella), and K (capsule) (Figure 1). A total of 173 O antigens, 56 H antigens, and 103 K antigens have been identified as of 2005 (Bettelheim and Thomas, 2005). Diarrheagenic *E. coli* are characterized into specific groups, or pathotypes, based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O:H serotypes. These categories include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC).

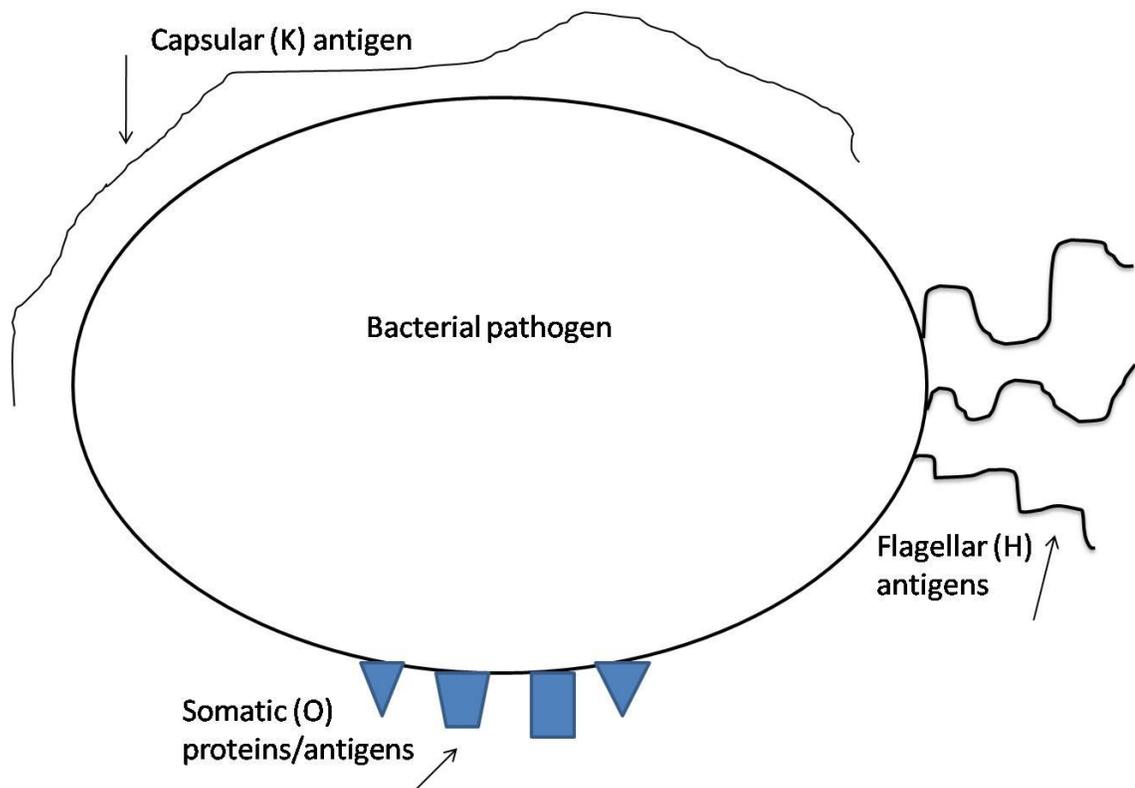


Figure 1. Antigenic features of interest on *E. coli*

Enterohemorrhagic *E. coli* (EHEC)

EHECs were first recognized as a human pathogen in 1982 when *E. coli* O157:H7 was identified as the cause of two outbreaks of hemorrhagic colitis. Other strains have also been identified as EHEC since, such as O157:NM, O111:NM, O111:H8, and O26:H1, but O157:H7 is the predominant cause of EHEC related disease in the United States. All EHEC produce cytotoxins that are virulent to African green monkey kidney (Vero) cells, and are therefore known as verotoxins. Verotoxins are also called Shiga-like toxins (Stx) because of their relatedness to the Shiga toxin produced by *Shigella dysenteriae* (Kaper et al., 2004). EHEC cause attachment and effacement and also release a Stx toxin, which is absorbed by the host cell and can cause life threatening complications. EHEC causes bloody diarrhea (hemorrhagic

colitis), non-bloody diarrhea, and haemolytic uremic syndrome (HUS). HUS can be serious, leading to lifelong problems of hypertension, chronic renal failure, other disabilities, and even death. The main reservoir for EHEC is the bovine intestinal tract. Early outbreaks were associated with consumption of undercooked hamburgers (Bell et al., 1994; Griffin and Tauxe, 1991). However, large outbreaks have been linked to salami, sausage, milk, apple cider, and unpasteurized juice (Griffin and Tarr, 1995; Besser et al., 1993; Tauxe, 1991). Bovine feces is the major vehicle for EHEC transmission. EHEC can also be spread by person to person contact, by contaminated drinking water, and by exposure during swimming (Swerdlow et al., 1992; Griffin and Tauxe, 1991). The infectious dose is extremely low (10-100 cells), and virulence is established through both the production of Stx and attaching and effacing lesions (Kaper et al., 2004).

Association of *E. coli* O157:H7 with Fresh Produce

Due to the association of *E. coli* O157:H7 with the feces of livestock, contamination of produce growing near livestock is of contamination concern. The vegetables most commonly implicated in *E. coli* O157:H7 outbreaks are leafy greens (Brandl, 2006), but *E. coli* O157:H7 has also been a problem in apple juice and cider (Heaton and Jones, 2008). Since 2002, 350 outbreaks of *E. coli* O157:H7 were reported, 52% of cases were foodborne. Of the foodborne cases, 21% were due to contaminated produce (Rangel et al., 2005). Since 2001, *E. coli* O157:H7 has caused multistate outbreaks due to contamination of romaine lettuce, alfalfa sprouts, spinach, and vegetable salad base (CDC, 2011).

Salmonella: Basic Characteristics

Salmonellae are pathogens with a huge impact on public health. They infect not only humans but also domestic animals used in the human food chain. Salmonellosis is one of the most prevalent foodborne infectious agents in both developed and developing countries. All *Salmonella* strains are potential pathogens.

Salmonella species are facultative anaerobic, Gram-negative, non-spore forming, rod-shaped bacteria (Curtis and Lawley, 2003). They are motile with peritrichous flagella, although nonflagellated variants exist, such as *S. enterica* serovar Pullorum and *S. enterica* serovar Gallinarum (Jay et al., 2005). They can grow from a temperature range of 7°C to 48°C, with optimum growth at 35-37°C (Curtis and Lawley, 2003). *Salmonella* species can survive in pH ranging from 3.6 to 9.5, although they prefer pH 6.5-7.5. Depending on the strain, they can live in water activities as low as 0.93 (Curtis and Lawley, 2003).

Salmonella are classified by serology and phage susceptibility. The genus *Salmonella* contains two lineages that diverged early in its evolution: the species *S. enterica* and *S. bongori* (Reeves et al., 1989; Le Minor and Popoff, 1987). Serological typing is based on surface antigens to *Salmonella*-specific antibodies. These include somatic (O), lipopolysaccharides (LPS) on the external surface of the outer membrane, flagellin (H) antigens of peritrichous flagella, and the capsular (K) antigen (Le Minor, 1981).

Salmonella infections lead to a variety of diseases known collectively as salmonellosis. Infection is caused by consumption of raw or undercooked contaminated food. The outcome of the infection depends on the serovar and the type of host in which it is infecting (Baumler et al., 1998). The infection associated with food consumption, known as “non-typhoid salmonellosis”

or gastroenteritis, is self-limiting and affects the intestinal epithelium (Miller et al., 1995). Non-typhoid salmonellosis is characterized by diarrhea, abdominal pain, mild fever, nausea, and occasional vomiting (Baird-Parker, 1990). Illness is more severe in very young individuals, the elderly and immunocompromised individuals. Death can occur in these groups as a result of infection.

Symptoms of salmonellosis usually occur 6 to 48 h after consumption of contaminated food and may last 2 to 5 days. The disease is usually self-limiting and rarely results in hospitalization. A person may be a carrier up to 3 months or longer after infection (D'aoust, 1997). The carrier state is an important cause of foodborne salmonellosis as it leads to spreading the pathogen to the farm, food, food-handlers, and consumers. The infective dose is usually 10^5 to 10^7 CFU/g but can be as low as 10-1000 CFU/g in susceptible groups (D'aoust, 1997; Kapperud et al., 1990).

Salmonella enterica infection begins after consumption of contaminated food or water. Bacterial cells must pass through the stomach, which is highly acidic. A high infective dose is required to survive pH of the stomach. Once *S. enterica* have successfully passed through the stomach, they colonize the distal ileum. The M cells of the Peyer's patch are *S. enterica*'s vehicle for cell entry. Binding of *S. enterica* to M cells is mediated by specialized fimbriae, named Ipf (Baumler et al., 1998). Once *S. enterica* have invaded the M cell, lymphocytes and phagocytic cells are recruited to the site of invasion, causing inflammation of the area. The inflammatory response leads to secretion of large amounts of fluid into the intestinal lumen. When *S. enterica* invade cells other than M cells, such as epithelial cells, they induce cell ruffling by cytoskeletal rearrangement of host cells in order to gain entrance to cells (Finlay, 1994). Once inside, *S. enterica* remain inside the vacuole and replication begins within a few hours of internalization.

The infected vacuoles move from the apical to the basal pole of the host cell. *S. enterica* are released into the lamina propria. *S. enterica* in the lamina propria are then phagocytosed by macrophages. At this point, cells may pass through the mesenteric glands to the bloodstream. If this occurs, the organisms may spread to the liver, gall bladder, spleen, bone marrow, lymph nodes, lungs and the kidneys and further divide.

Association of *Salmonella enterica* with Fresh Produce

Foods commonly associated with salmonellosis are eggs, poultry, meat, dairy products, and any product contaminated by fecal matter. Major outbreaks have been seen with milk, poultry, and eggs. More recently, outbreaks of salmonellosis have been link to produce. *Salmonella enterica* contamination of tomatoes, cantaloupe, and watermelon has caused outbreaks because they were not washed before being placed into salad bar containers. Unpasteurized orange juice has also been the source of an outbreak (D'Aoust and Maurer, 2007). Recently *S. enterica* has gotten press time due to the contamination of peanut butter at the Peanut Corporation of America, Blakely, GA branch that was widely distributed throughout the United States (CDC, 2009). *Salmonella* is a bacterium of utmost concern in food safety capable of contaminating a wide variety of foods.

Plants as a Host for Pathogenic Foodborne Bacteria

It is somewhat surprising to learn that enteric pathogens like *S. enterica* and *E. coli* thrive with plant tissue as host. Plants are not a truly inhospitable environment for all bacteria. There are many bacteria that prefer life on a plant. These include species such as *Pseudomonas* and *Erwinia* (Brandl, 2006). Enteric pathogens are able to survive the pH and oxygen conditions of

the intestines and attach, adhere and infect gastrointestinal cells; as well as survive the harshness of the plant environment. *Salmonella* has made the transition from intestine to plant quite readily and has caused problems with fruit and vegetable safety for years. *Salmonella* outbreaks have been closely associated with produce including melons (Munnoch et al., 2009; CDC, 1991), tomatoes (Greene et al., 2008; Gupta et al., 2007; CDC, 2005), seed sprouts (Werner et al., 2007; Emberland et al., 2007; Winthrop et al., 2003), and leafy greens (Nygard et al., 2008; Denny et al., 2007; Horby et al., 2003). The occurrence of peppers as an outbreak vehicle was the first of its kind in 2008. More recently, the Herring Produce Company located in Lake Park, Georgia recalled a lot of Anaheim peppers from Wegman's grocery stores due to positive confirmation of *Salmonella* on the peppers (FDA, 2009). No known illnesses resulted. Although it appears that peppers are becoming more at risk in the produce market, little is known about the potential interactions between pathogenic bacteria and peppers.

Summer 2008 Outbreak of *Salmonella* Associated with Jalapeños

The source of foodborne outbreaks are often a mystery when investigators begin their inquiries. The large number of factors that could have contributed to contamination makes for a complicated situation. An example of a recent outbreak that baffled the food safety community was the case of *S. enterica* serovar Saintpaul and the contamination of jalapeño peppers. In May of 2008, it became evident that persons around the United States were suffering from an outbreak of *S. enterica*. What began as four people from New Mexico infected with the same pulsed field gel electrophoresis (PFGE) pattern of *S. enterica* Saintpaul eventually turned into the largest foodborne disease outbreak identified in the United States in the past decade (CDC, 2008). The greatest mystery throughout the course of the outbreak was the identity of the vehicle causing all

the infections. As of August 25, 2008 a total of 1442 persons had been reported infected with the outbreak strain, at least 286 had been hospitalized, and the infection may have contributed to two deaths (CDC, 2008). The vehicle implicated in the outbreak was eventually determined to be jalapeño peppers, although serrano peppers could have contributed (CDC, 2008). It is possible that tomatoes were a vehicle of infection early in the outbreak. The manner in which the produce became infected is unknown; it may have originated on the farm or during processing or distribution.

According to a report from the CDC (2008), as case studies were being conducted in areas where the outbreak occurred, raw tomatoes were the first suspect. It was advised that persons stop eating certain varieties of tomatoes. Soon, however, case studies showed that infection was more likely associated with jalapeños. Many of the cases included persons who had eaten salsa containing raw tomatoes and raw jalapeño peppers. In one particular case in Texas, a salsa from a local Mexican restaurant was implicated which contained raw jalapeño peppers and processed commercially canned tomatoes. Jalapeño peppers became the number one suspect at this point. An outbreak in North Carolina found infections to be highly associated with eating guacamole. The guacamole contained avocado, raw roma tomatoes, raw red onions, raw serrano peppers, cilantro, salt, and lime juice, but no jalapeño peppers. This study showed that not all outbreak illnesses could be linked to jalapeño peppers. In the end, it was believed that jalapeño peppers and perhaps serrano peppers were carriers of this outbreak strain. However, sometimes tomatoes were involved, and jalapeño peppers were not involved in every case. In July, the FDA reported that they isolated the outbreak strain from a jalapeño pepper sample obtained from a distributor in Texas that got their produce from a farm in Tamaulipas, Mexico. This farm also grew serrano peppers and roma tomatoes. The FDA did not isolate the outbreak

strain from this farm, but they were able to isolate the outbreak strain from another farm in Tamaulipas, Mexico that also grew jalapeño peppers, but not tomatoes. The produce from these two farms were sent to a common packing facility in Mexico that exports to the United States.

All the confusion caused by this particular outbreak caused major damage to the tomato industry due to the premature conclusion that tomatoes were the vehicle for the outbreak. The National Restaurant Association reported that its member's losses were approximately \$100 million (The Tampa Tribune, 2008). The Florida Tomato Growers Exchange reported losses of \$500 million, the value of a full year's crop (The Sarasota Herald-Tribune, 2008). Jalapeño and serrano peppers have never been identified as an outbreak vehicle for *Salmonella*. Little is known about the survival and growth characteristics of *Salmonella* on these types of peppers.

Capsicum: Fact and Folklore

The term "hot pepper" can bring a number of uses to mind; vegetable, spice, medicine, condiment, decoration. In fact, the uses for a pepper are far and wide. Hot peppers are the second most commonly used seasoning agent in the world, after salt (Andrews, 1995). A hot pepper is scientifically known as a *Capsicum*. There are five common domesticated species of *Capsicum*-*annuum*, *baccatum*, *chinense*, *frutescens*, and *pubescens*, and a vast array of wild species (Andrews, 1995). The most common domesticated species found in the United States is *Capsicum annum*, which includes such peppers as jalapeños, serranos, bell peppers, pimentos, banana peppers, ancho, and paprikas, to name a few. *Capsicum* fruits are referred to in casual conversation as peppers, chiles, capsicums, chilis, aji, and paprikas (Bosland and Votava, 2000). The *Capsicum* is known for its pungent fruit which can be eaten fresh or dried into a powder. It is known that the pungency causing compound that is found only in *Capsicum* species, known as

capsaicin, carries the "heat" element that defines a pepper. *Capsicum* use as a crop has become a "new fascination" (Bosland and Votava, 2000) in the United States due to its richness in diversity, as peppers can be used as food, flavoring agents, medicine, and decoration. Peppers are rich in vitamin C, low in calories, flavorful, and stimulate saliva flow and gastric juices which promotes healthy digestion. It is said that peppers can raise body temperature, relieve cramps, improve complexion, reverse inebriation, cure a hangover, soothes gout, and increase passion (Bosland and Votava, 2000). These things, of course, can be attributed to folklore, but there is some evidence that the *Capsicum* has antimicrobial properties.

Pepper Production and Harvesting Measures

Peppers are grown on all continents in the world except Antarctica (Bosland and Votava, 2000). In the wild, peppers are found as perennial shrubs, but most domesticated peppers are grown as annuals. Peppers are commonly grown in both greenhouse or field environments. Chile peppers are warm season crops, and therefore susceptible to chilling injury. Growth conditions are ideal when day temperatures reach 75°F-85°F, and night temperatures range from 50°F-60°F (Bosland and Votava, 2000). Nearly all *Capsicum* plants are transplanted, although direct seeding is not unheard of. Peppers can be grown in a variety of soils. The ideal soil would be a deep, well-drained, medium textured sandy loam or loam soil that holds moisture and has some organic matter. It is important that the soil drains well to avoid root rot due to fungal growth. Crop rotation is recommended when growing peppers in consecutive years, and rotation should be to non-solanaceous crops like wheat, maize, lucerne and legumes. In southern California, peppers are usually grown in double rows on raised beds, 60 to 72 inches wide. Fumigation of the entire field is commonly performed before transplantation. Plastic mulch and drip irrigation

is common. For drip irrigation, lines are typically buried 2 to 10 inches deep with one to two drip lines per bed. Growers may also place the tape on the surface of the soil to improve root development and to aid in the ease of removal and installation of the tape. Furrow irrigation is also a common practice. Peppers are a self-pollinating plant, although pollination with bees reduces the days from fruit set to harvest (Bosland and Votava, 2000). As a pepper plant proliferates, it needs added support. Support systems are typically made of string tied to the stems and supported from overhead wires that are 2.5 to 3.0 meters above. The stem must be twisted around the string stock wire every 10-14 days to keep providing support. An ideal pepper plant will put forth one fruit for every two leaves produced.

Jalapeño peppers are harvested at physiological immaturity as a green fruit and sold for fresh market use. There is also a market for jalapeños grown to be sold to processors. Fresh market fields are harvested two to four times at 10 to 15 day intervals. The majority of peppers are harvested by hand to maintain a quality product, and placed into bulk bins or trailers for transit to packing or processing facilities. Hand-picked peppers are of higher quality because those peppers that are moldy, under ripe, overripe, or damaged can be instantaneously rejected. Fewer leaves and stems are also incorporated into the harvest when picked by hand. Mechanical harvesting does exist to a limited extent and is more prevalent for use of harvesting peppers for processing. Mechanical injuries such as cuts, abrasions, and bruises can lower the market grade and reduce shipping life of the peppers.

The post-harvest handling practices of the pepper fruits are just as important as growth and harvest. Harvested chili peppers are often washed with water containing 75 to 100 ppm chlorine and cooled before shipment via forced air or room cooling (Boyette et al., 1990). Optimal storage and shipping conditions for peppers are 45°F to 50°F (7°C to 10°C) with a

relative humidity of 90 to 95%. Maintaining these ideal conditions can extend a fresh peppers shelf life up to 10 days. Refrigeration extends shelf life by decreasing respiration, water loss, color change, and post-harvest disease development. Storage temperature warmer than 50°F (10°C) promotes ripening and color change. Chilling injury occurs when temperature fall below 45°F (7°C). Chilling injury is characterized by pitting of the surface, fruit discoloration, and excessive decay (Smith et al., 1996). Most fresh peppers can be stored under refrigerated conditions for two weeks. Ethylene produced by other fruits or vegetables will hasten ripening in chile peppers and reduce shelf life. Therefore, peppers should be kept separate from other crops such as tomatoes, apples, and melons during shipping and storage. Peppers are typically stored and shipped in waxed corrugated boxes and can be found shipped to the market in half bushel boxes and 10 to 20 pound food service cases (Smith et al., 1996).

Properties Associated with *Capsicum* species

Capsicum species are known for their pungency. Pungency is a word used to describe the taste of heat or hot flavor (Bosland and Votava, 2000). Initially, the pungency of chiles was thought to be caused by one compound, capsaicin (Ishikawa, 2003). Upon further investigation, it was found that pungency is caused by seven or more homologous branched-chain alkyl vanillylamides that are collectively known as capsaicinoids (Table 1) (Torabi, 1997). Capsaicinoids are simple phenolic amides found only in *Capsicum* plants (Ravishankar et al., 2003). They are produced in glands within the placenta of their fruit (Bosland and Votava, 2000) and localized in the vacuole of the epidermal cells within the placenta (Fujiwake et al., 1982). Capsaicinoids are synthesized from phenylpropanoid intermediates through the cinnamic acid

pathway (Figure 2) (Manirakiza et al., 2003). Seeds do not produce capsaicinoids, but may absorb them due to their proximity to the placenta.

Table 1. A selection of known capsaicinoids found in *Capsicum* species.

Capsaicin	Dihydrocapsaicin
Homocapsaicin	Homocapsaicin II
Homodihydrocapsaicin	Norcapsaicin
Homodihydrocapsaicin II	Nornorcapsaicin
3-nor-dihydrocapsaicin	Bis-homocapsaicin
Nordihydrocapsaicin	Tris-homocapsaicin
Nornordihydrocapsaicin	Tetra-homocapsaicin
Tetra-homodihydrocapsaicin	Bis-homodihydrocapsaicin

Adapted from Bosland and Votava (2000)

Capsaicinoid content varies from pepper to pepper based on genetics, maturity and the environment. It is known that the more stress a pepper endures while in the field, the higher level of capsaicinoids they will produce (Harvell and Bosland, 1997). Capsaicin levels within the fruit are known to increase steadily with time for up to 40 days after flowering, and then decrease drastically (Suzuki et al., 1980). It is suggested that pepper peroxidase, which is located mainly within the placenta of pepper fruits, oxidizes capsaicin (Bernal et al., 1993) and may be responsible for the degradation of capsaicinoids. *Capsicum* fruits show a variety of capsaicinoid content and the proportion between capsaicinoids are nearly impossible to predict (Zewdie and

Bosland, 2001). It is believed that capsaicin and dihydrocapsaicin represent nearly 80% of the capsaicinoid content within any fruit (Manirakiza et al., 2003).

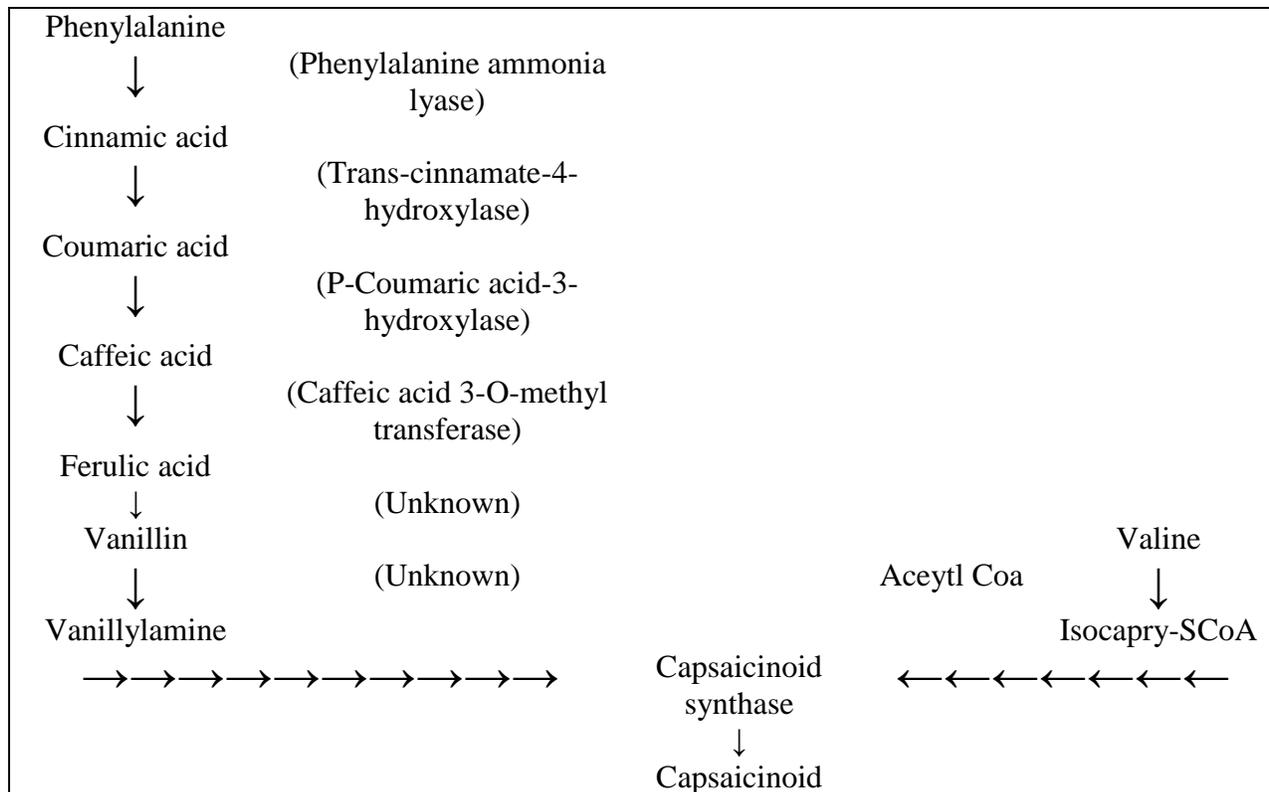


Figure 2. Capsaicinoid synthesis via the cinnamic acid pathway

The two most common ways to measure pungency level of a pepper are the Scoville organoleptic test and through high-performance liquid chromatography (HPLC) (Collins et al., 1995). Pepper pungency is expressed in Scoville Heat Units (SHU). A SHU is defined as the number of parts of sugar-water needed to neutralize the heat of one part sample extract. For example, if the heat of a cayenne pepper is 30,000 SHU, then 30,000 parts of sugar-water are needed to dilute one part of cayenne pepper extract to the last point that hotness can be detected (Manirakiza et al., 2003). This test was developed using a panel of five human subjects who tasted pepper samples and recorded the heat levels. A sample was diluted until pungency could

no longer be detected (Bosland and Votava, 2000). Limitations to this test are great. Although it has been reported that the different capsaicinoids are perceived differently during sensory (Krajewska and Powers, 1988), the Scoville test cannot distinguish between the levels of each capsaicinoid. The most common instrumentation for measuring capsaicinoid content and type is HPLC. HPLC is the standard method for routine analysis by the processing industry (Boland and Votava, 2000). To estimate the amount of SHU a pepper will have, you may multiply the capsaicinoid content in ppm by 15 (Bosland and Votava, 2000).

Capsaicinoid content is important in many industries because of its many uses. Capsaicin has proved useful because of its interactions with nerve receptors. When capsaicin comes into contact with nerve receptors, the receptors release a messenger called Substance P. Substance P signals to the brain that pain is present, and the nervous system then signals the brain to flood the nerve endings with endorphins. The pharmaceutical industry is interested in capsaicin as a topical treatment for sore muscles and joint pains. When applied to skin, capsaicin depletes the area of Substance P and prevents the nerves from making more Substance P. This diminishes pain signals or even completely eliminates pain (Carmichael, 1991). Capsaicin is also the main ingredient in many personal defense aerosols. The spray can cause an assailant to gasp for breath and be blinded for at least 20 minutes (Bosland and Votava, 2000). *Capsicum annuum* crude extracts are able to actively inhibit infection from the parasite *Schistosoma mansoni* which is estimated to infect over 200 million people worldwide. Because this study was done with a crude extract, capsaicin cannot solely be credited with anti-parasite activity.

***Capsicum annuum* as a Source for Natural Antimicrobials**

The potential uses for capsaicinoids may extend further than listed above. A small number of studies have shown extracts from *Capsicum* species fruit to possess antimicrobial activity. Fruits from several *Capsicum* species including *C. annuum*, *C. bacatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* were ground in a blender, centrifuged, and the supernatant was used to perform a filter disk assay to assess antimicrobial capability. These crude extracts were found to have inhibitory effects against *Bacillus cereus*, *Bacillus subtilis*, *Clostridium sporogenes*, *Clostridium tetani*, and *Streptococcus pyogenes* (Cichewicz and Thorpe, 1996). Jalapeño fruits were looked at specifically in this study and found to be inhibitory to *S. pyogenes*, *C. sporogenes*, and *C. tetani* and stimulate growth of *B. cereus* and *B. subtilis*. Interestingly, capsaicin (98% purity) purchased from Sigma was tested using the filter disk assay against the same bacteria, and no antimicrobial activity was found (Cichewicz and Thorpe, 1996). In another study, three *C. annuum* varieties were chosen for investigation of antimicrobial properties against *L. monocytogenes*, *S. aureus*, *S. enterica*, and *B. cereus* (Dorantes et al., 2000). Extracts of habanero, serrano, and pimiento morrón peppers were used for a sterile disk assay to determine antimicrobial activity of each pepper. *Listeria monocytogenes* was most sensitive to the extracts, followed by *B. cereus*, *S. aureus*, and *S. enterica* serovar Typhimurium. Interestingly, the pepper with the highest antimicrobial activity was the pimiento morrón, which does not have the highest capsaicin content. The extracts were separated using reverse phase HPLC analysis to determine the capsaicinoid content of each pepper. Phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin, and dihydrocapsaicin (Figure 3) were all eluted and tested as inhibitors for growth of the four bacteria. Capsaicin and dihydrocapsaicin did not show inhibitory effects on the bacteria, and coumaric and cinnamic acids caused the greatest inhibitory affect (Dorantes

et al., 2000). It is suggested that cinnamic acid may cause antimicrobial effects by inhibiting glucose uptake and ATP production with a bacterial cell (Dorantes et al., 2000).

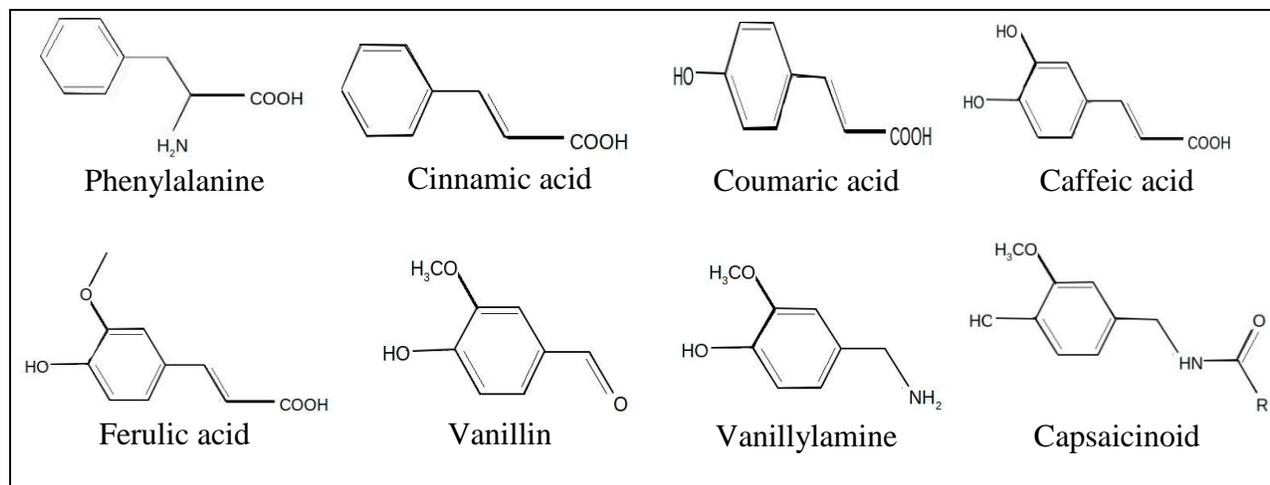


Figure 3. Structures of capsaicinoid intermediates produced via the cinnamic acid pathway

Although both of these studies suggest that the antimicrobial effect that is seen with some *Capsicum* fruits is not due to capsaicin, some studies show that capsaicin does have antimicrobial properties. Molina-Torres et al., (1999) purchased capsaicin from Sigma that consisted of 74.63% capsaicin, 15.79% dihydrocapsaicin, and 4.43% nordihydrocapsaicin and nonivamide. *E. coli*, *P. solanacearum*, *B. subtilis* and *Saccharomyces cerevisiae* were grown in liquid culture and capsaicin extract was added to the flasks containing the culture. Growth was monitored using a spectrophotometer. Capsaicin completely inhibited the growth of *B. subtilis* (a Gram-positive bacteria). Capsaicin inhibited the growth of *S. cerevisiae* after 4 h but showed little effect on the growth of *E. coli* and *P. solanacearum* (Molina-Torres et al., 1999). Alcohol steam distillation of *Capsicum frutescens* was performed by Ponce et al., (2008) to extract capsaicin, and an agar disk diffusion assay was used to determine that *L. monocytogenes* was inhibited by the extract. When the extract was incorporated with a chitosan film, *L.*

monocytogenes was not inhibited (Ponce et al., 2008). Synergistic effects were seen when *Capsicum* extracts were added to cheese to inhibit the growth of *S. aureus* by adjusting *Capsicum* extract levels and pH (Dorantes et al., 2008). *Staphylococcus aureus* was inhibited at all levels, but the most inhibition was seen at pH 5 and 5% *C. annuum* extract. Capsaicin has been observed as a potent inhibitor of *Helicobacter pylori* (Jones et al., 1997). An ethanol extract of the dried *C. annuum* spice proved to be antimicrobial against *B. subtilis*, *E. coli*, and *S. cerevisiae* (De et al., 1999). Extract from a bell pepper, which contains little capsaicin, was applied to raw beef in order to see the effect it would have against *S. enterica* and *Pseudomonas aeruginosa*. Ground beef was enriched in broth and qualitative results indicated that *Salmonella* was inactivated when 1.5 ml of extract was added to 100 g of raw ground beef, which was a lower MIC than observed for *P. aeruginosa* (3 ml extract per 100 g raw ground beef) (Careaga et al., 2003). In recognition that crop plants support a microflora of their own, Flagan and Leadbetter (2006) tested the effect of capsaicin and vanillylamine on the growth of bacteria that are hosted by plants. It was found that the bacteria that were naturally localized to the surfaces of *C. annuum* were capable of degrading capsaicin and using it as a growth substrate. This study showed that treatment of *C. annuum* plants with capsaicin would likely encourage growth of the plants natural microflora while potentially inhibiting the growth of non-native pathogens (Flagan and Leadbetter, 2006).

Foodborne Pathogenic Bacterial Interactions with Peppers

When it comes to bacterial attachment to a plant surface, there are many factors that can contribute to successful attaching and adhering. These factors may include polysaccharide expression, outer membrane protein expression, presence of flagella, fimbriae, and pili, cell

surface charge and hydrophobicity (Ukuku, 2002; Burnett, 2000). The conditions a plant is exposed to early in life may also have a role in bacterial interaction with a plant. For example, factors like water, manure, soil, insects, and animals may dictate the surface molecules that the plant will express. A single plant exhibits a variety of morphologies on its surfaces, such as roots, stems, leaves, flowers, and fruits that can provide a wide range of diverse environments for microorganisms to compete and survive. It has been noted that human pathogens like *E. coli* O157:H7 prefer to attach to the cut edges of lettuce leaves as opposed to the intact leaf (Takeuchi and Frank, 2000). A bruised or cut surface may leak fluids that contain not only nutrients, but also organic acids that could serve as antimicrobials. For some bacteria, the released fluid may serve as a chemotaxin. Therefore the survival of bacteria on a plants surface is not readily predictable.

The natural microflora of most vegetables consists of Gram-negative bacteria, and fruits are often naturally inhabited by yeasts and molds (Splittstoesser, 1987). It is possible for bacteria to attach and infiltrate plant tissue through stomata, lenticels, and bruises or cracks on the skin surface of a fruit or vegetable (Dingman, 2000). When a human pathogen infiltrates a plant surface, it can be found in intracellular spaces between plant cells. The plant does not react as if harmed when the infiltration occurs, nor does it try to fight or resist the human pathogen. The infiltration of human pathogens has been demonstrated sufficiently in apples (Burnett et al., 2000; Buchanan et al., 1999) and has also been observed in other produce products such as tomatoes (Guo et al., 2002), bean sprouts (Warriner et al., 2003), lettuce (Solomon et al., 2002; Watchel et al., 2002), and radish (Itoh et al., 1998). Internalization of pathogens presents an added obstacle for food safety, as these bacteria may be protected from sanitizers and rinses.

The conclusion that jalapeño peppers were a vehicle of pathogen transmission in the 2008 *S. enterica* Saintpaul outbreak was the first of its kind. This outbreak emphasized that additional measures of food safety are needed to reduce illnesses that occur from ingestion of raw produce (CDC, 2008). In fact, little is known about the survival and growth characteristics of *S. enterica* on jalapeño peppers (CDC, 2008).

On jalapeño peppers dip inoculated with *S. enterica* Saintpaul and held for 48 h, the bacteria was recovered from the stem and calyx portion in over 90% of peppers tested, and on the fleshy pod on less than 10% of peppers tested (Liao et al., 2010). After jalapeños were inoculated with 6 logs CFU/g on the stem and calyx and 3 log CFU/g on the fleshy pod, *S. enterica* Saintpaul rapidly decreased by 2 log CFU/g during the first week of storage at 4°C for both inoculation sites, but remained detectable on both the stem and calyx and the fleshy pod for all eight weeks (Liao et al., 2010). When held at 4°C, 12°C, and 21°C, a five strain *Salmonella* cocktail did not grow but survived on intact jalapeños (Ma et al., 2010). In chopped jalapeños, *S. enterica* grew readily at 12°C and 21°C, increasing 4 log CFU/g at each temperature over 8 days. At 4°C, *S. enterica* populations increased approximately 2 log CFU/g in chopped jalapeños. The behavior of both *S. enterica* and *E. coli* associated with jalapeños was investigated by Castros-Rosas et al., (2011). On whole, intact jalapeño peppers stored at both 4°C and 25°C, no bacterial growth was observed for either pathogen over six days of storage. When jalapeños were sliced and stored for 6 days, no growth for either pathogen was noted for jalapeños stored at 4°C, and both pathogens increased approximately 2 log CFU/g when stored at 25°C. The above studies are the only ones which have investigated the growth of *S. enterica* and pathogenic *E. coli* in association with jalapeños at different storage temperatures and lengths at the present time.

Listeria monocytogenes growth and survival on jalapeño peppers has not been investigated at this point in time.

A small number of studies have evaluated the survival of some foodborne pathogens on bell peppers. Stine et al. (2005) used bell peppers to study the survival of *E. coli*, *S. enterica*, *Shigella*, and *Clostridium* on produce with varying textures, amounts of foliage and overall plant structure. The inactivation rates of *S. enterica* on bell peppers, unlike cantaloupe and lettuce, were markedly higher in humid conditions opposed to dry conditions. The overall highest survival rate of all pathogens was associated with cantaloupe. In another study, bell pepper disks were used as a model to investigate the attachment of *S. enterica* Chester on plant tissue and to evaluate the effectiveness of trisodium phosphate as a sanitizing agent (Liao and Cooke, 2000). This study showed the *S. enterica* Chester rarely attached to unbroken skin surfaces of bell peppers, but were most closely associated (84%) with the surfaces of intentionally injured (cut) fruit skins. Less than 1% of *S. enterica* that were attached to pepper disks were associated with the outer skin. Cells attached to injured tissues were not effectively removed even with repeated washes of phosphate buffered saline (PBS), while 99.9% of the *S. enterica* cells introduced to the outer skin of the pepper disks were removed with two consecutive washes of PBS. This data suggests that the injuries peppers sustain not only from mechanical injury during harvest, but also during dicing or cutting can significantly increase vulnerability to contamination. In a study by Richert et al., (2000), *E. coli* O157:H7 was reduced by 2 Logs when inoculated into diced green peppers and held at 4°C for 7 days. Contradictory results were found when *S. enterica* was introduced to both bell pepper and jalapeño fruit extract, and grew readily in each (Nutt et al., 2003). It is evident that conflicting results have been gathered from various studies on bacterial interaction with peppers and their extracts and more work is needed in this area.

Although *Capsicum* fruits have only been associated with one large scale disease outbreak, it has been reported that *S. enterica* has been isolated from diced green peppers within a salad mix (Beuchat, 1996). *Listeria* species and *Yersinia* species have been associated with whole green peppers (Pingulkar et al., 2001). An assortment of fresh vegetables, including a variety of peppers, in marketplaces in New Jersey were sampled and analyzed for the presence of *S. enterica* (Wells and Butterfield, 1997). Healthy peppers were found to be associated with *Salmonella*-suspect bacteria in nearly 34% of the samples, while peppers that were soft rotted were associated with *S. enterica* in nearly 68% of the samples tested. This shows that although it is possible for *S. enterica* to be present on a healthy looking pepper fruit, the presence of soft rot bacteria significantly affected the presence of *S. enterica*. It was stated that the incidence of suspected *S. enterica* on produce affected by soft rot bacteria was twice that of healthy produce (Wells and Butterfield, 1997). In the same respect, a variety of peppers were tested along with other produce to determine if the incidence of *S. enterica* was affected by fungal rot or physical injuries (Wells and Butterfield, 1999). Bell peppers and cubanelle peppers were two times more likely to harbor suspect-*Salmonella* bacteria in mechanically injured fruits than sound, intact fruit. Fungal rot did not significantly increase the likelihood of *S. enterica* infection in most produce products studied.

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CHAPTER 3

Effect of Storage Temperature on Survival and Growth of Foodborne Pathogens on Whole, Damaged, and Internally Inoculated Jalapeños (*Capsicum annuum* var. *annuum*)

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ABSTRACT

There is a lack of general knowledge regarding the behavior of foodborne pathogenic bacteria associated with jalapeño peppers. The survival and growth behaviors of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica* on the interior and exterior of jalapeño peppers were determined under different storage conditions. Jalapeños were inoculated with a five strain cocktail of *L. monocytogenes*, *E. coli* O157:H7, or *S. enterica* on the intact external surface, injured external surface, or intact internal cavity of jalapeño peppers and held at 7°C or 12°C for a period of 14 days. Populations of each pathogen were determined at 0, 1, 2, 5, 7 10 and 14 days throughout storage. The uninjured, intact external surface of jalapeño peppers did not support growth of the pathogens tested under both storage conditions, with the exception of *L. monocytogenes* at 12°C. Populations of *E. coli* and *S. enterica* declined on the external injured surface of peppers at 7°C, but populations of *L. monocytogenes* remained consistent throughout the length of storage. At 12°C, *L. monocytogenes* and *S. enterica* populations increased throughout storage, and *E. coli* populations remained unchanged on injured surfaces. The uninjured internal cavity of the jalapeño supported growth of all pathogens at 12°C. Overall, *L. monocytogenes* was the microorganism most capable of growth and survival in association with jalapeño peppers for the scenarios tested. Results emphasize the importance of jalapeño pepper quality and proper storage conditions in preventing or reducing pathogen survival and growth.

The importance of fresh produce in the American diet is increasing, as consumers look to fruits and vegetables as a source of essential vitamins, minerals, and antioxidants (6). However, the presence of human pathogens on fresh produce is a recurring problem that has become more prevalent in the past decade, accounting for 12.3% of foodborne outbreaks between the 1990 and 2007 (16, 29). Produce is most commonly grown outdoors in a natural environment, which leaves it exposed to a variety of potential contamination sources including soil, water, manure, and animals in or near the field (28). Contamination of produce with pathogens can happen during growth in the field, during harvest, post-harvest handling, processing, and distribution (4). Fresh produce is most often consumed with little to no processing, eliminating any chance for a kill step to reduce pathogenic bacterial loads (17).

Recently, authorities misidentified the vehicle of a national outbreak of *S. enterica* serovar Saintpaul as tomatoes imported from Mexico. Trace backs found that the actual carriers of the contamination were jalapeño and serrano peppers (14). Salsas that contained multiple ingredients, including both fresh cut tomatoes and fresh cut jalapeños, were commonly the source of infection for those affected in the outbreak. Jalapeños were not considered initially, as they have not previously been identified as an outbreak vehicle (14). Although *Capsicum* fruits have only been associated with one large scale disease outbreak, *Salmonella* has been isolated from diced green peppers within a salad mix (4), and *Listeria* spp. and *Yersinia* spp. have been associated with whole green peppers (27). Previous studies have shown that healthy looking pepper fruits, as well as mechanically and biologically damaged peppers, are capable of harboring pathogenic enteric bacteria (36, 37). It is clear that jalapeño peppers are capable of carrying foodborne bacterial pathogens. However, little is known about how foodborne pathogens interact with jalapeño peppers under pre- and post-harvest conditions. The goal of this

research was to understand the growth behavior and survival capabilities of select foodborne pathogens of public health concern when introduced to jalapeño peppers and stored under abusive (12°C) and recommended storage temperatures (7°C).

MATERIALS AND METHODS

Bacterial cultures and culture conditions. Bacterial cocktails containing five strains of *E. coli* O157:H7, five strains of *L. monocytogenes*, or five serovars of *S. enterica* were used in this study. Detailed information on each can be found in Table 1. Each strain was made nalidixic acid resistant (50 ppm) and preserved in Tryptic Soy broth (TSB, Bacto, Difco, Becton Dickinson, Sparks, MD) containing 30% glycerol and 50 ppm nalidixic acid and stored at -80°C until use. Cells were activated by three successive 24 h transfers into TSB containing 50 ppm nalidixic acid (TSBN) and incubated at 37°C. After cells were activated, equal volumes of culture from each of five strains were combined to create a cocktail of approximately equal populations of each strain. The cocktail was then centrifuged (Sorvall Legend RT+, Thermo Scientific, Waltham, MA) at 2000 x g for 10 min at 22°C, the pellet resuspended in 0.1% peptone water, and washed twice more to yield a bacterial inoculum of approximately 10⁸ CFU/ml. This procedure was followed for each cocktail of *L. monocytogenes*, *E. coli* O157:H7, and *S. enterica*.

Raw material preparation. Unwaxed jalapeño peppers (*Capsicum annuum* var *annuum*, Jalapeño, cv. 'Del Mar') were purchased from a local grocery store from March through December 2010. The peppers were used the day of purchase. Peppers were submerged into 100 ppm chlorinated water for two minutes, rinsed twice with tap water, and patted dry with paper towels. Individual peppers were placed on sterile plastic trays, placed under a biosafety hood,

and allowed to equilibrate to room temperature for 30 min. Peppers were then designated into three groups (to be inoculated on the surface, in damaged portions, or internally) at random.

Inoculation of jalapeño peppers. The five strain bacterial cocktails were serially diluted in sterile 0.1% peptone water to achieve a cell population of approximately 10^7 CFU/ml for surface inoculated peppers and 10^5 CFU/ml for injured and internally inoculated peppers. For surface inoculation, each intact fruit was inoculated with 50 μ l of inoculum from one cocktail which was evenly distributed (10 μ l per spot) in five separate areas in a vertical line so the inoculum would not drip. For injured peppers, jalapeños were scored with a sterile scalpel in order to disrupt the surface of the fruit and expose the pericarp. Five horizontal cuts were made on the fruit and 50 μ l of inoculum was distributed evenly along the cut lines. Jalapeños were inoculated internally with a sterile syringe so that the needle pierced the surface and pericarp, and inoculum was deposited into the cavity of the fruit that holds the core and seeds. Fifty μ l of inoculum was deposited into the fruit through one insertion spot. Following inoculation, jalapeño fruits were placed in a laminar flow hood (NuAire, Plymouth, MN) for 1 h at room temperature ($25\pm 0.5^\circ\text{C}$) to dry. The final target population of all inoculated peppers was 10^3 CFU/g, which was achieved once bacterial populations decreased during drying. In preliminary experiments, the difference between bacterial populations at initial inoculation and final target populations after drying was determined using serial dilution and plating methods, and this data was used to choose the initial inoculation levels above for the different jalapeño inoculation conditions. The pathogen inoculum levels were chosen to represent a realistic contamination level of peppers while still allowing for recovery and enumeration of the pathogens using plating methods. All fruits were stored in polypropylene containers with polyvinyl chloride lids (Rubbermaid, Huntersville, NC) and placed in an environmental chamber (Precision, Thermo Scientific,

Waltham, MA) at 7°C or 12°C. Humidity within the containers was monitored throughout the study and ranged from 85-92% RH. Control samples were prepared as described above using sterile deionized water in place of bacterial cultures at inoculation sites.

Microbiological counts. Peppers were sampled at days 0, 1, 2, 5, 7, 10, and 14 of the study. Each test interval consisted of one control pepper (no bacteria added) and two inoculated peppers to be enumerated for the pathogen. Whole peppers were removed from storage and placed into sterile stomacher bags (Whirl-Pak, Nasco, Ft Atkinson, WI), weighed, and diluted 1:10 with 0.1% peptone water. Samples were then pummeled in a stomacher (Easy Mix, AES Laboratoires, Princeton, NJ) for 2 min. Homogenates were serially diluted into 0.1% peptone blanks and pour plated with Tryptic Soy agar (Difco, Becton Dickinson, Sparks, MD) supplemented with 50 ppm naladixic acid (TSAN) in duplicate. Plates were incubated at 37°C for 48 hours. When levels of pathogenic bacteria studied were expected to be lower than the detection limit, peppers were placed into enrichment broth containing 50 ppm nalidixic acid and incubated for 24 h at 37°C before being streaked onto appropriate selective and differential agar (Table 1) for confirmation of presence of the pathogen. In order to confirm the identity of bacteria recovered, colonies were picked at random from TSAN plates and streaked onto selective and differential agar. Presumptive positives were picked from selective and differential agar and confirmed with latex agglutination tests (RIM *E. coli* O157:H7, Remel, Lenexa, KS; *Salmonella* Latex Test, Oxoid, Basingstoke, Hants, UK; *Listeria* Latex Agglutination Kit, Microbiology International, Frederick, MD). Each experiment was repeated in triplicate (n=6).

Statistical analysis. Each experiment was replicated in triplicate, with duplicate samples tested at each sampling (n=6). Enumeration data were transformed into log values prior to analysis. Data were analyzed with analysis of variance with treatment and day of sampling as the

independent variables. Significant differences ($p < 0.05$) between means were determined using Tukey's honestly significant difference test. For each pathogen tested, means were compared for each treatment and day. Population means were compared for each pathogen within a treatment for each day. All analysis was performed using JMP version 9.0 software (SAS Institute, Inc., Cary, NC).

RESULTS

Uninoculated (control) jalapeños produced no growth on TSAN agar plates for all experiments. Colonies selected at random for identity confirmation via growth on selective and differential agar and latex agglutination confirmed pathogen identity on all TSAN agar plates in these experiments. Initial mean populations of pathogens inoculated onto jalapeños after the 1h drying period can be found in Table 2.

Growth and survival of *Listeria monocytogenes*. The population of *L. monocytogenes* associated with jalapeños inoculated on the surface and stored at 7°C decreased a total 1.36 log CFU/g over 14 days, with a final population of 1.94 log CFU/g. Jalapeño peppers inoculated with *L. monocytogenes* at injured sites decreased in population from day 0 to day 1 ($p=0.0008$), yet populations returned to original levels on day 5 ($p<0.0001$) and did not differ from Day 0 means throughout the remainder of the study (Figure 1a). For internally inoculated jalapeños stored at 7°C, the population of *L. monocytogenes* at Day 14 was 4.9 log CFU/g, an increase of 1.84 log CFU/g from Day 0. *Listeria* survival after 14 days of storage was significantly more pronounced when inoculated internally compared to surface ($p<0.0001$) or injured inoculated peppers ($p<0.0001$) stored at 7°C.

Listeria monocytogenes populations associated with jalapeños stored at 12°C inoculated at the surface did not change until day 14 (Figure 1b), where the population increased ($p=0.0226$)

to 4.35 log CFU/g, a 1.07 log CFU/g increase from day 0. Injured jalapeños supported growth of *L. monocytogenes* throughout storage (Figure 1b). At day 14, *L. monocytogenes* populations reached 4.08 log CFU/g, increasing 1.42 log CFU/g overall. Populations of *L. monocytogenes* did not change from the initial level for internally inoculated jalapeños stored at 12°C until day 7 (p=0.0059). The final *L. monocytogenes* count was 4.93 log CFU/g, an overall increase of 1.83 log CFU/g.

Growth and survival of *Escherichia coli* O157:H7. Populations of *E. coli* O157:H7 stored at 7°C survived without change for 2 days on the surface of the pepper, and populations decreased below the detection limit (<10 CFU/g) by day 5 (Figure 2a). *Escherichia coli* O157:H7 populations on injured jalapeño surfaces fell to levels below the detection limit by day 7. Survival of *E. coli* O157:H7 was more pronounced in internally inoculated peppers, as population were enumerable throughout day 10, and were detectable by enrichment at day 14.

Population of *E. coli* O157:H7 on surface inoculated peppers after the 14 day storage at 12°C was 1.63 log CFU/g, a reduction of 1.50 log CFU/g from initial levels. Jalapeño peppers inoculated on injured surfaces supported the survival of *E. coli* O157:H7, but no significant change in population was noted at any point throughout the 14 day storage period (Figure 2b). Populations of *E. coli* O157:H7 inoculated within jalapeños did not change from day 0 until day 10, and increased (p=0.0387) to a final population of 4.32 log CFU/g.

Growth and survival of *Salmonella enterica*. Populations of *S. enterica* inoculated on the surface and stored at 7°C fell below detectable levels on day 14 (Figure 3a). *S. enterica* introduced to injured jalapeño surfaces decreased by 2.01 log CFU/g from the initial population after 14 days. Internally inoculated jalapeños supported *S. enterica* survival until day 10, when populations fell below the detection limit.

Salmonella populations decreased to 1.36 log CFU/g on intact peppers held at 12°C, a decrease of 1.61 log CFU/g from initial populations. Jalapeños that were injured at the surface supported growth of *Salmonella* (Figure 3b), as populations reached 4.67 log CFU/g, an increase of 1.62 log CFU/g from Day 0 populations. Internally inoculated peppers supported a rapid growth increase ($p < 0.0001$) of *Salmonella*, as populations reached 4.35 log CFU/g after 1 day of storage. After 14 days, *Salmonella* populations reached 5.68 log CFU/g, a total increase of 2.51 log CFU/g.

DISCUSSION

Although *Salmonella* is the bacterial pathogen most associated with fresh produce related infections (20), other enteric pathogens such as *Listeria* and pathogenic *E. coli* have been associated with fresh produce (13, 15, 21, 22, 30, 33, 35). This research was performed to better understand the behavior of select foodborne pathogens in association with jalapeño peppers held at recommended (7°C) and abusive (12°C) storage temperatures. The outcome of this research may give insight on the potential of jalapeño peppers as a food safety risk in the future, and steps that can be taken in the future to reduce the risk of another outbreak.

In general, the surface of fresh produce is considered a harsh environment for foodborne pathogen survival (8). Our results indicate that jalapeño peppers with healthy, intact surfaces do not provide an environment for which the bacterial pathogens studied can grow at the storage temperatures examined, with the exception of *L. monocytogenes* held at 12°C. All other pathogens on the surface decreased in population throughout the duration of the study at both 7°C and 12°C. Although the populations of *E. coli* O157:H7 (7°C) and *Salmonella* (7 and 12°C) fell below the detection limit toward the end of the study, both pathogens were recovered after enrichment of the peppers. Other research has shown that intact surfaces of healthy produce

tissue may not provide the environment needed for foodborne pathogens to thrive (5). One study found that jalapeño peppers inoculated with *S. enterica* and stored at 4°C for 8 weeks resulted in *S. enterica* populations that declined throughout the study although populations did not fall below the detection limit (25). Populations of *E. coli* and *Salmonella* declined at both 4°C and 25°C over 6 days in a study by Castro-Rosas et al (10). In contrast to our study and those mentioned above, another study found that populations of *S. enterica* did not change when introduced to the surface of jalapeño peppers and held at 4°C and 12°C for one week (26). Differences in results between these studies may be due to a number of varying factors, including inoculation method, inoculation concentration, treatment of jalapeños (cleaning and washing), non-abusive storage temperature range (4°C vs. 7°C), or jalapeño cultivar.

Injured surfaces of fruits and vegetables make nutrients readily available for microorganisms, and injured produce is generally considered more of a food safety risk than intact produce. However, a bruised or cut surface may leak fluids that contain not only nutrients, but also organic acids that could serve as antimicrobials. Therefore the survival of bacteria on a plant's surface is not readily predictable. It has been noted that human pathogens like *E. coli* O157:H7 prefer to attach to the cut edges of lettuce leaves as opposed to the intact leaf (34), and exposure to injured surfaces may result in large pathogen population increases over a short time (8). Our results indicate that injured jalapeño surfaces do not provide the environment needed for survival of *E. coli* O157:H7 and *Salmonella* when stored at 7°C. *L. monocytogenes* populations were maintained at original inoculum levels throughout storage. This indicates that at recommended storage temperatures (7°C), *Listeria* present on the pepper may persist and cause safety concerns. At abusive storage temperatures, *L. monocytogenes* and *S. enterica* populations increased throughout storage, and *E. coli* populations remained unchanged on injured surfaces.

This indicates that abusive storage temperatures may be permissive to bacterial pathogen survival and growth if pepper surfaces become compromised. In a study by Ma et al, *S. enterica* associated with chopped jalapeños (as opposed to those with injured surfaces) did not change over 7 days at 4°C (26), compared to nearly a 2 log reduction at 7°C in our study. At 12°C, populations increased 5.3 log CFU/g. in the chopped jalapeño study, where populations in our study only increased by 1.74 log CFU/g. *Salmonella* was capable of better survival and growth in chopped jalapeños, as chopping exposes more surface area of the injured tissue to the bacteria, offering more nutrient availability. Liao observed that *Listeria* populations increased approximately 4 log CFU/g on green bell pepper disks immersed in *Listeria* and then held at 10°C for 8 days (25). Populations of *E. coli* O157:H7 (approx. 2.5 log cfu/g) decreased when held at 5°C over 14 days of storage when inoculated on shredded lettuce, sliced cucumber, and shredded carrot, but the pathogen was still detectable by enrichment on lettuce and shredded carrots (1). Populations of *E. coli* O157:H7 increased for each vegetable observed when stored at 12°C for 14 days (1). Our results add to the collection of knowledge emphasizing the importance of discarding unhealthy fresh produce starting at harvest and continuing to consumer purchase.

Not only are foodborne pathogens capable of surviving on plant surfaces, they have also been found to survive internally within some plants (3, 12, 18, 19, 24). The infiltration of human pathogens has been demonstrated sufficiently in apples (9, 11) and has also been observed in other produce such as tomatoes (19), bean sprouts (36), lettuce (32, 37), and radish (23). Our research shows that the internal cavity of jalapeños is an especially habitable environment at abusive temperatures, with all pathogens tested increasing in population as time progressed. In contrast, only *L. monocytogenes* was capable of growth in the cavity at 7°C, while *E. coli* and *S. enterica* population decreased below the detection limit. It is especially important that jalapeños

are held at the correct storage temperature (7°C) if internal contamination occurs, limiting the survival potential of enteric pathogens that might otherwise be inactivated by external washing or exposure to sanitizers.

The only scenario tested in our study where *L. monocytogenes* populations did not increase was on healthy, uninjured surfaces of jalapeños held at 7°C. Even in these conditions, *L. monocytogenes* populations remained in the detectable range. The only incidence of populations increasing at 7°C storage was *L. monocytogenes* inoculated internally. This study also demonstrated the importance of holding jalapeños at recommended storage temperature. Although temperature is a parameter that is easy to control and can be highly influential in controlling microbial growth, the temperature range at which a jalapeño can be held has restrictions. It is recommended that jalapeños be stored between 7 and 10°C (7, 31). If peppers are stored below 7°C, chill damage can occur (2), causing the plant cells to leak fluid and potentially making more nutrients available for bacteria associated with the plant. Storage at 7°C prevented growth of *E. coli* and *S. enterica* in all regions of the jalapeño fruit. Pathogen growth was most prominent in the internal cavity of jalapeños held at 12°C. In order to assure that jalapeños are safe throughout the supply chain, only healthy, uninjured fruits held at recommended storage temperatures should make it to the consumer. Our results provide an understanding of how select pathogens behave in association with jalapeño peppers and may be useful when considering proper handling, holding, and risk assessment of jalapeño peppers.

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Table 1. Bacterial strains and identification methods used in this study

Genus	Species/serovar	Source	Culture Identification methods
<i>Salmonella</i>	<i>enteric</i> Saintpaul	UGA ^a -jalapeño outbreak	XLT-4 agar (Difco, Sparks, MD)
	<i>enteric</i> Anatum K2669	CDC ^b -tomato	API 20E (bioMérieux, Marcy
	<i>enteric</i> Baildon	UGA ^a -lettuce/tomato	E'toile, France)
	<i>enteric</i> Newport 1893	CDC ^b -tomato	<i>Salmonella</i> Latex Agglutination
	<i>enteric</i> Javiana 2675	CDC ^b -tomato	
<i>Escherichia coli</i>	O157:H7	UGA ^a -lettuce	Sorbitol MacConkey agar (Difco,
	O157:H7 F4546	UGA ^a -alfalfa sprouts	Sparks, MD)
	O157:H7	UGA ^a -cider	API 20E
	O157:H7 994	UGA ^a - beef	RIM <i>E. coli</i> Latex Agglutination
	O157:H7 E0019	UGA ^a -beef	
<i>Listeria</i>	<i>monocytogenes</i> ScottA	UGA ^c	Oxford medium base with
	<i>monocytogenes</i> V7	CDC ^b	Modified Oxford antimicrobial
	<i>monocytogenes</i> L-CDC	CDC ^b	supplement (Difco, Sparks, MD)
	<i>monocytogenes</i> D43	Unknown	API <i>Listeria</i> (bioMérieux, Marcy
	<i>monocytogenes</i> 2289	Unknown	E'toile, France) <i>Listeria</i> Latex Agglutination

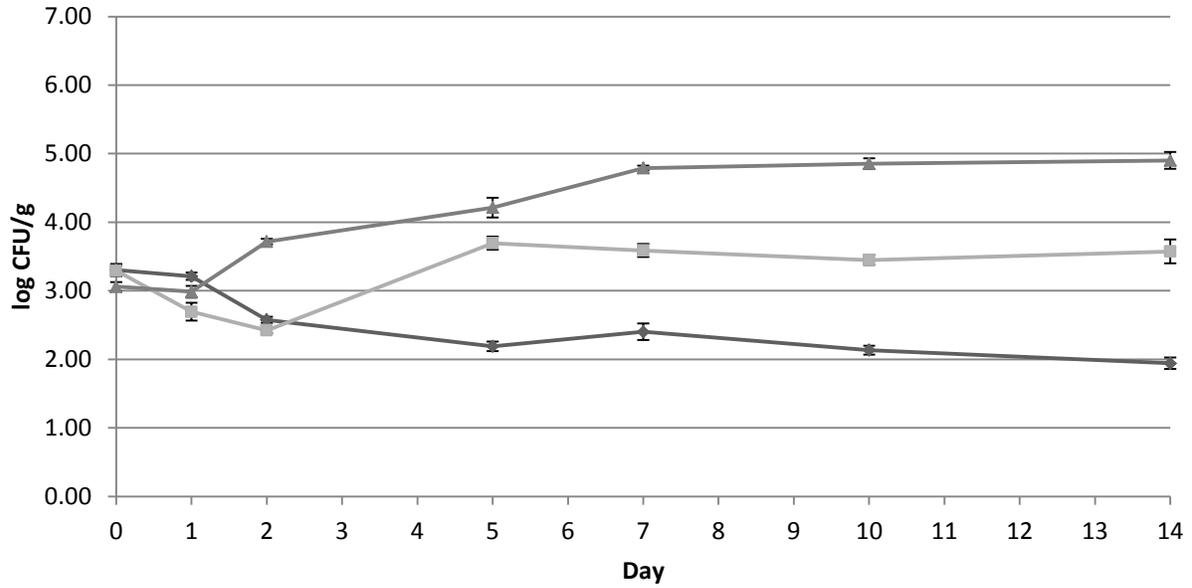
^a Provided by Dr. L. R. Beuchat at the University of Georgia, Griffin, GA. ^b Provided by the Center for Disease Control and Prevention, Atlanta, GA. ^c Provided by Dr. R E. Brackett while at the University of Georgia, Griffin, GA.

Table 2: Initial population (log CFU/g) of inoculated pathogens after 1h drying period

	7°C			12°C		
	Surface	Injured	Internally	Surface	Injured	Internally
	Inoculated	Inoculated	Inoculated	Inoculated	Inoculated	Inoculated
<i>Listeria</i>						
<i>monocytogenes</i>	3.30±0.09	3.29±0.08	3.06±0.07	3.28±0.13	2.67±0.15	3.10±0.16
<i>Escherichia</i>						
<i>coli</i> O157:H7	2.85±0.11	2.71±0.05	3.18±0.08	3.13±0.05	3.07±0.03	3.21±0.05
<i>Salmonella</i>						
<i>enterica</i>	2.60±0.07	3.25±0.03	3.15±0.06	2.87±0.09	3.05±0.04	3.16±0.06

Figures

A)



B)

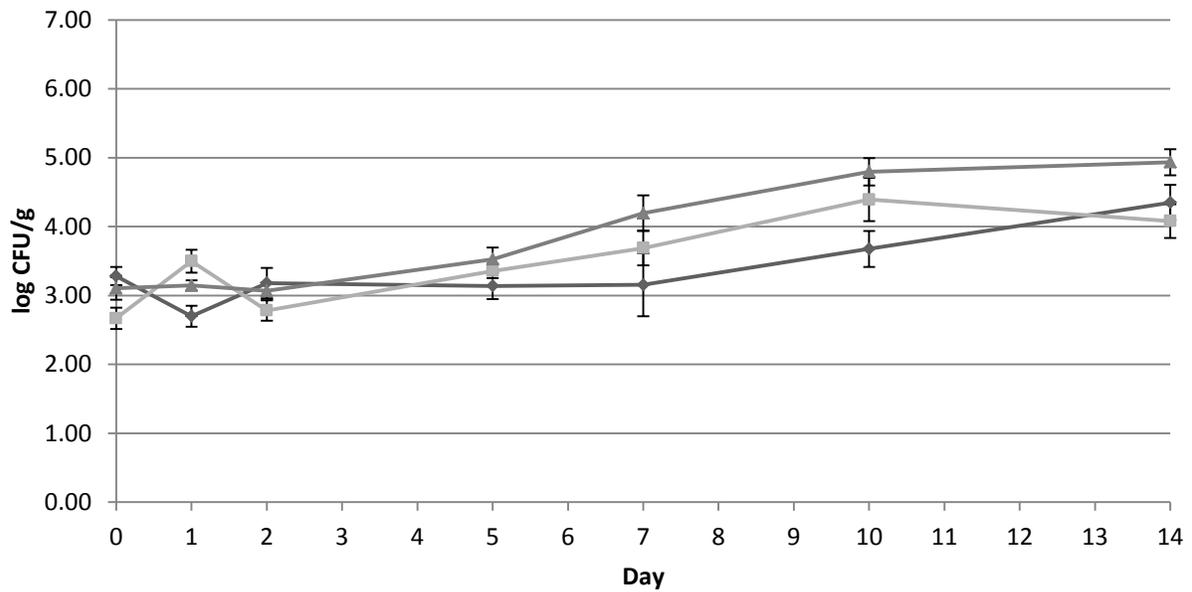
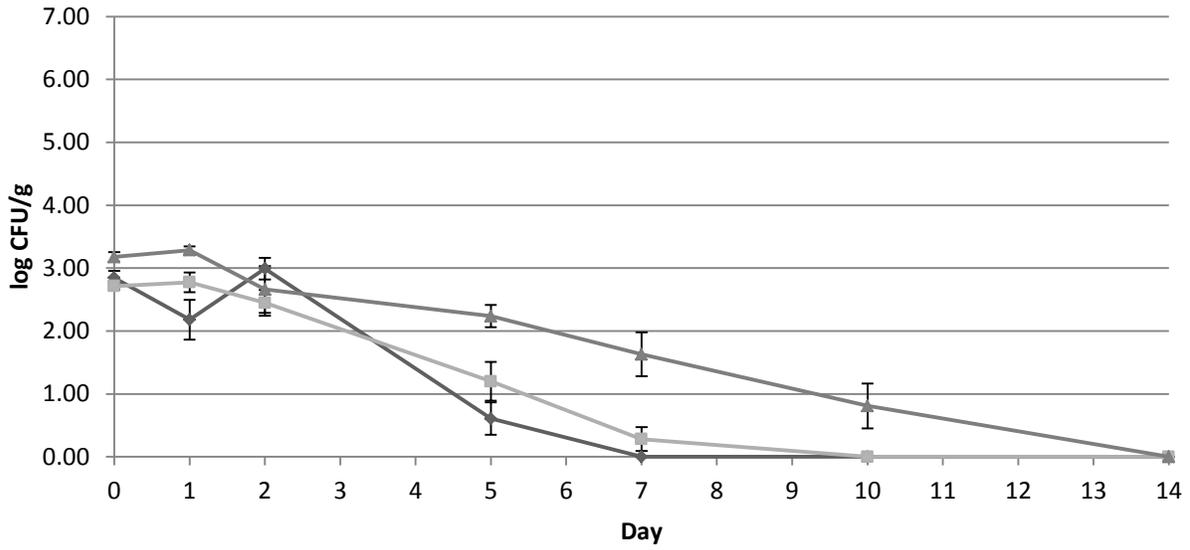


FIGURE 1. Behavior of a five strain *Listeria monocytogenes* cocktail inoculated on the intact surface (◆), injured surface (■), or internal cavity (▲) of jalapeño fruit and stored for 14 days at (A)7°C and (B)12°C (n=6). Error bars represent standard error of the mean.

A)



B)

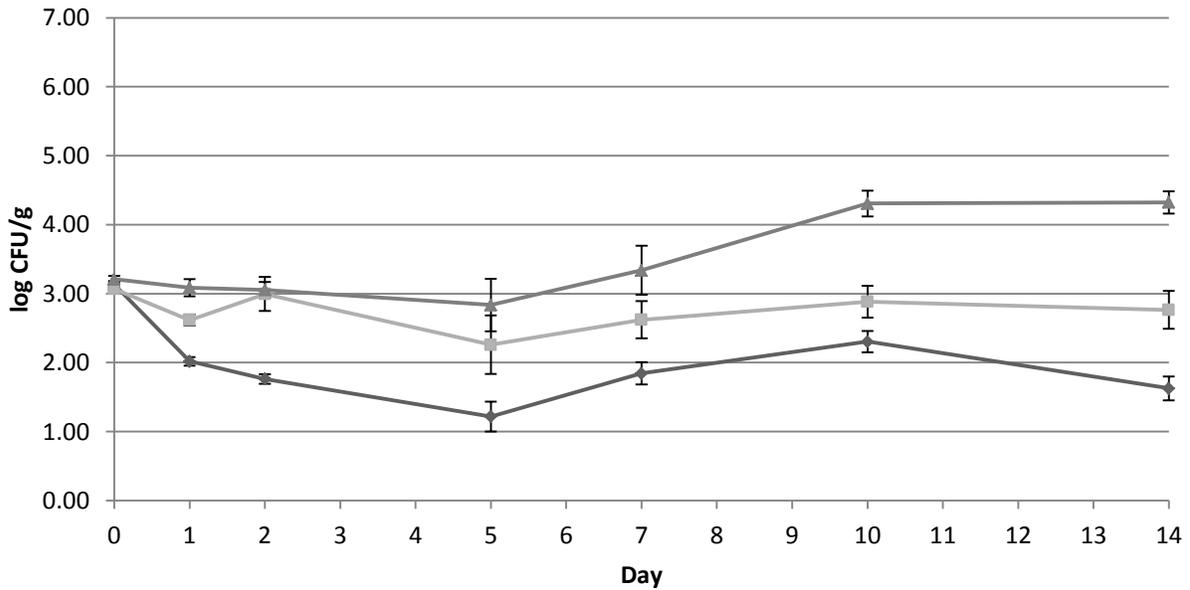
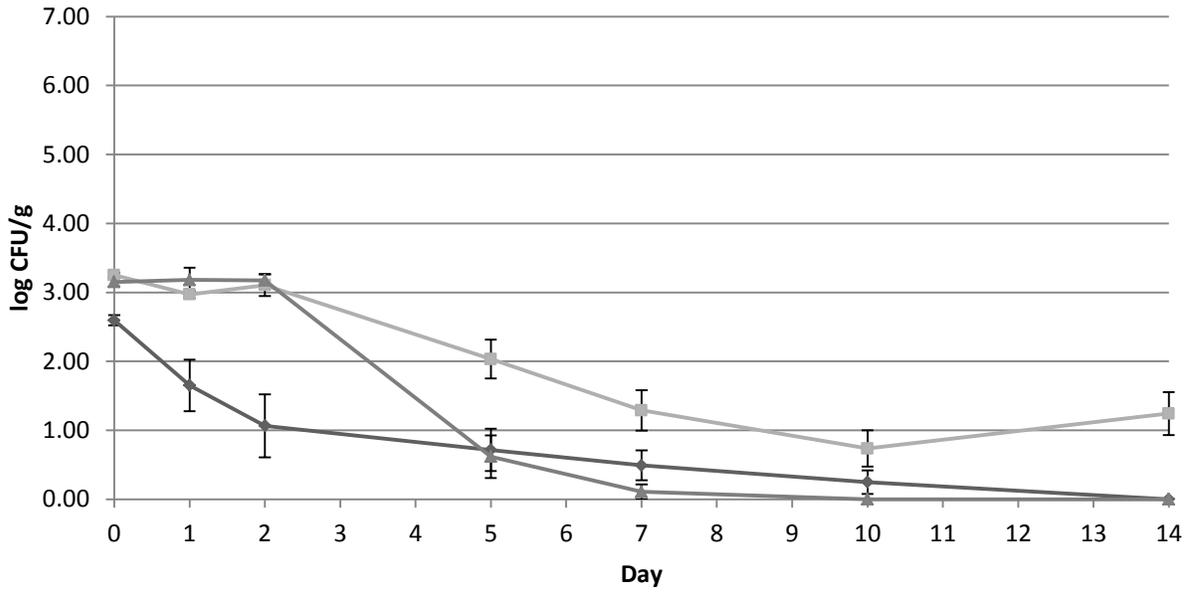


FIGURE 2. Behavior of a five strain pathogenic *Escherichia coli* cocktail inoculated on the intact surface (◆), injured surface (■), or internal cavity (▲) of jalapeño fruit and stored for 14 days at (A)7°C and (B)12°C (n=6). Error bars represent standard error of the mean.

A)



B)

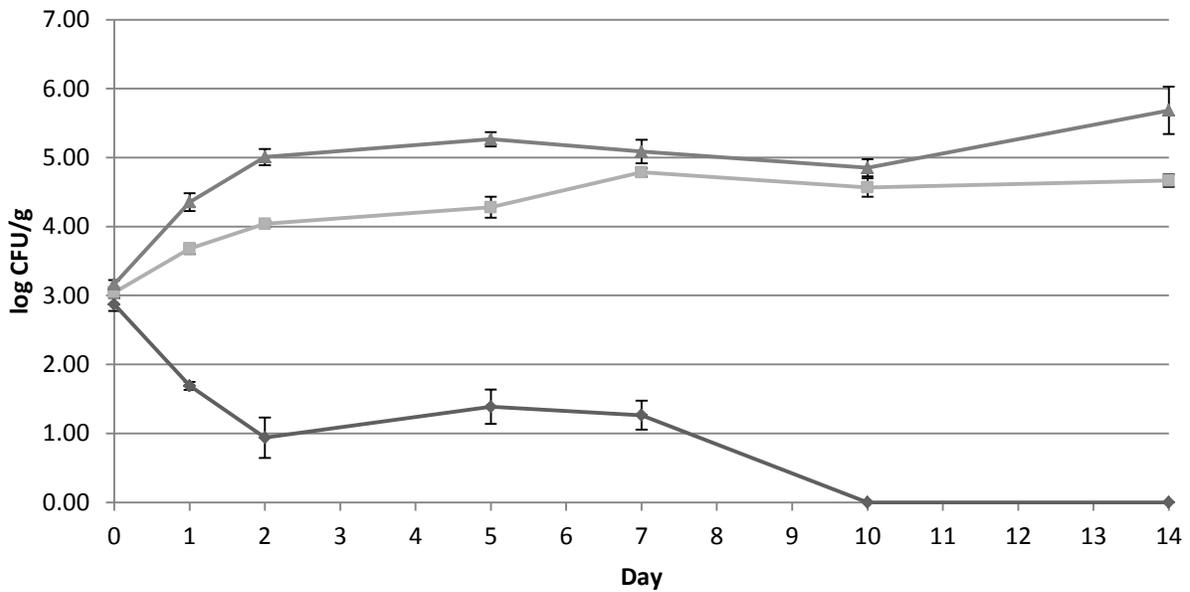


FIGURE 3. Behavior of a five strain *Salmonella enterica* cocktail inoculated on the intact surface (◆), injured surface (■), or internal cavity (▲) of jalapeño fruit and stored for 14 days at (A)7°C and (B)12°C (n=6). Error bars represent standard error of the mean.

CHAPTER 4

Evaluation of the Antibacterial Potential of Jalapeño Peppers using Different Extraction Solvents

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Running Title: Evaluation of extraction solvents for antibacterial compounds using jalapeño
peppers.

Key words: Antibacterial, *Capsicum annuum*, jalapeño, solvent, extraction

Abstract

A small number of studies have indicated that chile peppers (*Capsicum* spp.) possess antibacterial properties, although the compound most commonly investigated from peppers, capsaicin, appears to not be responsible. The complete chile pepper extract should be evaluated for its antibacterial effect. The type of solvent used for extraction can affect the compounds that are isolated. The purpose of this study was to identify which solvent is most successful at extracting unknown antibacterial compounds from jalapeño peppers. Fresh jalapeño peppers were chopped, weighed, and placed in a blender with a solvent (either sterilized hot water, 70% methanol, 95% methanol, 70% ethanol or 95% ethanol) at a 1:1 ratio (g/g) and blended until the mixture was completely homogenized, followed by shaking for 15 min. The slurry was centrifuged; supernatant was removed and immediately used for antibacterial testing using disk diffusion assay against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica*. The diameter of growth inhibition was measured and statistically evaluated using ANOVA to determine the most antibacterial extract. Solvents were tested alone as a control. There was greater bacterial inhibition from methanol and ethanol extracts than hot water extracts. The strongest inhibition was seen from the ethanol extract against *E. coli*. Each solvent extract was then analyzed using HPLC and fractions were collected and used for disk diffusion analysis against *L. monocytogenes*. Although it was clear from the disk diffusion assay that the fractions that contained the most antibacterial activity were Fraction E and Fraction F, no differences were noted between solvents used ($p=0.05$). Further research should be performed to fractionate the extract and identify exact antibacterial compounds. This research could possibly lead to identification of new natural antibacterial treatments for foods.

To combat contamination and spoilage, the food industry often relies on food additives to suppress unwanted microbial growth (Davidson and Taylor, 2007). Recently, there has been a resurgence of interest in natural antimicrobial compounds due to consumer concern for all-natural or organic food products. Currently, foods are commonly preserved by compounds such as nitrite, sodium benzoate and sodium metabisulfite that have been tested and proven safe (Gould and Russell, 2003). However, there are occasional reports of allergic reactions to these preservatives, and even potential formation of carcinogenic by-products like nitrosamines from nitrite (Roller, 2003). Essential oils isolated from some plant sources have been found to be effective antimicrobial agents (Rupasinghe et al., 2006; Cerrutti et al., 1997; Ngarmsak et al., 2006; Nascimento et al., 2000), and there is ongoing research to identify more antimicrobial plant sources.

Extracts from *Capsicum annuum* fruit (hot peppers) have been investigated to some extent, and antimicrobial properties have been reported with mixed results. The belief that hot peppers possess healing powers is well known in multicultural folklore (Andrews, 1995), but little scientific fact exists to support these claims. Crude tissue extracts from several different *C. annuum* varieties have growth inhibiting properties against species of *Bacillus*, *Clostridium*, and *Streptococcus* (Cichewicz and Thorpe 1996). Extracts from *C. annuum* fruits using isopropanol as a solvent were also found to inhibit the growth of *L. monocytogenes*, *Salmonella enterica*, *Bacillus*, and *Staphylococcus* in liquid media to varying degrees (Dorantes et al., 2000). Contradictory results found that both bell pepper and jalapeño fruit extract failed to inhibit *S. enterica* (Nutt, 2003). Differences in results may be due to differences in the type of *C. annuum* plants tested (ex: bell pepper, pimiento pepper, scotch bell, etc.), the different methods used to test for inhibition, and differences in preparing extract samples. Little is known about

antimicrobial properties of *C. annuum* extract when tested against various foodborne pathogens. The purpose of this study was to explore the antimicrobial potential of jalapeño peppers using a disk diffusion assay, and to determine the best solvent type and concentration for extraction of unknown antibacterial compounds from jalapeño peppers.

Materials and Methods

Bacterial cultures and culture conditions. Fifteen bacterial cultures were used in this study; five *Listeria monocytogenes*, five *Escherichia coli* O157:H7, and five *Salmonella enterica* isolates. Details of each strain used can be found in Table 1. Bacterial cultures were preserved in a Tryptic Soy broth (TSB, Bacto, Difco, Becton Dickinson, Sparks, MD) solution containing 30% glycerol and stored at -80°C until use. Cells were activated by three successive 24 h transfers into TSB containing 50 ppm nalidixic acid (TSBN) and incubated at 37°C. Activated cells were centrifuged (Sorvall Legend RT+, Thermo Scientific, Waltham, MA) at 2000 x g for 10 min at 22°C, the pellet resuspended in 0.1% buffered peptone water, and washed twice more to yield a bacterial cocktail of approximately 8.0 log CFU/ml. Cultures were diluted ten-fold into sterile peptone water and this dilution was used in the disk diffusion assays.

Preparation of jalapeño extracts using no solvent. Extract made using no solvent was prepared following procedures outlined by Cichewicz and Thorpe (1996) with modifications. Fresh jalapeño peppers were purchased from a local grocery store in Blacksburg, VA and rinsed with 100 ppm chlorine water for 2 min while shaking by hand. Peppers were then rinsed with sterile water and diced with a knife. Ten jalapeño peppers (approximately 200 g) were placed into a Waring blender (Waring, New Hartford, Conn.) and blended until a homogenous slurry was obtained (approx. 1 min). The slurry was placed into a filter lined stomacher bag and 50 ml of filtered liquid extract was removed from the bag using a 10 ml pipette. The extract was placed

into a centrifuge tube and centrifuged at 15,000 x g for 10 min. The supernatant was collected and centrifuged twice more under the same conditions. The supernatant was removed and passed through a 0.45µm pore size filter to sterilize (Whatman Inc., Piscataway, NJ). Extracts were used immediately following preparation.

Preparation of jalapeño extracts using a solvent. Solvents used for this study were boiling (98°C) water, ethanol (70% and 95% (v/v)), and methanol (70% and 95% (v/v)). Jalapeños were purchased, rinsed, and diced as described above. Jalapeños were added to a Waring blender, and solvent was added at a ratio of 1:1 (wt/wt). Jalapeños and solvent were blended for approximately 1 min until a homogenous slurry was obtained. The slurry was poured into a 500 ml beaker. Controls for each solvent extract were prepared as described above, replacing jalapeño weight with charcoal and omitting the blending step. Both experimental and control beakers were covered with aluminum foil and placed in an orbital shaker for 24 h at room temperature. After 24 h, extracts were poured into filter lined stomacher bags and prepared as described above. Extracts were immediately used for disk diffusion assays.

Reverse phase high performance liquid chromatography of jalapeño extracts. Analyses of the solvent and non-solvent extracts were performed using a reverse phase HPLC technique employing an Agilent 1200 Series HPLC (Santa Clara, CA) consisting of degasser, quaternary solvent pump, autosampler with refrigeration, column oven, and a diode array detector and a Phenomenex (Torrance, CA) Luna 5µ C18 (250 x 4.6mm) column with a Phenomenex Security Guard column. An acetonitrile gradient consisting of two solvents: solvent A (0.1% aqueous acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile) was used. Flow rate was 1.0 ml/min. The sample injection was 100 µl. UV absorbance was recorded at 280 nm. Compounds from the jalapeño extract were collected as they eluted from the HPLC column

in five minute increments into clean glass centrifuge tubes. This was repeated for each of the different solvent extracts.

Preparation of HPLC fractions for disk diffusion assays. Collected fractions were placed under a fume hood and acetonitrile was evaporated from fractions using a stream of nitrogen gas. One ml of sterile deionized water was then added to each tube (to dilute any remaining acetonitrile), tubes were capped, and placed into the freezer ($-18\pm 2^{\circ}\text{C}$) for approximately 3 h until samples were frozen solid. Caps were then removed from tubes and cheese cloth was secured over the tube openings with a rubber band. Samples were placed into a freeze dryer (Virtis, The Virtis Company Inc., Gardiner, New York) and dried for approximately 18 h until all liquid was removed from the samples and an off-white powder could be detected at the bottom of the tubes (not all tubes had powder). Fractions were resuspended in sterile deionized water to achieve a concentration of approximately 100 ppm. Fractions were used immediately, or stored at 4°C until ready for use in antimicrobial assays.

Disk diffusion assays. A disk diffusion assay was performed following recommendation by Vigil et al. (2005) with some modifications. Whatman #2 filter paper (Whatman Inc., Piscataway, NJ) was used in this assay. A hole punch was used to produce 6.5 mm diameter filter disks. The disks were collected and autoclaved prior to use. Bacterial cultures grown overnight in TSB broth were diluted in 0.1% sterile peptone water to achieve a concentration of approximately $7.0 \log \text{cfu/ml}$ (confirmed by serial dilution and pour plating with TSA). Cultures were spread plated (0.1ml) onto TSA plates. Plates were allowed to dry for 10 min. Flame-sterilized tweezers were used to place filter disks onto inoculated TSA plates, one disk in each of four equal quadrants. Each disk on the TSA agar was then impregnated with $10 \mu\text{l}$ of either liquid extract treatment or control (two control disks and two extract disks per plate). For

controls, 100 µl of relevant solvent was injected into the HPLC, collected, and processed as described above. Two plates were prepared for each unique culture and solvent combination (n=4). Plates were inverted and incubated for 24 h at 37°C. Zones of inhibition were measured in mm with a digital caliper. Each experiment was run three times (N=12).

In addition, a filter disk assay was performed in order to rule out the antimicrobial activity attributed to the pH of the extract. The pH of the crude jalapeño extract was determined to be 5.67. Based on this measurement, an experimental sample was prepared by filter sterilizing deionized water with an adjusted pH of 5.67. A control test sample was made using sterile deionized water (pH 7.40). Both experimental and control samples were used for a disk diffusion assay, and inhibition results were compared. No inhibition was observed due to pH.

Statistical analysis. The diameter of growth inhibition was statistically evaluated using ANOVA as well as Tukey's Honestly Significant Difference to compare mean zones of inhibition for jalapeño extracts and controls. All analysis was performed using JMP 7.0 statistical software (SAS Institute, Cary, NC).

Results and Discussion

A small number of studies have reported antimicrobial activity from *Capsicum* species fruit (Dorantes et al., 2000; Cichewicz and Thorpe, 1996). *Capsicum* fruit extracts were used in a filter disk diffusion assay, and inhibitory effects were observed against *Bacillus cereus*, *B. subtilis*, *Clostridium sporogenes*, *Cl. tetani*, and *Streptococcus pyogenes* (Cichewicz and Thorpe, 1996). Extracts from jalapeño fruits were specifically inhibitory to *S. pyogenes*, *Cl. sporogenes*, and *Cl. tetani* but stimulated growth of *B. cereus* and *B. subtilis*. Capsaicin (98% purity)

purchased from Sigma was tested using the filter disk assay against the same bacteria, and no antimicrobial activity was found (Cichewicz and Thorpe, 1996).

In another study, extracts of *C. annuum* varieties were used for a sterile disk assay (Dorantes et al., 2000). *Listeria monocytogenes* displayed the most inhibition due to the extracts, followed by *B. cereus*, *S. aureus*, and *S. enterica* Typhimurium. The extracts were separated using reverse phase HPLC analysis to determine the content of compounds found in the capsaicinoid pathway for each pepper type. The content of phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin, and dihydrocapsaicin were all determined, and tested as inhibitors for growth of the four bacteria. Capsaicin and dihydrocapsaicin did not show inhibitory effects on the bacteria, and coumaric and cinnamic acids caused the greatest inhibitory affect (Dorantes et al., 2000). It is suggested that cinnamic acid may cause antimicrobial effects by inhibiting glucose uptake and ATP production within a bacterial cell (Dorantes et al., 2000).

Our results showed that jalapeño extracts displayed antibacterial activity. The mean diameters of the inhibition zones of all solvent extracts against *L. monocytogenes*, *S. enterica*, and *E. coli* are shown in Figures 1-3. There were significant differences between solvent extracts and controls ($p < 0.05$) for each solvent extract tested with the exception of water extract. The jalapeño extract obtained without using a solvent exhibited antibacterial activity, especially evident against *L. monocytogenes* (Figure 1a).

In agreement with the above studies, Dorantes et al. (2000) found that *L. monocytogenes* was the most sensitive to extracts of different *C. annuum* peppers when compared to *B. cereus*, *S. aureus*, and *S. enterica* Typhimurium. Our results showed that *L. monocytogenes* cultures were the most consistently inhibited by the extracts, producing measurable zones of inhibition for each solvent tested except for hot water. The largest zones of inhibition associated with *L.*

monocytogenes (11 mm) were observed with jalapeño extract obtained without solvent, as well as with 95% methanol and 95% ethanol extracts. The extract using 95% ethanol solvent was the most successful inhibitor of *E. coli* and *S. enterica*. Methanol was the most promising solvent for extracting anti-Listerial compounds based on differences between experimental and control zones of inhibition. Both concentrations of the methanol solvent tested were successful, but the 95% methanol solvent resulted in the greatest differences in inhibition between controls and extracts (Figure 2).

The one solvent tested that was effective at inhibiting all bacteria in this study was the 95% ethanol solution (Figure 3b). Controls were run in parallel to ensure inhibition can be properly attributed to the extract rather than the alcohol solvent. However, a large amount of inhibition was seen with the controls for this experiment. Although it is possible that the ethanol solvent is extracting antimicrobial compounds that are accounting for the large zones of inhibition observed, the use of 95% ethanol for further studies must be cautioned due to the high antibacterial activity of the control. For this reason, 95% ethanol will not be used to pursue studies in this area.

Of the different solvents and concentrations tested, there was no one combination that inhibited all bacteria tested (*L. monocytogenes*, *S. enterica*, and *E. coli*) in the most efficient manner. Inhibition response to extracts was not uniform across members of the same bacterial genus. The most sensitive species for each genus were *L. monocytogenes* 2289, *Salmonella* Anatum, and *E. coli* Cider, respectively.

The extracts were analyzed with HPLC to display the differences in compound extraction achieved by using different solvents. A comparison of the different HPLC chromatograms produced by the different solvent extracts can be seen in Figure 4. The chromatogram shows that

the solvents did cause some variation between extraction methods. However, the overall chromatograms look similar. In order to test whether the different solvents made a difference when the extracts were collected as fractions off the HPLC column, fractions of each solvent extract were collected and tested against *L. monocytogenes* using the disk diffusion assay. *Listeria monocytogenes* was chosen due to the sensitivity it displayed in disk diffusion assays with the unfractionated compounds.

Results for the disk diffusion assay using the fractionated extracts for *L. monocytogenes* revealed that Fraction E and Fraction F contained the most active inhibiting compounds (Figure 5). Comparison of the different solvents used and inhibition revealed the 70% MeOH fractions did not produce zones of inhibition for *L. monocytogenes*. All other extracts produced zones of inhibition for *L. monocytogenes*. Because the same amount of inhibition was displayed for the extract using no solvent as the extracts that were made using solvents (with the exception of 70% MeOH), it was determined that all future studies would be carried out using jalapeño extract without the use of a solvent to prepare the extract in order to remove an extra step in the preparation process.

Conclusion

Jalapeño peppers are a promising resource for natural antibacterial components, especially for inhibition of *L. monocytogenes*. Our study builds upon the research to indicate that jalapeño peppers contain potential antimicrobial compounds. Although a number of solvents were tested for extraction of these antimicrobial compounds, HPLC analysis and disk diffusion assays showed that there is little difference in the antimicrobial activities between the solvents used for extraction. This indicates that the differences seen between control and experimental

unfractionated extracts were likely due to the solvent attributing to inhibition rather than the compounds extracted. *Listeria monocytogenes* exhibited the highest sensitivity to the extracts in all the studies conducted. Therefore, further studies should focus on the isolation and identification of compounds that may be contributing to inhibition of pathogenic foodborne bacteria, especially *L. monocytogenes*.

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Tables

Table 1. Bacterial strains and identification methods used in this study

Genus	Species/serovar	Source	Culture Identification methods
<i>Salmonella</i>	<i>enteric</i> Saintpaul	UGA ¹ -jalapeño outbreak	XLT-4 agar (Difco, Sparks, MD) c API 20E (bioMérieux, Marcy E'toile, France) <i>Salmonella</i> Latex Agglutination
	<i>enteric</i> Anatum K2669	CDC ² -tomato	
	<i>enteric</i> Baildon	UGA ¹ -lecttuce/tomato	
	<i>enteric</i> Newport 1893	CDC ² -tomato	
	<i>enteric</i> Javiana 2675	CDC ² -tomato	
<i>Escherichiacoli</i>	O157:H7 H1730	UGA ¹ -lettuce	Sorbitol MacConkey agar (Difco, Sparks, MD) API 20E RIM <i>E. coli</i> Latex Agglutination
	<i>coli</i> O157:H7 F4546	UGA ¹ -alfalfa sprouts	
	<i>coli</i> O157:H7	UGA ¹ -cider	
	<i>coli</i> O157:H7 994	UGA ¹ - beef	
	<i>coli</i> O157:H7 E0019	UGA ¹ -beef	
<i>Listeria</i>	<i>monocytogenes</i> ScottA	UGA ³	Oxford medium base with Modified Oxford antimicrobial supplement (Difco, Sparks, MD) API <i>Listeria</i> (bioMérieux, Marcy E'toile, France) <i>Listeria</i> Latex Agglutination
	<i>monocytogenes</i> V7	CDC ²	
	<i>monocytogenes</i> L-CDC	CDC ²	
	<i>monocytogenes</i> D43	Unknown	
	<i>monocytogenes</i> 2289	Unknown	

¹ Provided by Dr. L. R. Beuchat at the University of Georgia, Griffin, GA. ² Provided by the Center for Disease Control and Prevention, Atlanta, GA. ³ Provided by Dr. R E. Brackett while at the University of Georgia, Griffin, GA.

Figures

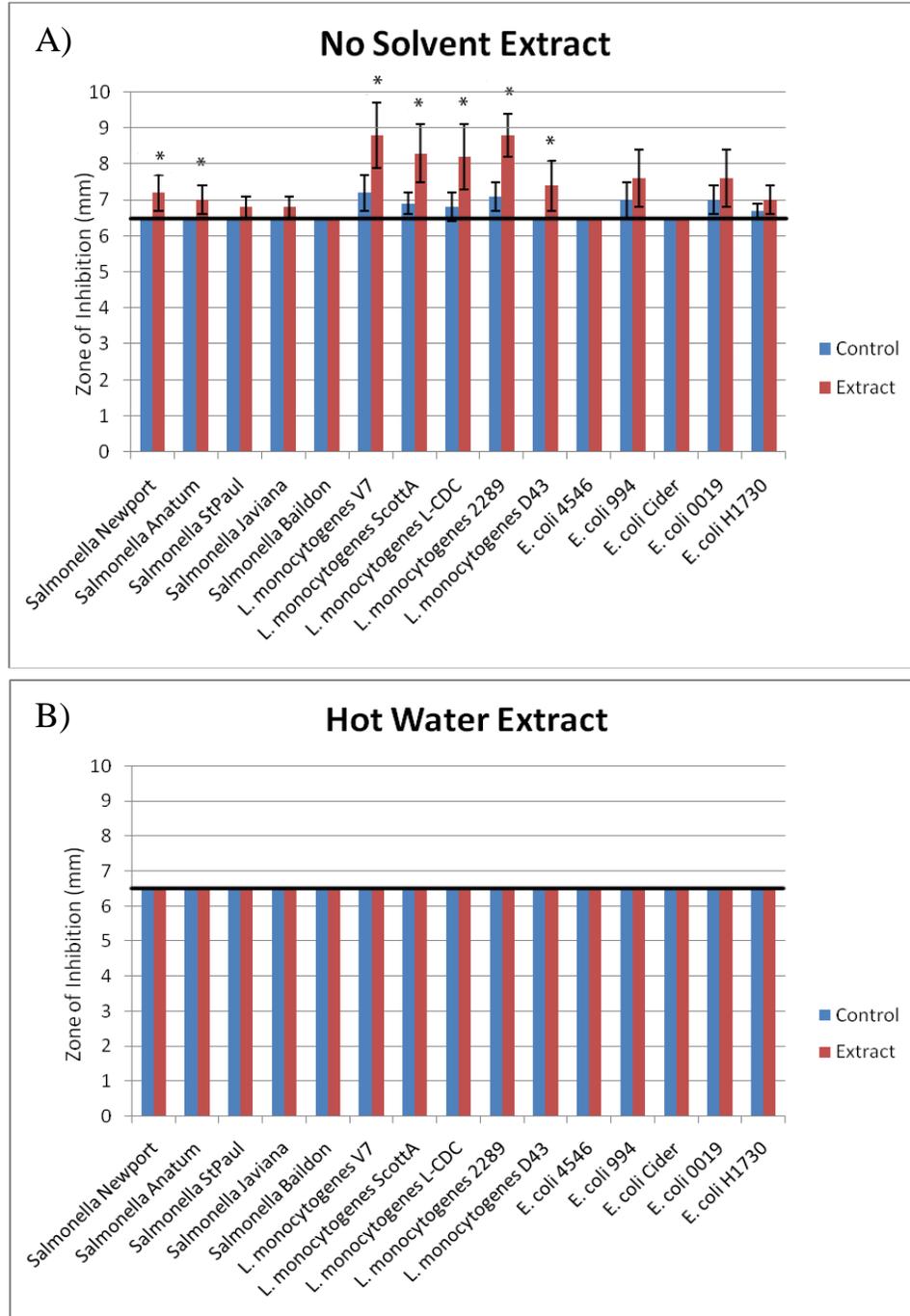


Figure 1. Mean zones of inhibition for bacterial cultures in association with jalapeño extract made with A) no solvent and B) hot water as a solvent. Sterile deionized water was used for the control in both assays. Asterisks represent measurements where zones of inhibition for solvent extracts are significantly different than zones of inhibition for their respective controls. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

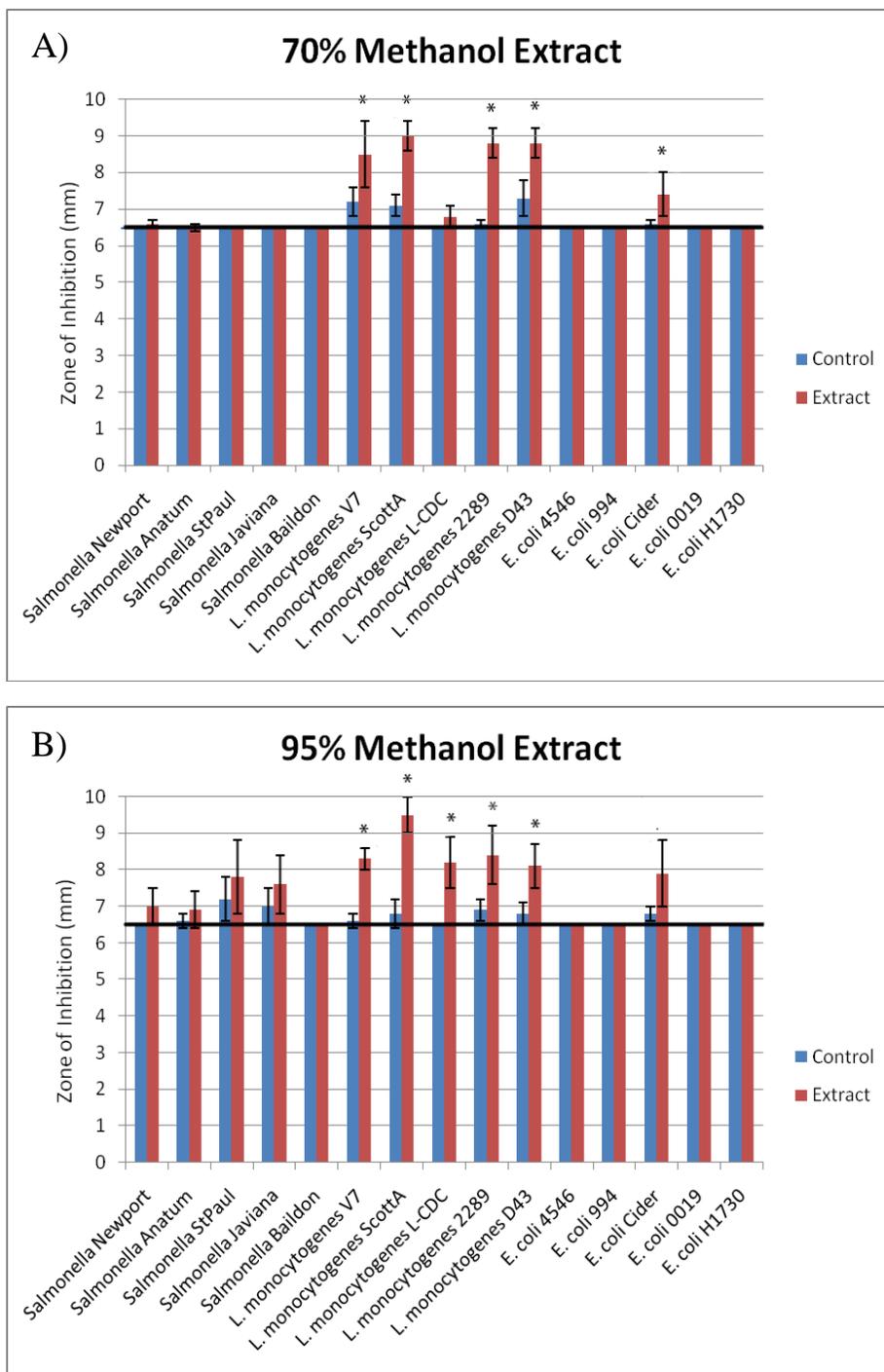


Figure 2. Mean zones of inhibition for bacterial cultures in association with jalapeño extract made with A) 70% methanol solvent and B) 95% methanol solvent. Controls are the solvent with water replacing jalapeño extract. Asterisks represent measurements where zones of inhibition for solvent extracts are significantly different than zones of inhibition for their respective controls. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

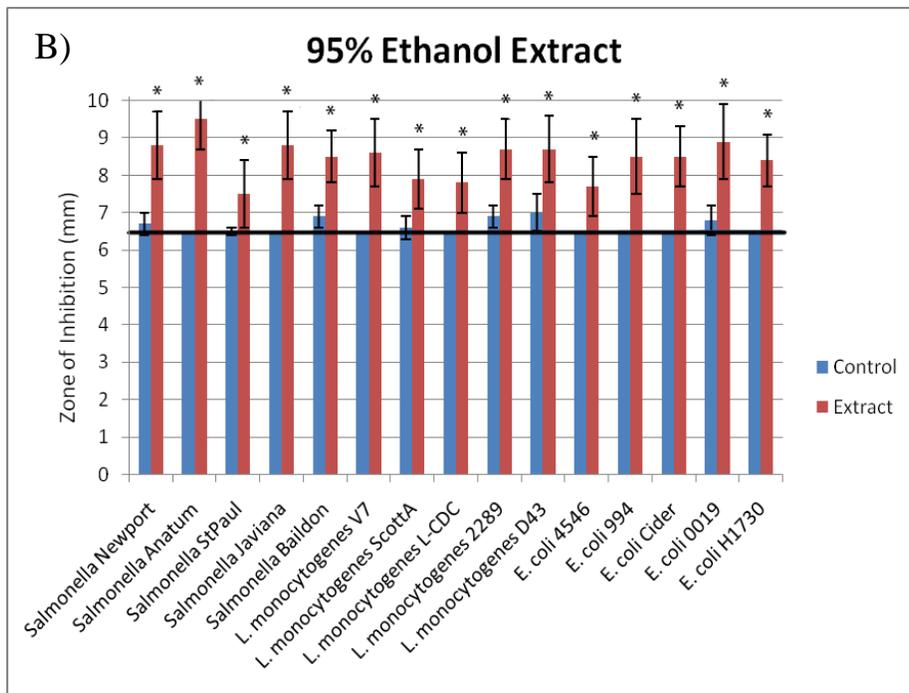
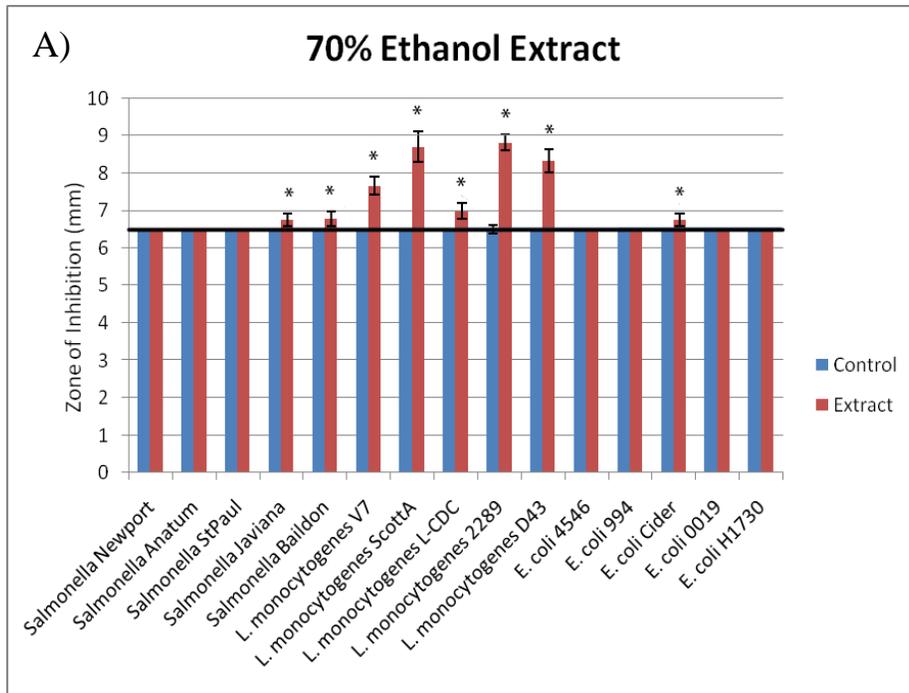


Figure 3. Mean zones of inhibition for bacterial cultures in association with jalapeño extract made with A) 70% ethanol solvent and B) 95% ethanol solvent. Controls are the solvent with water replacing jalapeño extract. Asterisks represent measurements where zones of inhibition for solvent extracts are significantly different than zones of inhibition for their respective controls. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

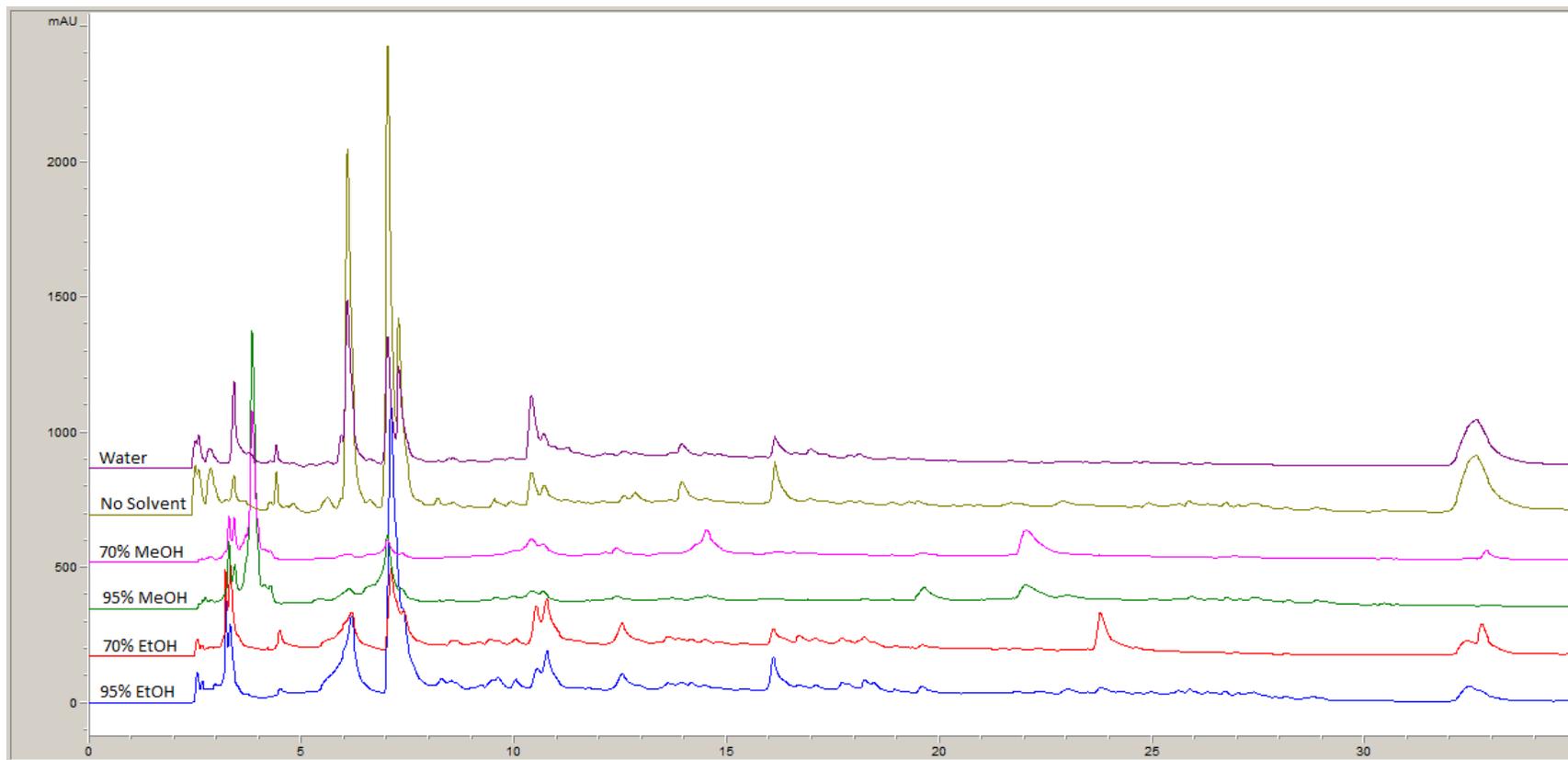


Figure 4. Representative HPLC chromatogram of jalapeño extracts using different solvents for extraction.

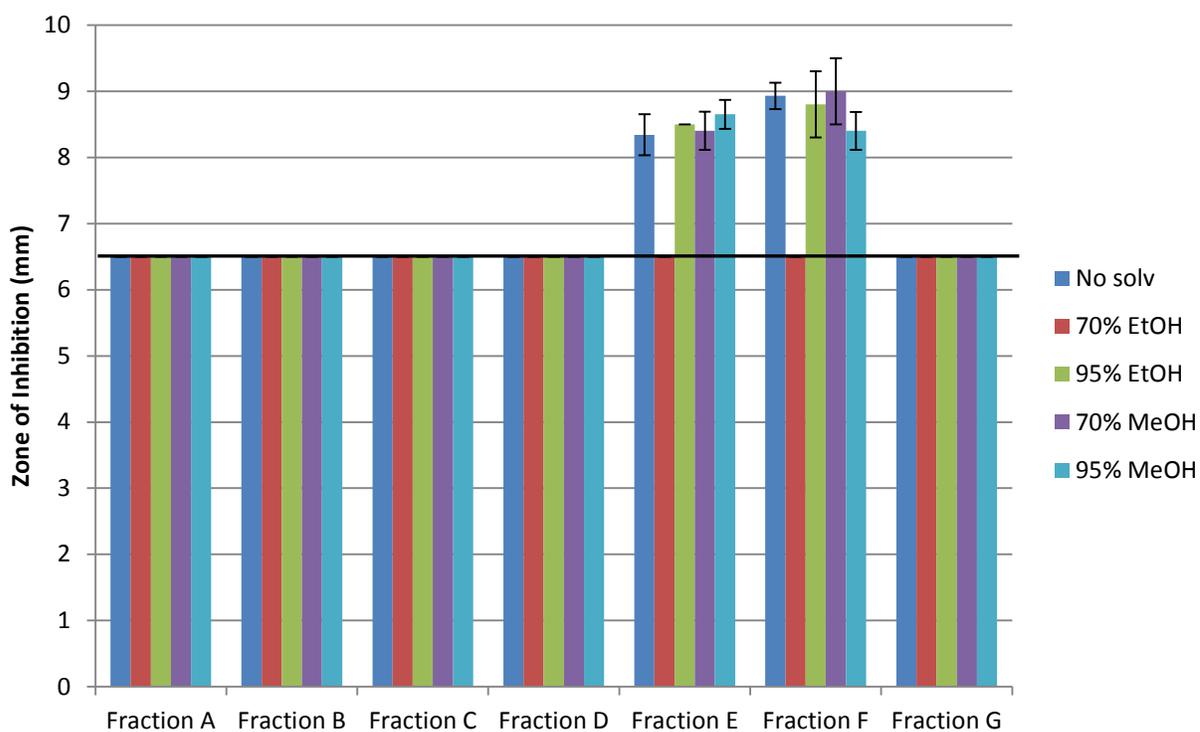


Figure 5. Comparison of the mean zones of inhibition for *L. monocytogenes* and fractionated jalapeño extracts using different solvents. Error bars represent standard error of the mean. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

CHAPTER 5

Anti-bacterial activity of jalapeño pepper (*Capsicum annuum* var. *annuum*) fractions against select foodborne pathogens

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Running Title: Jalapeño extract antimicrobial activity against select foodborne pathogens

Key words: *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli*, *Capsicum annuum*, antimicrobial activity, liquid chromatography, disk diffusion, growth curve

Abstract

Capsicum annuum fruits have been investigated for antimicrobial activity in a number of studies, and capsaicin or other cinnamic acid pathway intermediates like vanillin or cinnamic acid are often declared as the antimicrobial components with conflicting results. However, no research has been conducted to fractionate *C. annuum* extract to collect and isolate the compound(s) responsible for inhibition. In this study, we collected fractions from jalapeño pepper extracts using reverse phase HPLC and tested the fractions for antibacterial activity using disk diffusion and growth curve generation methods. In our initial fractionation, two fractions (Fraction A and Fraction C) showed growth promoting properties when associated with *Listeria monocytogenes* (Fraction A and Fraction C), *Escherichia coli* O157:H7 (Fraction A), and *Salmonella enterica* (Fraction A). In addition, two fractions (Fraction E and Fraction F) displayed antibacterial activity in conjunction with *L. monocytogenes*, *E. coli* O157:H7, and *S. enterica* ($p=0.05$). The elution time for a commercial standard of capsaicin placed it in Fraction G, a fraction which did not have an effect on microbial growth for the organisms tested. Fraction E and Fraction F were subject to further HPLC fractionation and growth analysis. The only fraction to display clear inhibition for each assay was Fraction E1 in association with *L. monocytogenes*. Fraction E1 was analyzed using HPLC-MS. The resulting mass spectra revealed Fraction E1 contained compounds belonging to a group of *C. annuum* specific compounds known as capsianosides. Little research is available concerning capsianosides, and a pure commercial standard is not available. In order to confirm the potential antimicrobial activity of the compound(s) isolated, methods need to be developed to isolate and purify capsianosides specifically from jalapeño peppers.

Since the discovery of antibiotics in the 1950s, the use of plant derived antiseptics has been comparatively nonexistent. However, with the development of many antibiotic resistant strains of microorganisms (Anderson, 2003; Montravers et al., 1996; Longenecker and Oppenheimer, 1982) and a trending consumer distrust of “unnatural” food ingredients, there has been a resurgence of interest in natural antimicrobials. At present, foods are typically preserved by compounds such as nitrite, sodium benzoate and sodium metabisulfite that have been tested and proven safe (Gould and Russell, 2003). However, there are occasional reports of allergic reactions to these preservatives, and even potential formation of carcinogenic by products like nitrosamines from nitrite (Roller, 2003).

Plants have been used for their healing powers since ancient times, and attempts to categorize the antimicrobial activities of plants in a laboratory date back to the early 1900s (Hoffman and Evens, 1911; Martindale, 1910). It is now known that plants and herbs have different properties and contain many classes of phytochemicals (Dorman and Deans, 2000). These phytochemicals include terpenoids, alkaloids, lectins, polypeptides, quinones, flavones, flavonoids, flavonols, tannins, coumarins, and many others (Cowan, 1999). There is currently much research in microbiology focused on plant essential oils and their ability to inhibit spoilage and pathogenic food bacteria. (Altundag et al., 2011; Du et al., 2011; Gao et al., 2011; Hsouna et al., 2011; Lazarevic et al., 2011; Lee et al., 2011; Prakash et al., 2011; Rahman et al., 2011; Sanchez-Gonzalez et al., 2011; Serrano et al., 2011; Viazis et al., 2011; Bevilacqua et al., 2010; El-Baroty et al., 2010). Essential oils isolated from plant sources have been found to be effective antimicrobial agents, and there is ongoing research to identify more antimicrobial plant sources.

Extracts from *Capsicum annuum* fruit have been investigated to some extent, and antimicrobial properties have been reported with mixed results. Crude tissue extracts from

several different *C. annuum* varieties were shown to have growth inhibiting properties against species of *Bacillus*, *Clostridium*, and *Streptococcus* (Cichewicz and Thorpe, 1996). Extracts from *C. annuum* fruits using isopropanol as a solvent were also found to inhibit the growth of *L. monocytogenes*, *Salmonella*, *Bacillus*, and *Staphylococcus* in liquid media to varying degrees (Dorantes et al., 2000). Contradictory results were found when *Salmonella* was introduced to both bell pepper and jalapeño fruit extract, and cells grew readily in each (Nutt et al., 2003). Differences in results may be due to the type of *C. annuum* plant tested (ex: bell pepper, pimiento pepper, scotch bell, etc.), differences in antimicrobial methodology, and differences in preparing extract samples. Little is known about antimicrobial properties of *C. annuum* extract when tested against various foodborne pathogens and there is little to no known research concerning the antimicrobial activity of fractionated jalapeño extract.

Consumers increasingly demand natural and environmentally friendly ways to preserve their foods. This research will investigate the *C. annuum* var. jalapeño fruit as a source of natural antimicrobial(s) and potentially uncover a useful antimicrobial compound obtained from a natural and economically inexpensive source. Antimicrobials discovered could be used in the future to help keep the food supply safe and add an additional choice to the variety of steps and methods used to preserve food.

Materials and Methods

Preparation of jalapeño pepper extract. Unwaxed jalapeño peppers (*Capsicum annuum* var *annuum*, Jalapeño, cv. ‘Del Mar’) were purchased from a local grocery store in Blacksburg, VA. The pepper extracts were prepared the day of purchase. Peppers were submerged into 100 ppm chlorinated water for two min, rinsed twice with tap water, and patted dry with paper towels. Ten jalapeños (approximately 350 g) were used each time an extract was

made. Stems were removed and jalapeños were chopped and placed in a Waring blender. Peppers were blended for 2 min, creating a homogenous slurry. The slurry was placed into a filter lined stomacher bag. A pipette was used to remove 100 ml of jalapeño fluid from the filtered bag and placed into a sterile centrifuge tube. Extract was centrifuged (Sorvall Legend RT+, Thermo Scientific, Waltham, MA) at 15,000 x g for ten min, supernatant was removed, and centrifuged twice more. The supernatant was passed through a 0.45 µl filter syringe (Whatman Inc., Piscataway, NJ) and used immediately. Any extra extract was stored at 4°C.

Bacterial cultures and conditions. Three bacterial cultures were used in this study; *L. monocytogenes* V7, *Escherichia coli* O157:H7 isolated from apple cider, and *S. enterica* Baildon. Bacterial cultures were preserved in a Tryptic Soy broth (TSB, Bacto, Difco, Becton Dickinson, Sparks, MD) solution containing 30% glycerol and stored at -80°C until ready for use. Cells were activated by three successive 24 h transfers into TSB and incubated at 37°C. Activated cells were centrifuged (Sorvall Legend RT+, Thermo Scientific, Waltham, MA) at 2000 x g for 10 min at 22°C, the pellet resuspended in 0.1% buffered peptone water, and washed twice more to yield a bacterial cocktail of approximately 8 log CFU/ml. Cultures were diluted ten-fold into sterile 0.1% peptone water and this dilution was used in the following antimicrobial assays.

Fractionation of jalapeño extracts using high-performance liquid chromatography. The initial HPLC work was performed with an Agilent (Santa Clara, CA) 1200 Series HPLC consisting of degasser, quaternary solvent pump, autosampler with refrigeration, column oven, and a diode array detector and a Phenomenex (Torrance, CA) Luna 5µ C18 (250 x 4.6 mm) column with a Phenomenex Security Guard column. An acetonitrile gradient consisting of two solvents: solvent A (0.1% aqueous acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile) was utilized (Table 1a). Flow rate was 1.0 ml/min. The sample injection was 100 µl.

UV absorbance was recorded at 254 and 280 nm. Commercial standards of phenylalanine, cinnamic acid, coumaric acid, caffeic acid, vanillin, and capsaicin (Sigma-Aldrich, St. Louis, MO) dissolved in 95% aqueous ethanol were used to determine separation characteristics of jalapeño extract. Peaks from known standards were identified by retention time and UV absorbance spectra. Compounds from the fresh jalapeño extracts were collected as they eluted from the HPLC column in five minute increments (total of 32 minutes collected) into clean glass centrifuge tubes. Each five-minute fraction was labeled A-G (Figure 1).

After jalapeño extract Fractions A through G were used in antimicrobial assays and analyzed, Fractions E and F were selected based on antibacterial performance for further fractionation using a different HPLC set-up. The HPLC equipment used was an Agilent 1260 consisting of degasser, quaternary solvent pump, autosampler with refrigeration, column oven, diode array detector, and an automated fraction collector and a Phenomenex Luna 5 μ C18 (250 x 4.6 mm) column with a Phenomenex Security Guard column. An acetonitrile gradient consisting of two solvents: solvent A (0.1% aqueous acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile) was utilized. The HPLC schedule for Fraction E and Fraction F can be seen in Table 1b and 1c, respectively. Fractions were collected automatically based on peak slope into fraction collection vials (Agilent, Santa Clara, CA). Nomenclature for fractions collected were assigned based on retention time and peak elution. Fractions were pooled into sterile glass tubes and processed for antimicrobial assay as described below.

Preparation of HPLC fractions for antimicrobial assays. Fractions eluted from the column were placed under a fume hood and acetonitrile was evaporated from fractions using a stream of nitrogen gas. Once acetonitrile was no longer detectable by smell, 1 ml of sterile deionized water was added to each tube (to dilute any remaining acetonitrile), tubes were capped,

and placed into freezer ($-18\pm 2^{\circ}\text{C}$) for approximately 3 h until samples were frozen solid. Caps were then removed from tubes and cheese cloth was secured over the tube openings with a rubber band. Samples were placed into a freeze dryer (Virtis, The Virtis Company Inc., Gardiner, New York) and dried for approximately 18 h until all liquid was removed from the samples and an off-white powder could be detected at the bottom of the tubes (not all tubes had powder). Fractions were resuspended in sterile deionized water to concentrations of 1.0 mg/ml of jalapeño extract. Fractions were used immediately, or stored at 4°C until ready for use in antimicrobial assays.

Disk diffusion assay for antimicrobial assessment. A disk diffusion assay was performed following recommendations by Vigil et al. (2005) with modifications. Whatman #2 filter paper (Whatman Inc., Piscataway, NJ) was used in this assay. A hole punch was used to produce 6.5 mm diameter filter disks. The disks were collected and autoclaved prior to use. Bacterial cultures were spread plated (0.1 ml) onto Tryptic Soy agar (TSA) (Difco, Becton Dickinson, Sparks, MD) plates. Plates were allowed to dry for 10 min. Flame-sterilized tweezers were used to place filter disks onto inoculated TSA plates, one disk in each of four equal quadrants. Each disk on the TSA agar was then impregnated with 10 μl of either extract fraction treatment or control (two control disks and two extract disks per plate). To prepare controls, 100 μl of water was injected into the HPLC column and the eluent was collected at the same time frame as the fraction to be tested. The controls were processed in the same manner as the extract fractions. Two plates were prepared for each culture and extract combination ($n=4$). Plates were inverted and incubated for 24 h at 37°C . Zones of inhibition were measured in mm with a digital caliper. Each experiment was run three times ($N=12$). Statistical difference between each extract fraction and its control was determined using a student's t-test performed with JMP software

(JMP 7.0, SAS Institute, Cary, NC). Statistical significance was determined when p-values were less than 0.05.

Automated growth curve analysis for antimicrobial assessment. To test the effect of jalapeño extracts on the growth of selected bacterial pathogens, a Bioscreen C Microbiology Reader (Growth Curves, Piscataway, NJ) equipped with an incubator and automated turbidimeter was used for determination of optical density (OD) over time. The Bioscreen measures microbial growth by vertical pathway and the changes in optical density in liquid medium are correlated with microbial populations in the samples. Optical density was determined between 420-540 nm. The liquid growth medium used was either TSB or 0.1% buffered peptone water. Jalapeño fractions were filter sterilized using a 0.45 µm syringe Whatman PTFE Puradisc (Whatman Inc., Piscataway, NJ). Each well of a honeycomb microwell plate (Bioscreen, Growth Curves, Piscataway, NJ) was filled with 125 µl of growth medium, 15 µl of prepared jalapeño extract fraction, and 10 µl of culture (containing approx. 10^3 cells). For controls, eluent was collected from the HPLC column using the same conditions as the experimental fraction, but water was injected into the column rather than jalapeño extract, and processed as described above. Control wells contained 15 µl of corresponding fraction controls to replace jalapeño extract. Microwell plates were incubated at 37°C for either 24 h (peptone) or 72 h (TSB), and OD was measured every 15 minutes with 10 seconds of shaking before each reading. The growth curve data was generated by using EZExperiment software (Growth Curves, Piscataway, NJ) and exported as a Microsoft Excel spreadsheet (Microsoft, Seattle, WA). Each experiment was replicated three times. Growth curves generated were analyzed for statistical difference of the means of trapezoidal area under the curve (AUC) values using a student t-test. All statistical analysis was

performed with JMP 7.0 software (SAS Institute, Cary, NC) and the difference was set to be significant when the p-value was less than 0.05.

HPLC-Mass Spectrometry Analysis. Fraction E1, which showed anti-listerial activity in our antimicrobial experiments, was analyzed using HPLC-MS. An Agilent (Palo Alto, CA) 1100 series HPLC coupled to a triple quadrupole mass spectrometer API 3200 (Applied Biosystem Sciex Instruments, Rotterdam, The Netherlands) equipped with a Turbo Ion Spray interface (Electrospray) was used for the analysis. Both positive and negative ion mass spectra were recorded. The MS operating parameters were as follows: capillary voltage (IS) -4200 V; declustering potential (DP) -50 V; and source temperature set to 420°C. A full scan of mass spectra from m/z 300 to 1800 was performed. HPLC separations were accomplished using a Kinetex (Phenomenex Torrance, CA), C18 (3 x 100 mm, 2.6 µm) column. A gradient was used consisting of solvent A (deionized water with 0.1% acetic acid) and solvent B (acetonitrile with 0.1% acetic acid) and a flow rate of 0.4 mL/min was used throughout the analysis. The gradient schedule is listed in Table 1d. The injection volume was 10 µl. The gradient was run for 30 min. After initial MS analysis, there was consistent appearance of high mass spec ions near 10 min into the gradient, and the method was altered to look only at m/z 900 to 1400.

Results and Discussion

Effect of Fractions A through G on the growth of foodborne pathogens

Preliminary studies showed that unfractionated extracts of jalapeño peppers contained the ability to inhibit some pathogenic bacteria tested using a disk diffusion assay (data not shown). These results are consistent with other studies that have examined the antimicrobial potential of a number of *C. annuum* varieties using disk diffusion methods (Dorantes et al., 2008; Careaga et

al., 2003; Dorantes et al., 2000; Cichewicz and Thorpe, 1996). These studies generally hypothesized that a phenylpropanoid intermediate produced during the cinnamic acid pathway was responsible for antimicrobial activity. In order to verify, several phenylpropanoid intermediate standards were purchased and used in disk diffusion assays to reveal antimicrobial activity. Our methodology differs from previous studies as we are not presuming that the antimicrobial activity is a function of a limited set of compounds present in jalapeños. We used reverse phase HPLC to separate components in the jalapeño extract based on polarity. In general when using reverse phase HPLC under the conditions that were used for this study, it is expected that more polar compounds would elute from the column toward the beginning of the run, and more non-polar compounds would elute towards the end, in a gradient fashion (Robbins, 2003). These fractions were used for antimicrobial assays to narrow down which fractions may hold antibacterial compounds.

Disk diffusion assays and jalapeño extract Fractions A through G. The disk diffusion assays were performed against three different foodborne pathogenic organisms (*L. monocytogenes*, *E. coli* O157:H7, and *S. enterica* Anatum). Overall, *L. monocytogenes* showed the highest antibacterial susceptibility to the fractionated extracts, with zones of inhibition significantly larger ($p < 0.05$) than the control for Fractions C, E, and F (Figure 2a). *Escherichia coli* O157:H7 displayed no significant differences in zones of inhibition from fractionated extracts and controls (Figure 2b). *Salmonella enterica* was affected only by Fraction F (Figure 2c).

Effect of jalapeño extract Fractions A through G on bacterial growth curves generated with Bioscreen. Growth curves generated by Bioscreen were analyzed using trapezoidal area under the growth curve (AUC) analysis (Figures 3-5). AUC analysis is

commonly used to compare the growth curves, as a whole, to one another (Lambert and Bidlas, 2007; Alakomi et al., 2006; Thomas et al., 2002; Lambert and Pearson, 2000; Raaska et al., 1999; Jaskari et al., 1998). The effects of the jalapeño extract fractions on the growth of the bacteria manifest as differences in curve shapes between bacterial treatments. These differences are quantified by reductions or increases in the area under the growth curve (Lambert and Bidlas, 2007).

Two separate types of media were used for the generation of growth curves, TSB and 0.1% peptone. Peptone is a media that is not rich in carbohydrate, containing only peptone, sodium chloride, disodium hydrogen phosphate dodecahydrate, and potassium dihydrogen phosphate. Peptone is capable of providing organisms with sugars, amino acids, purines, pyrimidines, vitamins and other nutrients (Schaechter et al., 2006), but using a concentration of 0.1% peptone in our experiment limited the amount of these nutrients available to the growing bacteria. This was an attempt to mimic an environment which might be like that of an uninjured plant surface, which offers little nutrients for growth due to natural plant barriers like wax layers and cell walls (Harris et al., 2003). The nutrient rich TSB was used to provide growth conditions in which bacteria were exposed to a nutritious environment meant to promote growth. Ingredients in TSB include pancreatic digest of casein, papaic digest of soybean meal, sodium chloride, dipotassium phosphate, and dextrose (Becton Dickson Diagnostics, 2010).

The two different media used in the growth curve experiments proved to elicit two very different responses. AUC values showed that Fractions A and C were stimulatory for *L. monocytogenes* in 0.1% peptone, and Fraction C was stimulatory in TSB (Figure 3). Fractions E and F were inhibitory for *E. coli* O157:H7 (Figure 4a) and *S. enterica* (Figure 5a) in 0.1% peptone, and Fraction A was stimulatory. No significant difference was noted in AUC values for

E. coli O157:H7 (Figure 4b) and *S. enterica* (Figure 5b) in TSB, although Fractions E and F displayed the smallest AUC values of all the fractions tested against *E. coli* O157:H7 and *S. enterica*.

After reviewing the effects of the fractionated jalapeño extracts on the bacteria using both disk diffusion assays and area under the growth curve analysis, it was determined that the fractions with the most potential to contain inhibitory compounds were Fraction E and Fraction F. Therefore, Fractions E and F were subjected to further HPLC analysis. It was also evident from the growth curve analysis that Fraction A contained compound(s) which created a stimulatory effect on all bacteria tested, and Fraction C stimulated the growth of *L. monocytogenes*. Although there may be some benefit to exploring which compound(s) were responsible for stimulating growth, we chose only to focus on the compounds which may inhibit growth in this study.

HPLC analysis of known phenylpropanoid standard compounds

A number of known phenylpropanoid standards were analyzed using the same reverse phase HPLC conditions performed for jalapeño extract fractionation, and the elution times were determined (Table 2). Only cinnamic acid and caffeic acid eluted at time points that would place them in a collected fraction that was considered “active” (possessing either inhibitory or stimulatory properties). Cinnamic acid eluted at 23.60 ± 0.16 min, placing the compound in Fraction E, which displayed inhibitory behavior. Cinnamic acid has been shown to display antimicrobial activity in the presence of both bacteria and fungi (Olasupo et al., 2003; Wen et al., 2003; Roller and Seedhar, 2002; Nascimento et al., 2000; Yao and Shelef, 1998). The minimum inhibitory concentration (MIC) of cinnamic acid for *L. monocytogenes* was found to be between

1000-2000 ppm (0.1% -0.2 %) in acidic conditions (Wen et al., 2003; Yao and Shelef, 1998). Our extract contained approximately 5 ppm of cinnamic acid according to a standard curve (data not shown).

Caffeic acid was eluted during our collection of Fraction C, which displayed stimulatory growth effects in this study. It has been shown that the gastrointestinal bacteria of some mammals are able to metabolize caffeic acid (Peppercorn and Goldman, 1972). This may contribute to why our extract containing caffeic acid helped promote the growth of bacteria in our study.

Effect of Fraction E subfractions on the growth of foodborne pathogens

Fractionation and collection of E0 through E4. Multiple collections of Fraction E were pooled and run through RP-HPLC under altered conditions, which may be found in Table 1b. These fractions were collected on a peak by peak basis rather than by time as was used to collect Fractions A through G. It is likely that there is more than one compound in each peak collected, but the compounds within each peak are expected to be similar to each other in terms of polarity. For Fraction E, there were six subfractions collected, labeled in numerical order as they eluted (Figure 6). Labeling of the E subfractions was noted by the researcher in one such way (Fractions E1, E2, E3, and E4) and then upon recognition that two fractions were overlooked, the labels E0 and E 2.5 were added as an afterthought.

Disk diffusion analysis of E subfractions against foodborne pathogens. Disk diffusion assays were performed using E subfractions against *L. monocytogenes*, *E. coli* O157:H7, and *S. enterica*. The only bacterium to show visible zones of inhibition for the disk diffusion assays was *L. monocytogenes* (Figure 7). All other bacteria and extract combinations yielded no difference

between controls and experimental extracts (data not shown). The E subfractions that produced zones of inhibition were E1 and E2. Of the two subfractions, E1 had the largest difference in zone of inhibition from the control.

Effect of E subfractions on bacterial growth curves. Growth curves generated by the Bioscreen C revealed that E subfractions had variable effects on *L. monocytogenes* grown in 0.1% peptone (Figure 8a). For growth in TSB, Fraction E1 had evident inhibitory effects on *L. monocytogenes*, while the remaining E subfractions did not appear to drastically effect growth of *L.monocytogenes* (Figure 8b). AUC values for *L. monocytogenes* in 0.1% peptone were smaller than the control for Fractions E1 and E2.5 (Figure 9a). AUC for E1 was 69% smaller than the control. In TSB, only Fraction E1 inhibited growth, which was 15% smaller than the control (Figure 9b). AUC was not significantly different between *E. coli* O157:H7 and *S. enterica* cultures grown in the presence or absence of E subfractions (Figures 10 and 11).

Elution of the known standard cinnamic acid

The antimicrobial agent cinnamic acid, as mentioned above, was found to elute in Fraction E. We were therefore concerned with the elution of cinnamic acid when Fraction E was separated further using HPLC. A known standard was run through the HPLC column using the same conditions as those used to subfractionate Fraction E. Cinnamic acid eluted at 7.94 ± 0.02 min. This elution time placed it in Fraction E3, which did not display antimicrobial activity for the bacteria tested. The concentration of cinnamic acid in our extract was approximated to be 4 ppm according to a standard curve (data not shown). Although cinnamic acid has been found to have an antimicrobial capacity, the concentration needed was much higher than that of our extract. Therefore, it is not surprising that no antibacterial activity was observed.

Effect of Fraction F subfractions on the growth of foodborne pathogens

Fractionation and collection of F0 through F7. Several collections of Fraction F were pooled together and run through the HPLC under altered conditions (Table 1c) in order to separate the compounds further. Fractions were collected peak by peak, with a total of 7 fractions collected. The F subfractions were labeled F1 through F7, with the number increasing with each subsequent fraction collected.

Disk diffusion analysis of F subfractions against foodborne pathogens. Much like the disk diffusion results for the E subfractions, only *L. monocytogenes* was sensitive to any of the F subfractions. Fractions F1 and F2 showed zones of inhibition larger than those of the control ($p < 0.05$) (data not shown). The F subfractions did not produce visible zones of inhibition when tested with *E. coli* O157:H7 and *S. enterica* (data not shown).

Effect of F subfractions on bacterial growth. AUC analysis showed there was no significant decrease in growth between any of the bacterial cultures studied associated with an F subfraction and that of the control for both 0.1% peptone and TSB (data not shown).

Synergistic, antagonistic, and additive interactions of antimicrobial compounds

While Fraction F consistently displayed antimicrobial activities in the disk diffusion and growth curve assays for the bacteria examined, the subfractions of F did not yield any strong and consistent antimicrobial results. Although the F fraction appeared to lose antimicrobial capacity when subfractionated, there are a number of possible reasons why this occurred. Antimicrobial compounds can often interact with one another and give either synergistic, additive, or antagonistic effects (Vigil et al., 2005). When additivity occurs, the combined compounds yield

antimicrobial activity that is equal to the sum of the activity of the two independent compounds. This is also known as indifference. When compounds are synergistic, they increase or enhance the overall antimicrobial activity beyond that of the sum of the individual compounds. Antagonistic compounds work together to reduce the efficacy of the compounds when compared to their individual results (Vigil et al., 2005). When we tested Fraction F, we were testing it with all the compounds that it contained interacting with one another and the result was an inhibitory combination. When the compounds were separated further using HPLC, we removed the compounds' abilities to interact with one another, and as a result saw less ability to inhibit bacterial growth. We can postulate that some of the compounds in Fraction F were likely working in combination with one another to produce the inhibitory effect. When those compounds were separated, they were not able to produce the synergistic effect that they once had in combination. For future studies, it may be beneficial to look at combinations of Fraction F subfractions to see if the combinations result in increased inhibition.

Mass spectrometry of Fraction E1

The antibacterial activity of Fraction E1 in the presence of *L. monocytogenes* was apparent in this study, and mass spectrometry (MS) was performed in attempt to identify compounds present in Fraction E1. The initial mass spectrometry runs were performed in positive and negative modes, scanning a wide mass range (300-1800 amu). This revealed limited signal. There was a consistent appearance of high mass ions at around 10 minutes in the gradient so a method looking at only 900-1400 amu was run. The LCMS data provided are of these analyses (Figure 13).

Review of the literature revealed that the compounds found within Fraction E1 are likely acyclic diterpene glycosides (Lee et al., 2009; De Marino et al., 2008; Lee et al., 2008; Lee et al., 2007; De Marino et al., 2006; Lee et al., 2006; Materska and Perucka, 2005; Iorizzi et al., 2002; Iorizzi et al., 2001). Throughout the literature, there are a number of acyclic diterpene glycosides known as capsianosides that have been isolated specifically from *C. annuum* plants, both sweet and spicy, that share the m/z found in our mass spec analysis (Table 3). Several known capsianosides have m/z values that match with the values we discovered in our MS scan. In order to make a positive identification of the compounds present in Fraction E1, either a known standard would need to be purchased and MS analysis run in parallel, or a pure compound needs to be isolated and analyzed. Capsianoside standards are not commercially available. Therefore, we were not able to verify our mass spec results with positive capsianoside controls or obtain an absolute identification.

Biological activity of known capsianosides

A limited number of studies have been conducted on various biological activities of capsianosides. The antimicrobial activity of Capsianoside II extracted from the sweet pepper *C. annuum* L. var *acuminatum* was tested using an agar dilution assay against both yeast and fungi (Iorizzi et al., 2002). The results of these assays revealed no antimicrobial activity displayed by Capsianoside II. The antioxidant activity of Capsianoside VIII and Capsianoside III was tested using the diphenyl picryl hydrazyl (DPPH) scavenging test, assays based on reduction of $\text{Cu}^{++}/\text{Cu}^+$, and lipid peroxidation (De Marino et al., 2006). All tests results were negative. Human Caco-2 cells treated with Capsianoside F displayed increased permeability in cellular tight junctions without causing toxicity to the cells (Shimizu, 2010; Hashimoto et al., 1996). Increased

permeability of the tight junctions may be useful to enhance the delivery of drugs or other hydrophilic substances across the intestinal membrane (Hashimoto et al., 1997), and many nutrients and compounds that are eaten on a regular basis, such as glucose, alanine, tryptophan, and milk whey proteins, have been proven to increase tight junction permeability as well (Hashimoto and Shimizu, 1993; Madara and Carleson, 1991; Pappenheimer, 1987). It is believed tight junction permeability is altered as capsiainosides pass through or are inserted into the cell membrane, after which they change the F-actin/G-actin ratio and cause dysfunction in tight junctions of human intestinal cells (Shimizu, 2010; Hashimoto et al., 1993). It has also been shown that Capsiainoside F has Ca^{2++} chelating activities that are about one-tenth as effective as EDTA (Hashimoto et al., 1997).

Effect of calcium on bacterial growth

Factors that may affect bacterial growth include nutrients, pH, temperature, aeration, salt concentration, and ion concentration (Brooks et al., 2004). It is well documented that metal cations are important to bacterial growth and adherence (Banin et al., 2006; Banin et al., 2005; Sarkisova et al., 2005; Weinburg, 2004; Ozerdem et al., 2003; Arakawa et al., 2000; Onoda et al., 2000; Ratledge and Dover, 2000; Norris et al., 1999; Smith, 1995; Dunn et al., 1992; Norris et al., 1991; Brubaker, 1985). Calcium in particular is important for growth of several bacteria (Arakawa et al., 2000; Onoda et al., 2000; Norris et al., 1999; Smith, 1995; Norris et al., 1991; Perry and Brubaker, 1987). For example, *E. coli* is inhibited by the calcium chelator ethylene glycol bis(beta-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) under alkaline conditions, but this inhibition is overcome with very small amounts (0.16 to 0.35 nmol per mg of protein) of available Ca^{2++} (Arakawa et al., 2000).

Calcium is essential to maintain chemical gradients across bacterial cell membranes and to facilitate enzymatic catalysis (Brooks et al., 2004). Calcium is also important in processes like chemotaxis (Ordal, 1977), sporulation (Shyu and Foegeding, 1989), virulence (Brooks, 2004), and transport of sugars and proteins (Vyas et al., 1987). Biofilm formation, maintenance, and stability are dependent on the presence of Ca^{2++} (Banin et al., 2006; Ozerdem et al., 2003; Dunne and Burd, 1992). Calcium chelators have successfully been used to disrupt biofilm formation and prevent biofilm related infections (Banin et al., 2006; Percival et al., 2005; Ozerdem et al., 2003; Raad et al., 2003).

Calcium is required as a component of the Gram-positive cell wall (Brooks, 2004; Doyle, 1989). The presence of Ca^{2++} and other cations in the peptidoglycan layer of Gram-positive bacteria like *L. monocytogenes* is necessary to provide the correct ionic environment for cation dependent membrane transport systems (Hughes et al., 1973). Gram-negative microorganisms, such as *E. coli* O157:H7 and *S. enterica*, do not have the cell wall requirement for calcium (Ferris, 1989). This difference in requirement for calcium between Gram-positive and Gram-negative bacteria may help explain why *L. monocytogenes* displayed some inhibition in the presence of Fraction E1 while *E. coli* O157:H7 and *S. enterica* were not as strongly affected. For this statement to be true, however, two major assumptions must be made: 1) Fraction E1 does in fact contain capsianoside(s) and 2) the capsianoside(s) present possess calcium chelating abilities.

Conclusions

Jalapeño extract has been examined in the past for antibacterial activity. Although some have reported that capsaicin and other cinnamic acid pathway intermediates are responsible for

antimicrobial activity, no research had been conducted that include fractionating the extracts and isolating the compounds responsible for inhibition. This research showed that using reverse-phase HPLC, the extract from jalapeño peppers was fractionated and the resulting fractions contained both bacteria growth promoting and growth inhibiting properties. After additional fractionation of selected growth inhibiting fractions, it was found that *L. monocytogenes* was susceptible to inhibition in the presence of Fraction E1. Upon HPLC-MS analysis, the m/z values of ions present in Fraction E1 matched those of a known group of *C. annuum* specific compounds known as capsianosides. Although little information is known about capsianosides, it has been discovered that they are potential calcium chelators. Therefore, inhibition of *L. monocytogenes* in the presence of Fraction E1 could be a result of calcium chelation, depriving the Gram-positive bacteria the necessary functionality on the surface needed for cation dependent transport required for growth. Because no commercial capsianoside standard is available for MS analysis, no positive control could be run to confirm capsianoside identity. A method should be developed to isolate capsianosides specifically from jalapeño peppers and purify the extract so that further analysis can be performed.

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Tables

Table 1: HPLC gradient schedule for analysis of jalapeño extracts.

HPLC gradient schedule for whole jalapeño extract			
A)	<u>Time(minutes)</u>	<u>%A</u>	<u>%B</u>
	0	100	0
	35	38	62
	40	0	100
	41	100	0
	45	100	0

HPLC gradient schedule for Fraction E			
B)	<u>Time(minutes)</u>	<u>%A</u>	<u>%B</u>
	0	60	40
	10	48	52
	11	0	100
	12	0	100
	15	60	40

HPLC gradient schedule for Fraction F			
C)	<u>Time(minutes)</u>	<u>%A</u>	<u>%B</u>
	0	50	50
	10	40	60
	11	0	100
	12	0	100
	15	50	

HPLC gradient schedule for LC-MS analysis of Fraction E1			
D)	<u>Time(minutes)</u>	<u>%A</u>	<u>%B</u>
	0	99.0	1.0
	10	99.0	1.0
	30	5.0	95.0
	40	5.0	95.0
	50	99.0	1.0

Table 2. Reverse-phase HPLC elution times of known phenylpropanoid intermediates produced during the cinnamic acid pathway for collecting Fractions A through G (n=3).

Compound	Elution time (min)	Theoretically contained in fraction
Phenylalanine	5.964±0.003	B
Caffeic acid	13.471±0.170	C
Coumaric acid	15.941±0.208	D
Vanillin	16.187±0.065	D
Cinnamic acid	23.595±0.155	E
Capsaicin	32.801±0.053	G

Table 3. Ion m/z detected in our MS scans that correlate to capsianosides isolated and identified in the literature from MS analysis.

Parent ion mass detected in Fraction E1	Capsianosides with matching m/z values reported in the literature	Reported m/z values*
937.6 [M – H] ⁻	Capsianoside IX (trivial name)	937 [M – H] ^{-A}
	Capsianoside XVII	961.47 [M + Na] ^{+D}
1123.9 [M + Na] ⁺	Capsianoside III	1123.51 [M + Na] ^{+A, C}
	Capsianoside VIII	1123.53 [M + Na] ^{+B}
	Capsianoside IX	1123.53 [M + Na] ^{+ B}
	Capsianoside XV	1123 [M+Na] ^{- B}
1285.8 [M + Na] ⁺	Capsianoside X	1285.59 [M + Na] ^{+B}
	Capsianoside XVIII	1285.57 [M + Na] ^{+E}

*Letter following corresponds to the publication in which the MS data is published.

^A De Marino et al., 2006; ^B Lee et al., 2006; ^C Lee et al., 2007; ^D Lee et al., 2008;

^E Lee et al., 2009

Figures

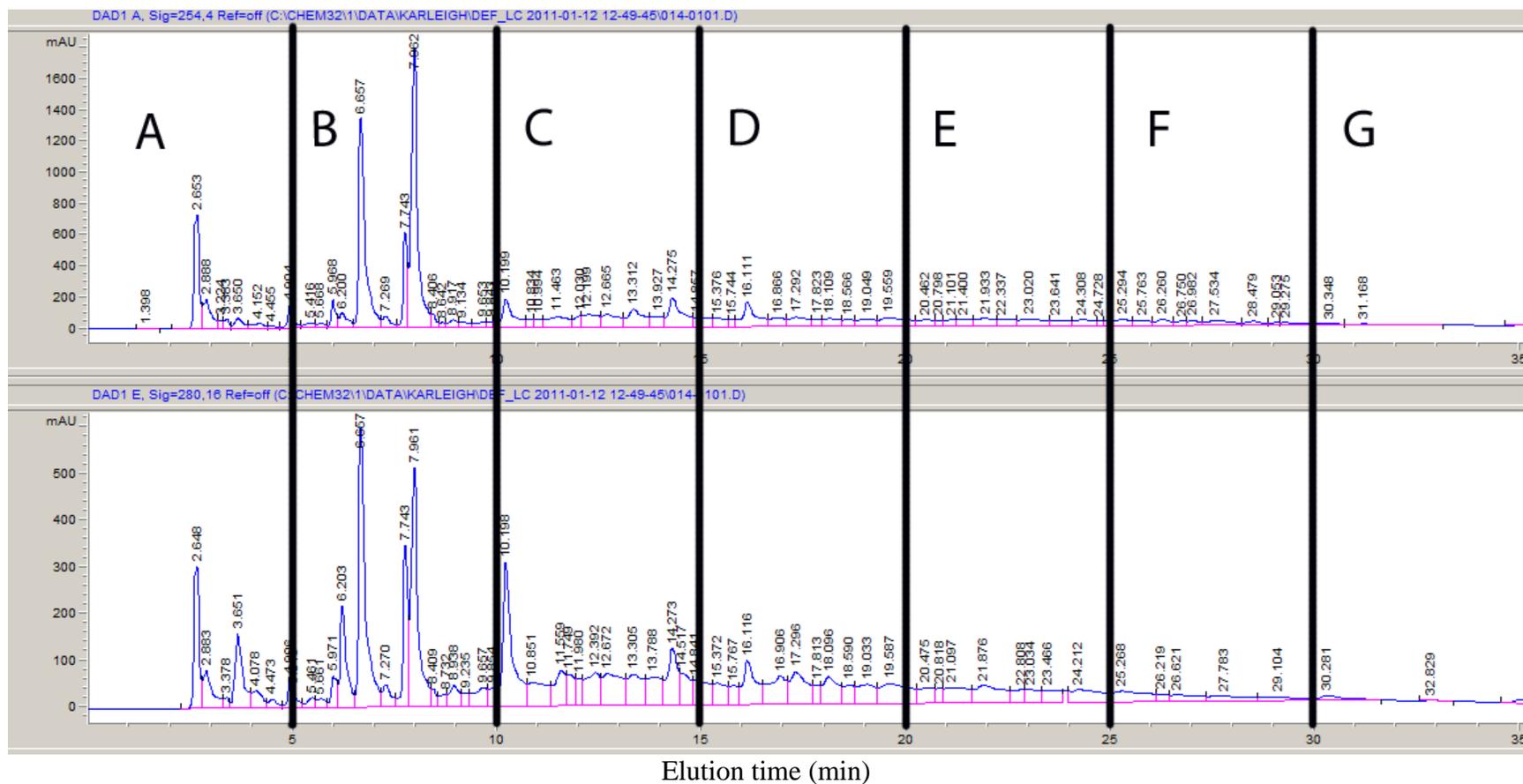


Figure 1. Reverse phase HPLC chromatogram of jalapeño extract with vertical indicators of fraction collected. Fractions were collected every five minutes and assigned alphabetical labels based on time of elution.

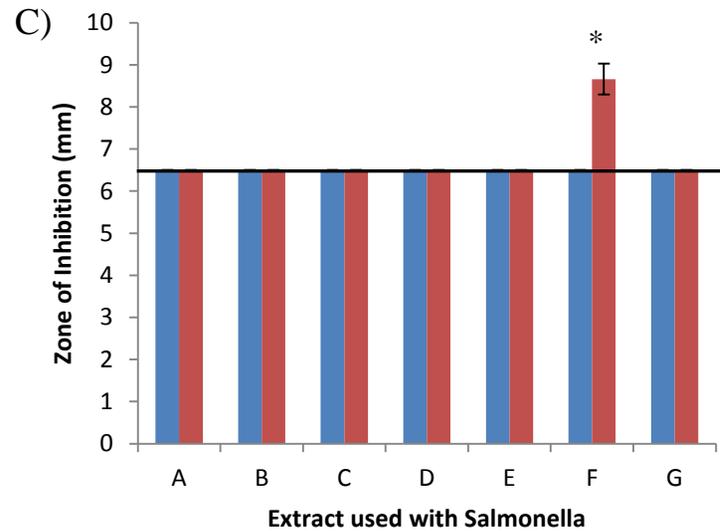
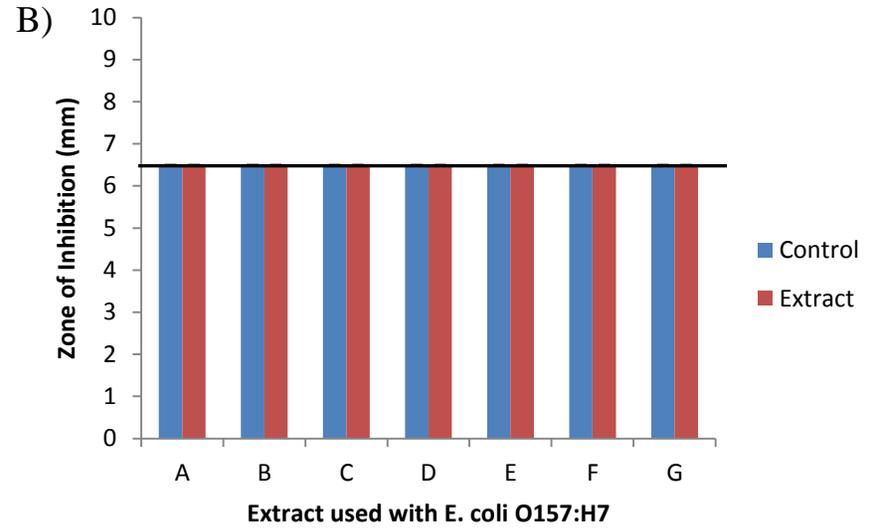
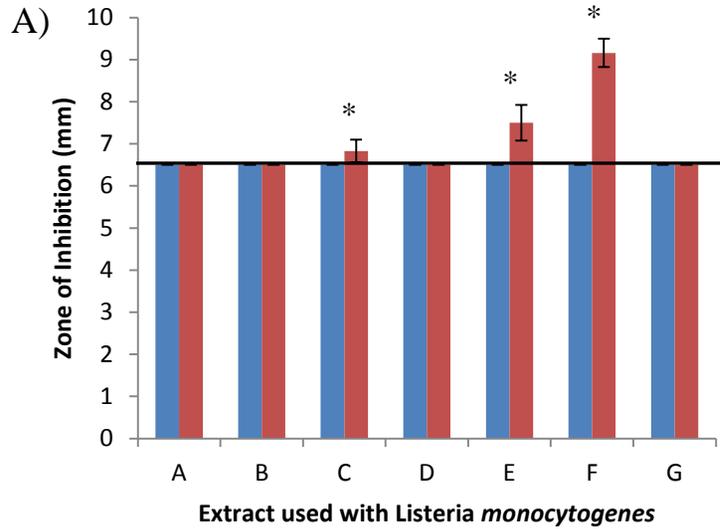


Figure 2: Disk diffusion results from jalapeño extract Fractions A through G. Zone of inhibition measurements are displayed for a) *L. monocytogenes*, b) *E. coli* O157:H7, and c) *S. enterica*. Error bars represent standard error of the mean. An asterisks indicates significant difference between the mean zones of inhibition observed for the jalapeño extract and control. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

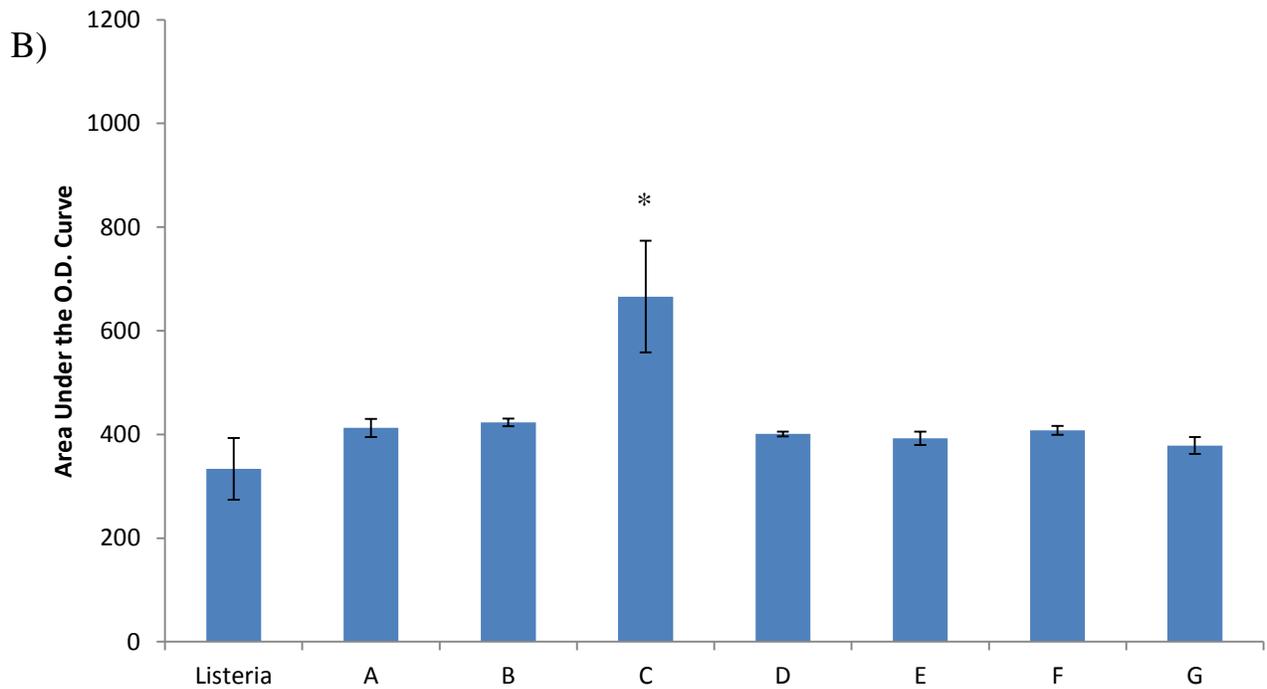
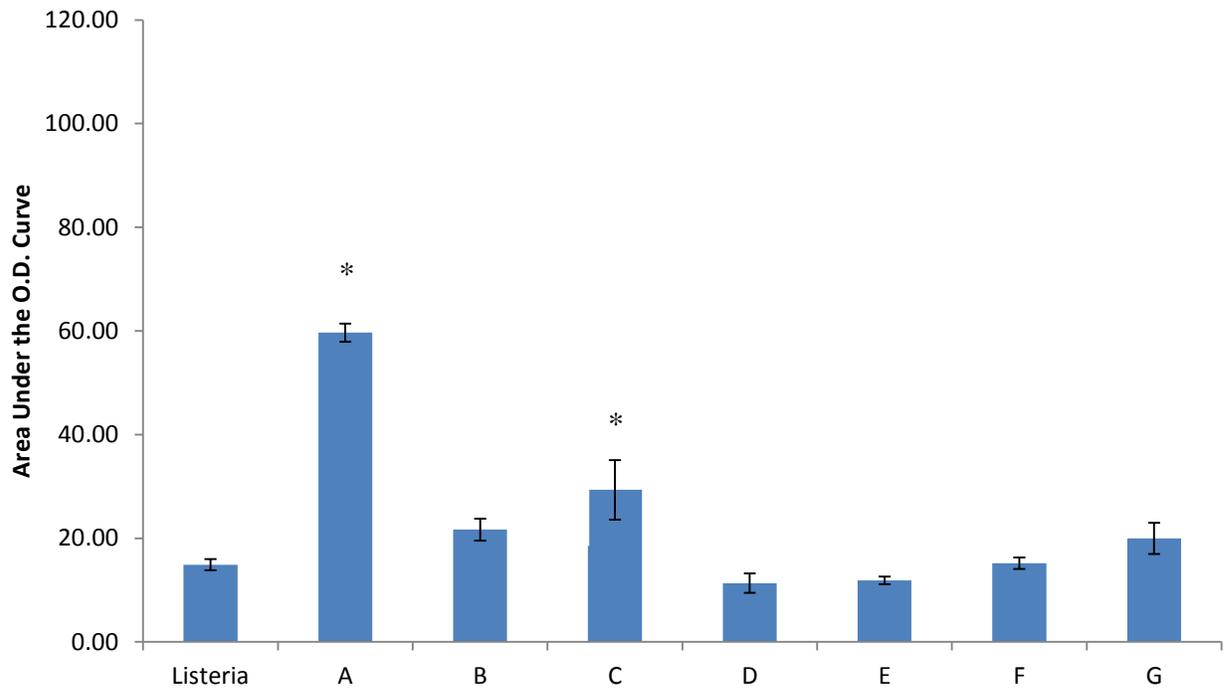


Figure 3. Area under the growth curve comparison for *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G. Error bars represent standard error of the mean. An asterisks depicts an AUC that is significantly different than the AUC of the *L. monocytogenes* control.

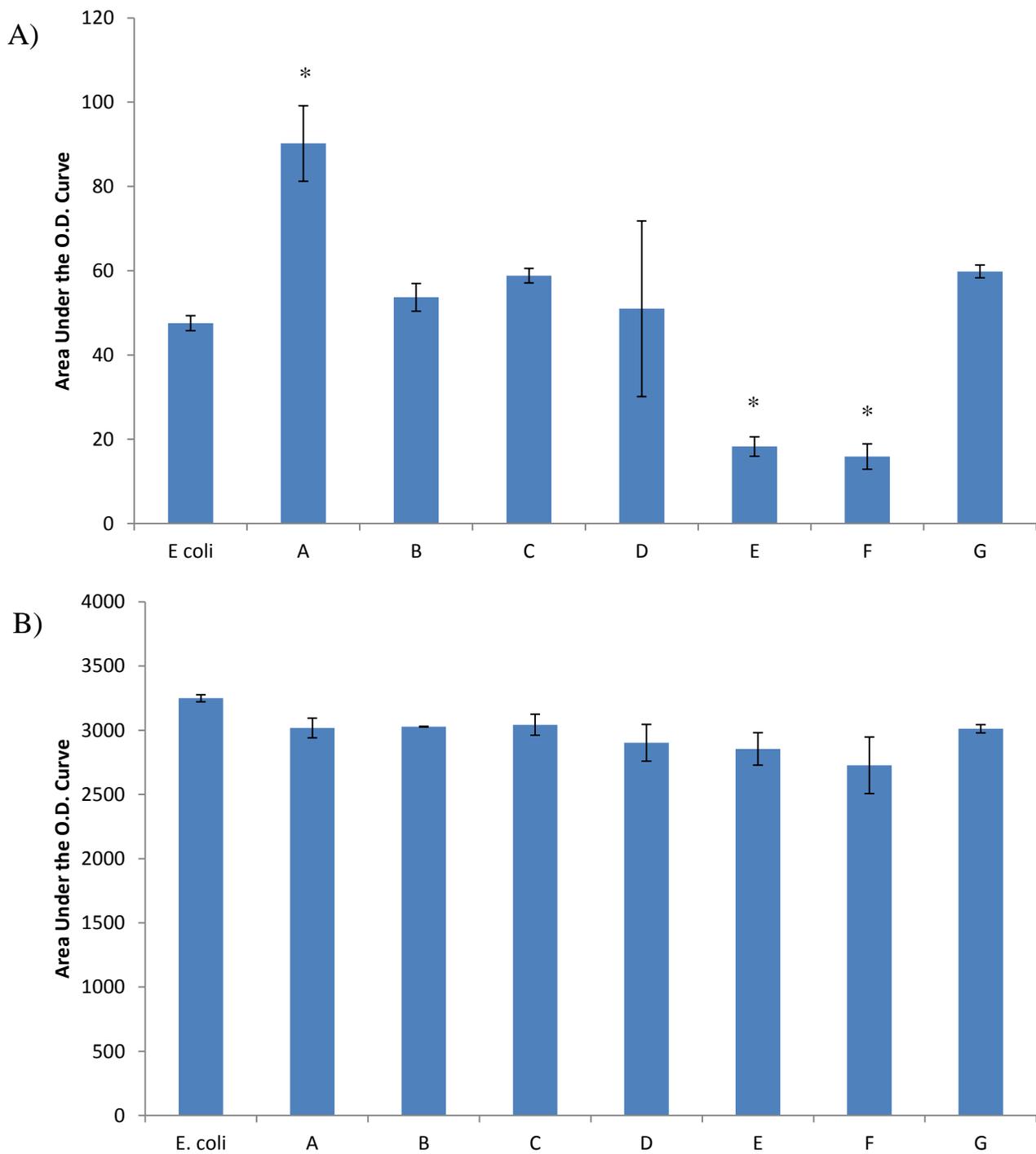


Figure 4. Area under the growth curve comparison for *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G. Error bars represent standard error of the mean. An asterisks depicts an AUC that is significantly different than the AUC of the *E. coli* O157:H7 control

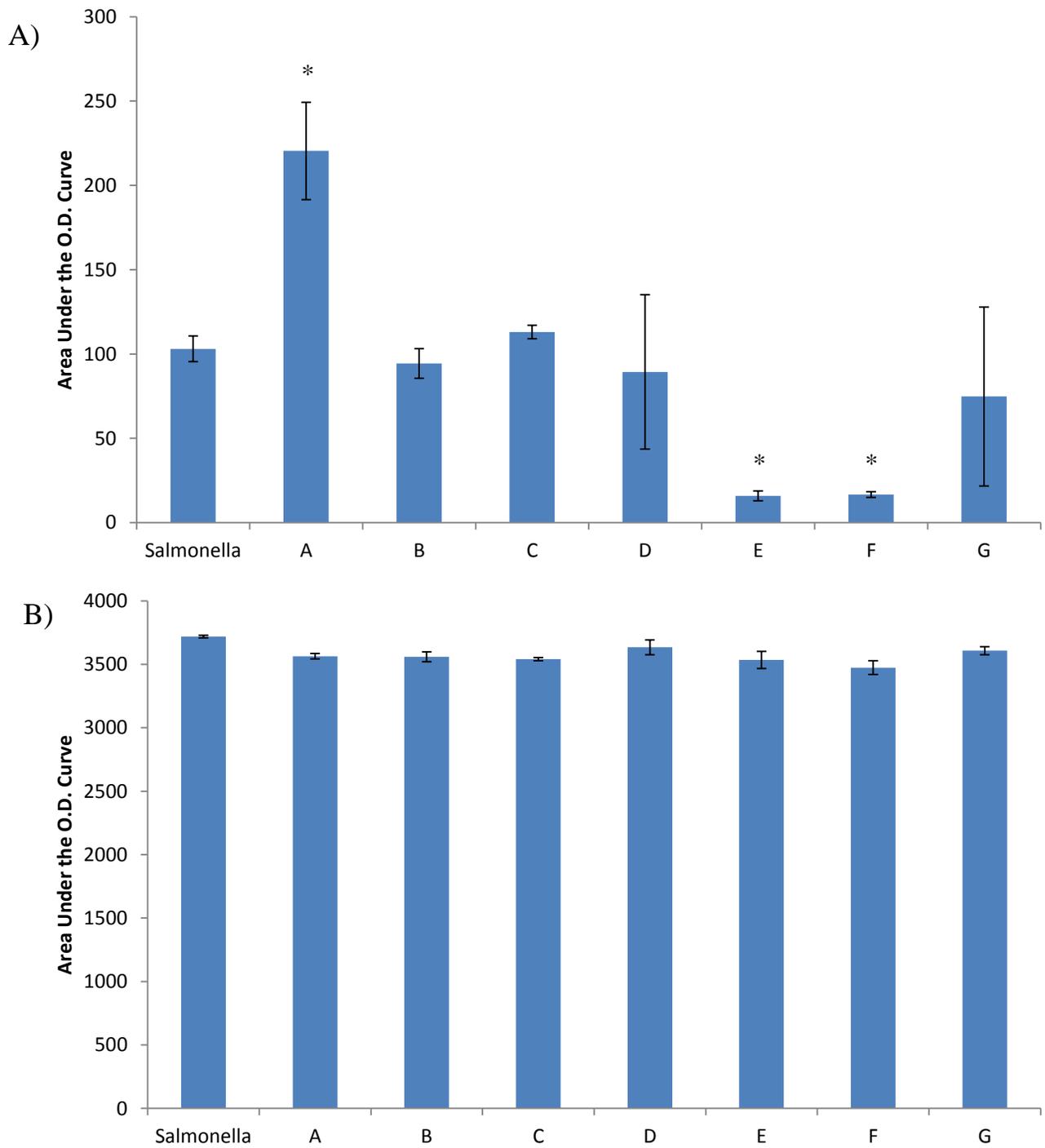


Figure 5. Area under the growth curve comparison for *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G. Error bars represent standard error of the mean. An asterisks depicts an AUC that is significantly different than the AUC of the *S. enterica* control

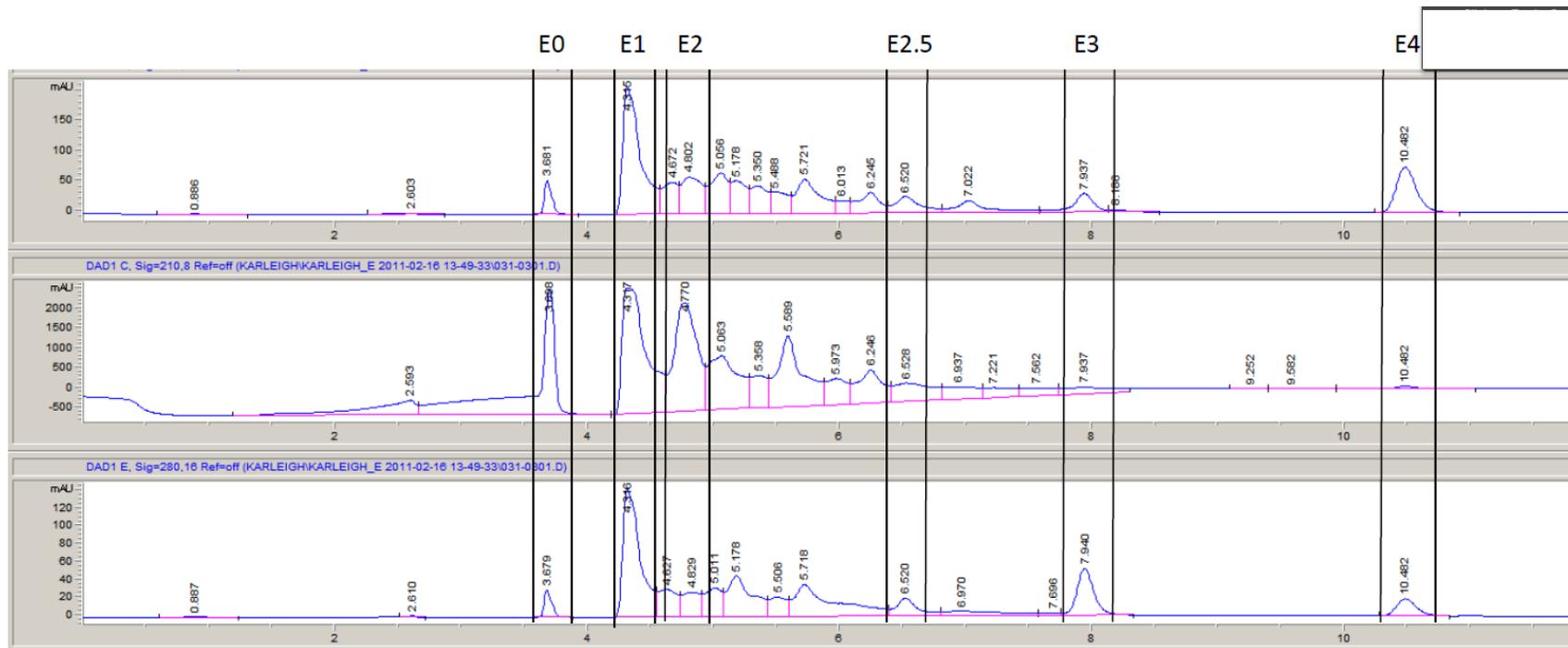


Figure 6. Reverse phase HPLC chromatogram of jalapeño extract Fraction E with vertical indicators of fraction collected. Fractions were collected based on a peak by peak basis and assigned numerical labels based on time of elution.

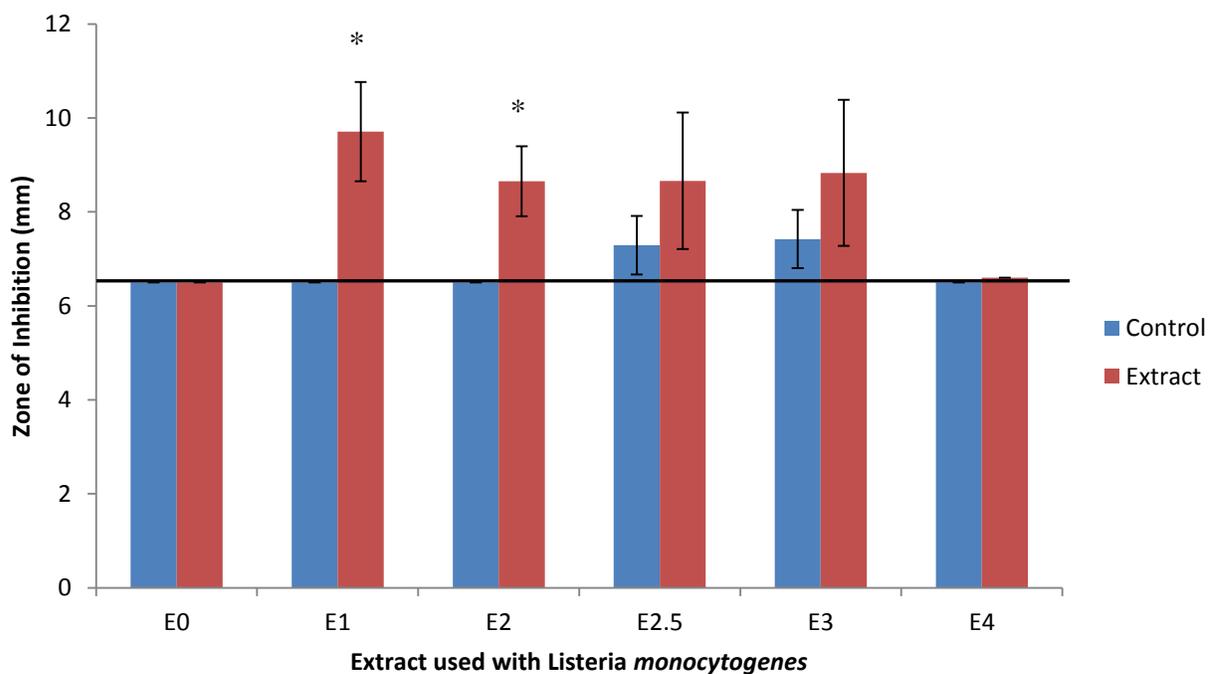


Figure 7: Disk diffusion results from subfractions of Fraction E against *Listeria monocytogenes*. Error bars represent standard error of the mean. An asterisk indicates significant difference between extract and control zones of inhibition. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned. The solid horizontal line represents the value 6.5 mm, which was the diameter of the disk used for the disk diffusion experiments. If no inhibition was seen, a value of 6.5 was assigned.

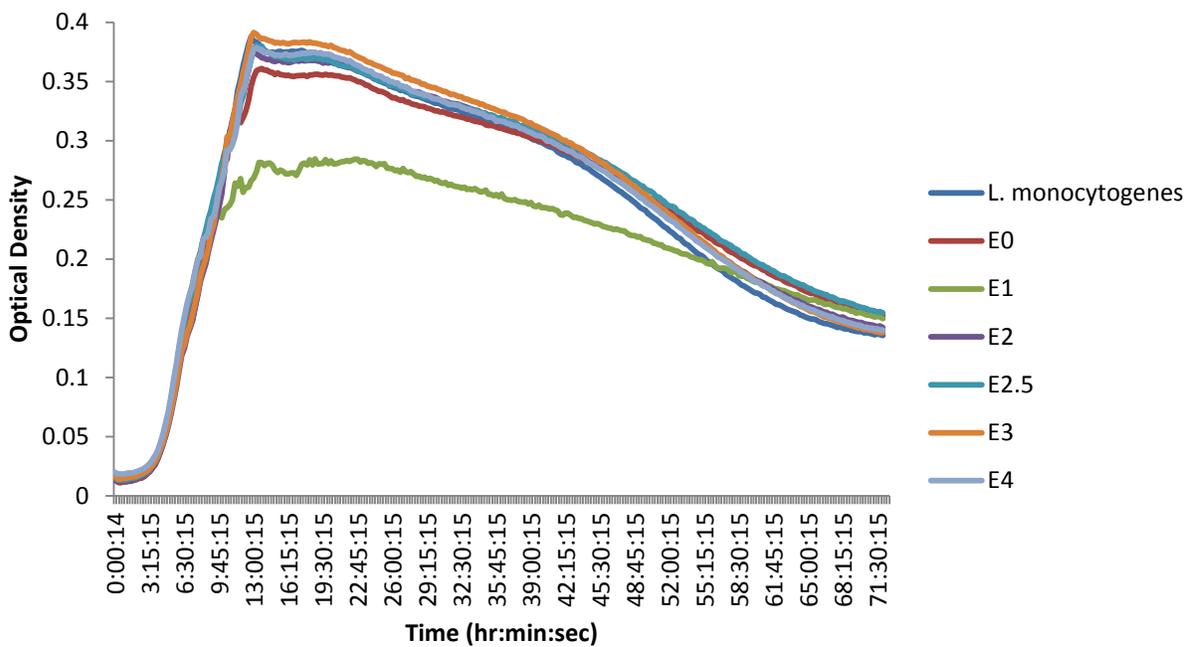
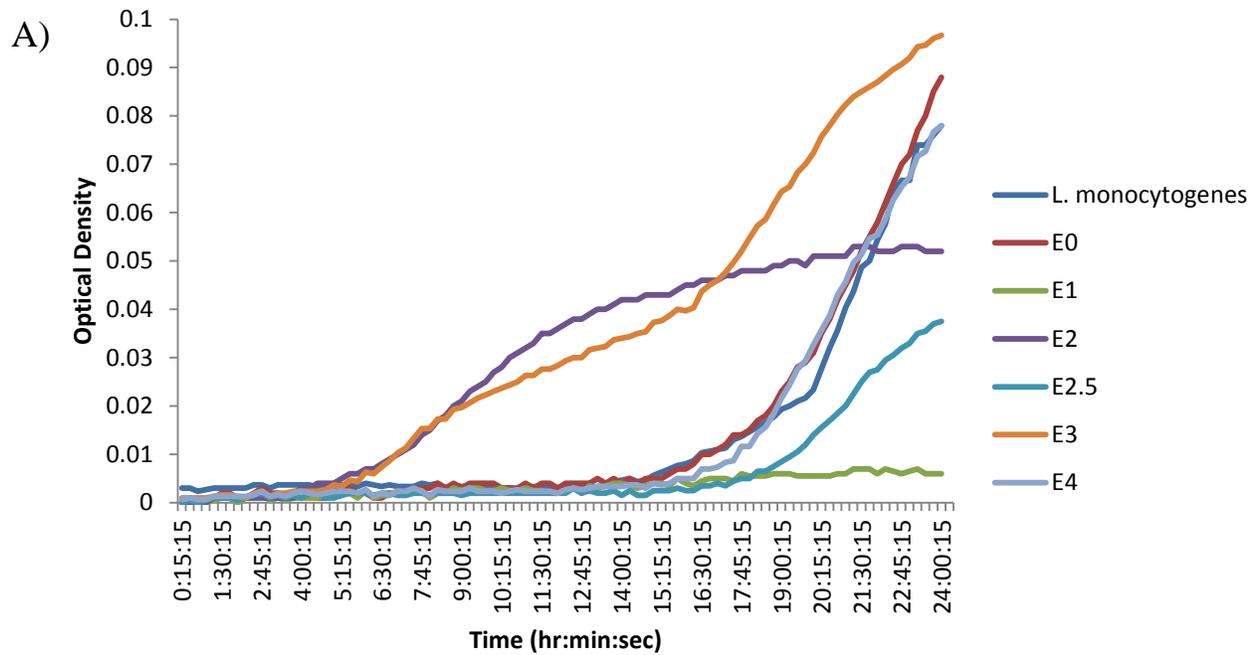


Figure 8. Growth curve OD values generated by Bioscreen C of *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4.

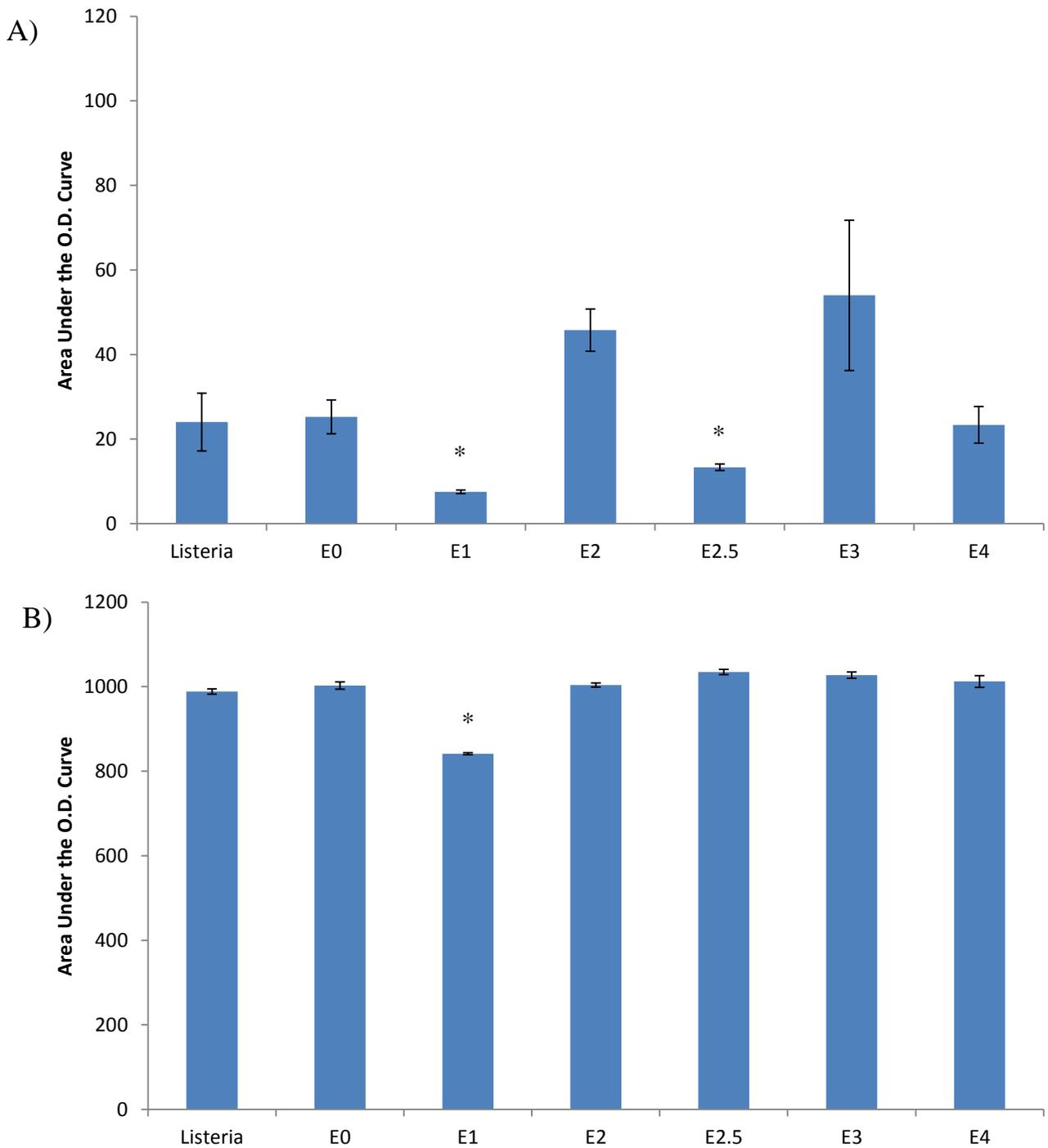


Figure 9. Area under the growth curve comparison for *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4. Error bars represent standard error of the mean. An asterisk indicates mean AUC values that are significantly less than the AUC value of the *L. monocytogenes* control.

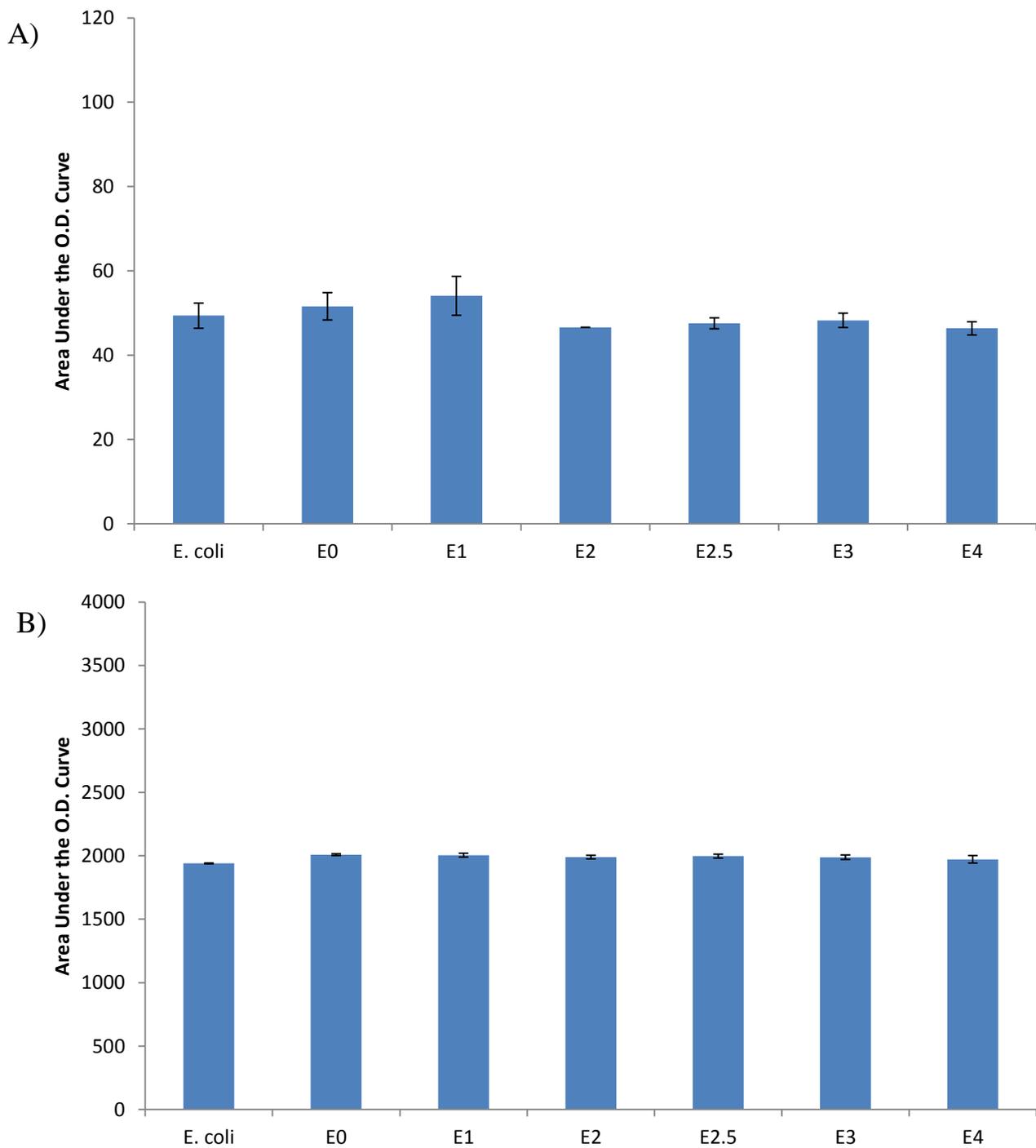


Figure 10. Area under the growth curve comparison for *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4. Error bars represent standard error of the mean.

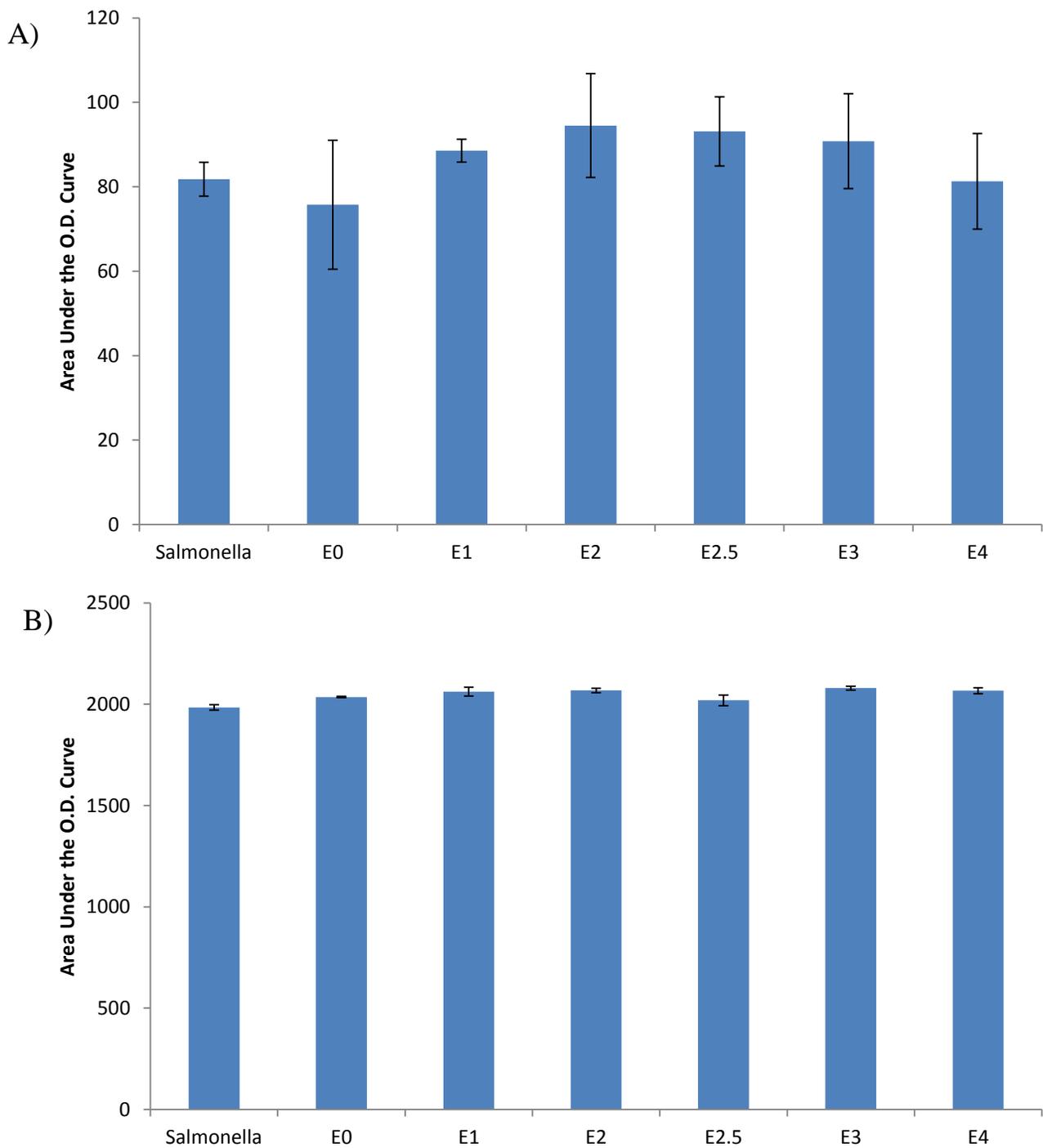
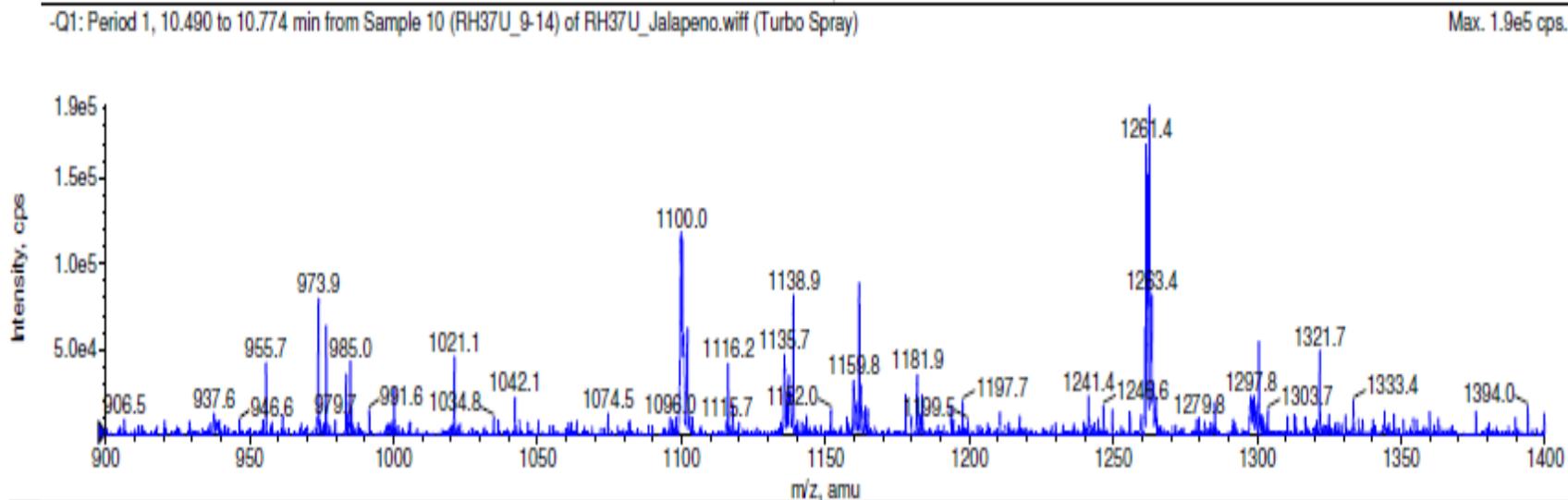


Figure 11. Area under the growth curve comparison for *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4. Error bars represent standard error of the mean.

A)



B)

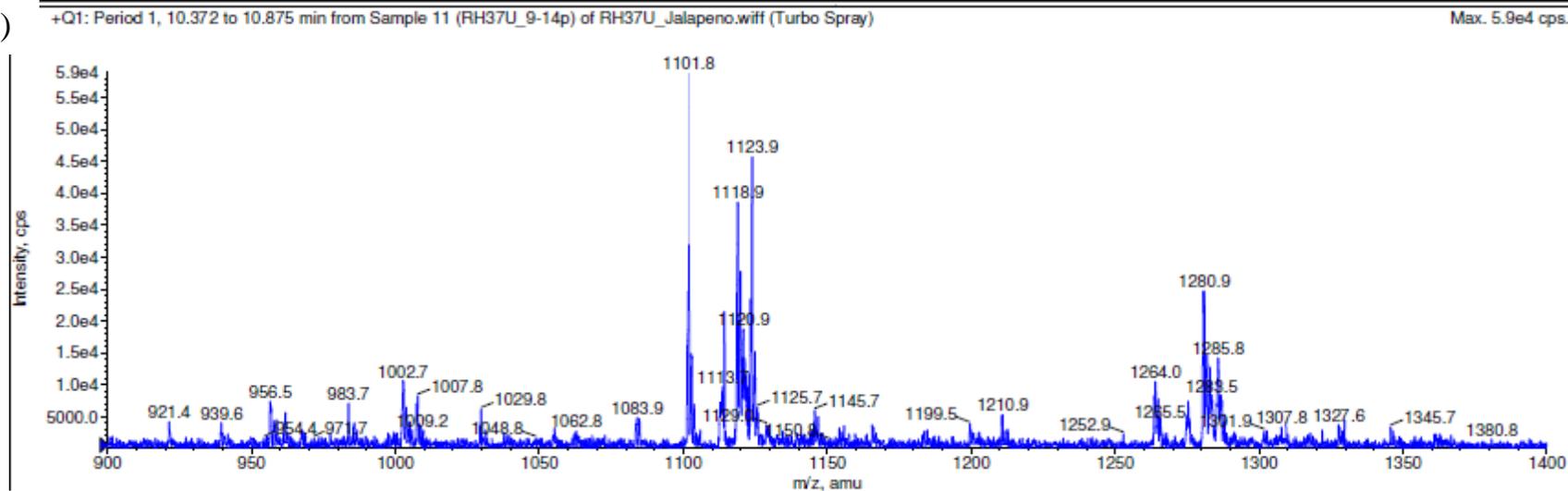


Figure 12. Mass scan of ions contained in Fraction E1 from 900 to 1400 amu. A) Negative ion scan. B) Positive ion (+Na) scan

CHAPTER 6

Conclusions and Future Directions

The purpose of this research was to better understand the role of jalapeño peppers in the field of microbial food safety. The hot pepper is gaining popularity in the United States as a both a vegetable and spice. Its role as a vehicle in a multistate food outbreak has revealed that it is not impervious to foodborne pathogenic bacteria. However, jalapeño peppers are also believed to possess some antimicrobial activity. This research was conducted to provide more information on the interactions of pathogenic bacterial pathogens and jalapeño peppers, and to reveal its bacterial survival, growth, and potential inhibitional properties.

This research revealed that an intact, uninjured jalapeño surface will not support the growth of the bacteria tested under storage conditions of 7°C. Jalapeño peppers in all the other situations tested became a food safety risk. *Listeria monocytogenes* proved especially adept at survival on stored jalapeño peppers, surviving at both recommended and abusive temperatures. *Listeria monocytogenes* was even capable of survival, but not growth on healthy jalapeño surfaces at 7°C. Storage at the recommended temperature (7°C) was successful at depriving the growth of *E. coli* and *Salmonella* for all conditions of the jalapeño fruit tested. The most successful growth environment for the enteric bacteria was the internal cavity of jalapeños held at 12°C. These results emphasize the importance of discarding jalapeños with damaged surfaces, which provide an environment favorable for survival of pathogens. This study also demonstrated the importance of holding jalapeños at recommended storage temperature. In order to assure that jalapeños are safe throughout the supply chain, only healthy, uninjured fruits held at proper storage temperatures should make it to the consumer.

It was also determined that extracts made from fresh jalapeño peppers were capable of inhibiting the growth of the pathogenic bacteria tested. *Listeria monocytogenes* was especially sensitive to the extracts. When jalapeño extracts were fractionated using HPLC, two fractions proved to stimulate bacterial growth, while two other fractions inhibited bacterial growth. Upon further fractionation of the inhibitory fractions, Fraction E1 displayed clear growth suppressing properties in association with *L. monocytogenes*. HPLC-MS analysis revealed that the mass of the ions in Fraction E1 matched with those of compounds known as capsianosides. Capsianosides are a group of compounds found exclusively within the *Capsicum* genus, and have been determined to be calcium chelators. It is possible that the inhibition of growth observed in *L. monocytogenes* was due to the presence of capsianosides in Fraction E1 and their ability to chelate calcium. Gram-positive organisms are especially sensitive to calcium chelation, as calcium is required to maintain the correct ionic environment for cation dependent membrane transport systems present on their cell wall. To prove that inhibition is being caused by capsianoside(s) and determine minimum inhibitory concentrations, a pure compound will need to be obtained.

An interesting paradox was revealed from the above research. It was shown that *L. monocytogenes* is the bacteria most capable of survival and growth on jalapeño peppers for the conditions tested, yet *L. monocytogenes* is also the bacteria most sensitive to jalapeño extract (both whole and fractionated). It may be that, although there are antimicrobial compounds present in the jalapeño peppers, those compounds are not readily available to interact with bacteria. It is also possible that the compounds that help stimulate bacterial growth, as seen in Fraction A and Fraction C, over power the compounds that can inhibit growth.

There are a number of opportunities for this research to be expanded upon in the future. For research concerning bacterial growth under select storage conditions, it would be interesting to observe the growth of the bacteria tested under room temperature, as well as under different humidity conditions. We saw from the antimicrobial studies that there are two fractions that actually stimulate the growth of bacteria. More research could be conducted to isolate those compounds. This would provide useful information in the future so that we may entertain the thought of selectively breeding peppers to make less of those stimulatory components. It was seen that Fraction F had antimicrobial properties, but further fractionation did not yield any antimicrobial portions. More research could be conducted to make combinations of F subfractions to look for synergistic interactions among compounds.

The most urgent step in the future of this research is to positively identify the compounds in Fraction E1. Although there is evidence that the compounds are capsiariosides, no positive control exists to affirm those assumptions. In the future, a method should be developed to isolate capsiariosides specifically from jalapeño peppers and produce a pure extract. The compounds isolated should be tested for antimicrobial activity and analyzed with mass spec and NMR to confirm the compounds' ionic mass and reveal their structure. Only then will we be able to say with certain that the inhibitory compounds present in Fraction E1 are capsiariosides.

Appendix A.

Comparison of methods to determine the inhibition values for jalapeño extract Fraction E1 using manual growth curve analysis and automated growth curve analysis.

To address the concern that optical density values may overestimate the amount of inhibitory ability a compound might possess, manual growth curves were generated along with automated growth curves for comparison using jalapeño Fraction E1 extract. The log values generated from the manual growth curve analysis was compared to the percent reduction of OD values collected at specific time points over a 24 h period.

Materials and Methods

Automated growth curve analysis for antimicrobial assessment. A Bioscreen C Microbiology Reader (Growth Curves, Piscataway, NJ) equipped with an incubator and automated turbidimeter was used for determination of optical density (OD). Optical density was determined between 420-540 nm. The liquid growth medium used was TSB. Jalapeño fraction E1 was filter sterilized using a 0.45 µm syringe Whatman PTFE Puradisc (Whatman Inc., Piscataway, NJ) and a honeycomb microwell plate (Bioscreen, Growth Curves, Piscataway, NJ) was filled with 125 µl of growth medium, 15 µl of prepared jalapeño extract fraction, and 10 µl of *Listeria monocytogenes* (containing approx. 10^3 cells). For controls, eluent was collected from the HPLC column using the same conditions as the E1 fraction, but water was injected into the column rather than jalapeño extract. Control wells contained 15 µl of corresponding fraction controls to replace jalapeño extract. Microwell plates were incubated at 37°C for 24 h, and OD was measured every 15 minutes with 10 seconds of shaking before each reading. The growth curve data was generated by using EZExperiment software (Growth Curves, Piscataway, NJ) and exported as a Microsoft Excel spreadsheet (Microsoft, Seattle, WA). Each experiment was

replicated three times. Growth curves generated were analyzed for statistical difference of the means using a student t-test for specific time points in the study. The trapezoidal area under the curve (AUC) was determined for each growth curve generated using an Excel worksheet, and the means were compared using a student t-test.

Manual growth curve analysis for antimicrobial assessment.

Growth curves for *L. monocytogenes* in the presence of Fraction E1 were also generated using traditional dilution and plating methods. In a sterile tube, 9 ml of TSB, 1 ml of jalapeño Fraction E1, and 100 μ l of *L. monocytogenes* were combined. Controls were prepared in a similar fashion, replacing jalapeño extract fractions with 1 ml of Fraction E control. All tubes were incubated at 37°C throughout the study. At hours 0, 3, 6, 12, and 24, 1 ml of the culture/media combination was removed from the tube, serially diluted in 0.1% peptone water, and pour plated using TSA. Plates were allowed to dry, inverted, and incubated at 37°C for 48 h. Plates were then removed from the incubator and colonies were counted and recorded.

Results

Results of the manual growth curve assays for E1 can be found in Table A1. Manual plating methods confirmed the antimicrobial effect of Fraction E1 on *L. monocytogenes*, showing growth is inhibited by 15% after 6 h. Optical density values show that growth is inhibited by 22.6 % and 18.2% after 12 h and 24 h, respectively. The difference in inhibition suggested by both assays is small. One obvious difference between the two assays was the time point in which the bacteria appeared to come out of lag phase. For the plating methods, bacterial populations started to rapidly increase after 3 h of incubation, where exponential growth for the bacteria in microwells took between 6 and 12 h. However, both assays started with a bacterial cell concentration of approximately 4 log CFU/ml. It has been noted that the Bioscreen OD reader has

low sensitivity and does not detect changes in OD until the bacterial population reaches approximately 10^6 - 10^7 CFU/ml (Nychas et al., 2003). Therefore, growth of bacteria between 4 log CFU/ml and 7 log CFU/ml would not be as readily detectable using OD values generated from Bioscreen as it would be for manual plating methods. This is supported by our results, in which bacterial populations were near 5 logs CFU/ml (3 h) and between 6 and 7 log CFU/ml (6 h) for the plating method, and little change in OD value was noted for those hours using the Bioscreen. Differences in populations may also be due to injured bacterial cells causing the OD reading to be lower, while those stressed but viable bacteria would likely grow when introduced to the nutrient rich TSA agar used for the plating method (Lambert and van der Ouderaa, 1999).

Table A1. Manual growth curve inhibition results for *L. monocytogenes* grown in TSB with Fraction E1 compared to inhibition results generated under the same conditions using Bioscreen OD values.

Time (h)	Log CFU			Optical Density		
	Control	E1 Extract	Percent difference	Control	E1 Extract	Percent Difference
0	4.09	4.07	0.50	0.00	0.00	0.00
3	5.16	5.01	3.02	0.01	0.01	0.00
6	6.86	5.77	15.78	0.01	0.01	0.00
12	8.85	8.66	2.08	0.31	0.24	22.6
24	8.89	8.89	0.04	0.33	0.27	18.2

References

- Lambert, R. J. W., and M.-L. H. van der Ouderaa. 1999. An investigation into the differences between Bioscreen and the traditional plate count disinfectant test methods. *J. Appl. Microbiol.* 86:689-694.
- Nychas, G.-J. E., and P. N. Skandamis. 2003. Antimicrobials from herbs and spices. p. 176-200. In S. Roller (ed.), *Natural antimicrobials for the minimal processing of foods*. CRC Press, Boca Raton, FL.

Appendix B.

Population and comparison of mean bacterial populations artificially inoculated on jalapeño peppers and stored at 7°C or 12°C over a 14 day period.

Table B1. Population change of bacteria on the surface of jalapeño peppers at 7°C

Experiment Day	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	3.30±0.09 ^A	N/A	2.85±0.11 ^{AB}	N/A	2.60±0.07 ^A	N/A
1	3.21±0.05 ^A	-0.09	2.18±0.32 ^B	-0.67	1.65±0.37 ^{AB}	-0.94
2	2.58±0.04 ^B	-0.73	2.99±0.17 ^A	+0.15	1.07±0.46 ^{BC}	-1.53
5	2.19±0.07 ^{CD}	-1.11	0.61±0.26 ^C	-2.24	0.72±0.31 ^{BC}	-1.88
7	2.40±0.12 ^{BC}	-0.90	<1.00 ^C	-2.85	0.49±0.22 ^C	-2.10
10	2.14±0.07 ^{CD}	-1.17	<1.00 ^C	-2.85	0.25±0.17 ^C	-2.35
14	1.94±0.08 ^D	-1.36	<1.00 ^C	-2.85	<1.00 ^C	-2.60

*Numbers followed by the same letter denotes no significant difference (p<0.05) between means as determined by Tukey's HSD comparison.

Table B2: Population change of bacteria on the injured surface of jalapeño peppers at 7°C

Experiment Day	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	3.29±0.08 ^A	N/A	2.71±0.05 ^A	N/A	3.25±0.03 ^A	N/A
1	2.69±0.13 ^B	-0.60	2.78±0.16 ^A	+0.06	2.97±0.06 ^A	-0.28
2	2.42±0.04 ^B	-0.87	2.45±0.20 ^A	-0.27	3.10±0.16 ^A	-0.15
5	3.69±0.10 ^A	+0.40	1.20±0.31 ^B	-1.51	2.03±0.28 ^B	-1.22
7	3.59±0.10 ^A	+0.30	0.28±0.19 ^C	-2.43	1.29±0.29 ^B	-1.96
10	3.45±0.07 ^A	+0.16	<1.00 ^C	-2.71	0.74±0.26 ^B	-2.51
14	3.57±0.17 ^A	+0.28	<1.00 ^C	-2.71	1.24±0.31 ^B	-2.01

*Numbers followed by the same letter denotes no significant difference (p<0.05) between means as determined by Tukey's HSD comparison.

Table B3: Population change of bacteria injected internally into jalapeño peppers at 7°C

Experiment Day	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	3.06±0.07 ^D	N/A	3.18±0.08 ^A	N/A	3.15±0.06 ^A	N/A
1	2.99±0.09 ^D	-0.08	3.29±0.06 ^A	+0.11	3.18±0.18 ^A	+0.03
2	3.72±0.04 ^C	+0.66	2.66±0.37 ^{AB}	-0.52	3.17±0.09 ^A	+0.02
5	4.21±0.15 ^B	+1.15	2.24±0.18 ^{BC}	-0.94	0.62±0.31 ^B	-2.53
7	4.79±0.03 ^A	+1.73	1.63±0.35 ^{CD}	-1.55	0.11±0.11 ^C	-3.04
10	4.85±0.08 ^A	+1.79	0.81±0.36 ^{DE}	-2.37	<1.00 ^C	-3.15
14	4.90±0.12 ^A	+1.84	<1.00 ^E	-3.18	<1.00 ^C	-3.15

*Numbers followed by the same letter denotes no significant difference (p<0.05) between means as determined by Tukey's HSD comparison.

Table B4: Population change of bacteria on the surface of jalapeño peppers at 12°C

Experiment	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
Day	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	3.28±0.13 ^B		3.13±0.05 ^A		2.87±0.09 ^A	
1	2.70±0.15 ^B	-0.58	2.02±0.06 ^{BC}	-1.11	1.69±0.06 ^B	-1.18
2	3.18±0.22 ^B	-0.10	1.76±0.07 ^{BCD}	-1.37	0.94±0.29 ^B	-1.93
5	3.14±0.019 ^B	-0.14	1.22±0.22 ^D	-1.91	1.39±0.25 ^{AB}	-1.48
7	3.15±0.46 ^B	-0.13	1.84±0.16 ^{BC}	-1.29	1.26±0.21 ^{AB}	-1.61
10	3.68±0.26 ^{AB}	+0.39	2.30±0.15 ^B	-0.83	<1.00 ^B	-2.87
14	4.35±0.26 ^A	+1.07	1.63±0.18 ^{CD}	-1.51	<1.00 ^B	-2.87

*Numbers followed by the same letter denotes no significant difference (p<0.05) between means as determined by Tukey's HSD comparison.

Table B5: Population change of bacteria on the injured surface of jalapeño peppers at 12°C

Experiment	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
Day	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	2.67±0.15 ^D		3.07±0.03 ^A		3.05±0.04 ^E	
1	3.50±0.17 ^{ABC}	+0.83	2.62±0.08 ^A	-0.45	3.68±0.07 ^D	+0.63
2	2.78±0.15 ^{CD}	+0.11	2.99±0.25 ^A	-0.07	4.04±0.04 ^{CD}	+0.99
5	3.36±0.10 ^{BCD}	+0.69	2.26±0.43 ^A	-0.81	4.28±0.15 ^{BC}	+1.23
7	3.69±0.25 ^{AB}	+1.02	2.62±0.27 ^A	-0.45	4.79±0.06 ^A	+1.74
10	4.39±0.31 ^A	+1.72	2.88±0.23 ^A	-0.19	4.57±0.13 ^{AB}	+1.52
14	4.08±0.25 ^{AB}	+1.41	2.76±0.27 ^A	-0.30	4.67±0.09 ^{AB}	+1.62

*Numbers followed by the same letter denotes no significant difference (p<0.05) between means as determined by Tukey's HSD comparison.

Table B6: Population change of bacteria injected internally into jalapeño peppers at 12°C

Experiment Day	Population (log CFU/g) of pathogen					
	<i>Listeria</i>		<i>Escherichia coli</i>		<i>Salmonella</i>	
	Log CFU/g*	Log change	Log CFU/g*	Log change	Log CFU/g*	Log change
0	3.10±0.16 ^C		3.21±0.05 ^B		3.16±0.06 ^D	
1	3.14±0.07 ^C	+0.04	3.09±0.13 ^B	-0.12	4.35±0.13 ^C	+1.19
2	3.07±0.11 ^C	-0.04	3.05±0.11 ^B	-0.15	5.01±0.12 ^{ABC}	+1.84
5	3.53±0.17 ^{BC}	+0.42	2.83±0.38 ^B	-0.37	5.27±0.10 ^{AB}	+2.10
7	4.19±0.26 ^{AB}	+1.09	3.34±0.36 ^B	+0.13	5.09±0.17 ^{AB}	+1.92
10	4.80±0.20 ^{AB}	+1.69	4.31±0.19 ^A	+1.10	4.85±0.12 ^{BC}	+1.69
14	4.93±0.19 ^A	+1.83	4.32±0.16 ^A	+1.11	5.68±0.34 ^A	+2.52

*Numbers followed by the same letter denotes no significant difference ($p < 0.05$) between means as determined by Tukey's HSD comparison.

Appendix C.

Comparison of mean OD values for pathogens grown in media containing jalapeño extract fractions at specific time points.

Table C1. Comparison of mean *Listeria monocytogenes* OD in peptone for treatments containing 10% jalapeño Fraction A through G extract over time.

	Time (h)				
	0	3	6	12	24
<i>Listeria monocytogenes</i> (no treatment)	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Fraction A	0.00±0.00	0.01±0.00*	0.02±0.00*	0.05±0.00*	0.07±0.00*
Fraction B	0.00±0.00	0.00±0.00	0.02±0.00*	0.02±0.00*	0.02±0.00*
Fraction C	0.00±0.00	0.01±0.00*	0.02±0.00*	0.03±0.00*	0.03±0.00*
Fraction D	0.00±0.00	0.01±0.00*	0.01±0.00	0.01±0.00	0.01±0.00
Fraction E	0.00±0.00*	0.01±0.00*	0.01±0.00	0.01±0.00	0.01±0.00
Fraction F	0.01±0.00*	0.01±0.00*	0.01±0.00	0.01±0.00D	0.01±0.00
Fraction G	0.00±0.00	0.01±0.00*	0.01±0.00*	0.01±0.00*	0.01±0.00*

*Denotes significant difference from OD value of *Listeria monocytogenes* control (p<0.05)

Table C2. Comparison of mean *Listeria monocytogenes* OD in tryptic soy broth for treatments containing 10% jalapeño Fraction A through G extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>Listeria monocytogenes</i> (no treatment)	0.00±0.00	0.00±0.00	0.02±0.00	0.16±0.00	0.14±0.00	0.11±0.00	0.06±0.00	0.04±0.01	0.03±0.01
Fraction A	0.00±0.00	0.00±0.00	0.02±0.00	0.15±0.00	0.12±0.00	0.10±0.00	0.09±0.01	0.08±0.01	0.07±0.00
Fraction B	0.00±0.00	0.00±0.00	0.02±0.00	0.16±0.00	0.14±0.00	0.12±0.00	0.07±0.00	0.06±0.00	0.06±0.00
Fraction C	0.00±0.00	0.00±0.00	0.02±0.00	0.23±0.02	0.24±0.3	0.18±0.03	0.11±0.02	0.10±0.02	0.10±0.02
Fraction D	0.00±0.00	0.00±0.00	0.02±0.00	0.25±0.09	0.35±0.22	0.37±0.26	0.35±0.27*	0.33±0.28	0.33±0.27
Fraction E	0.00±0.00	0.00±0.00	0.01±0.00*	0.13±0.00	0.12±0.00	0.10±0.00	0.08±0.00	0.07±0.00	0.07±0.00
Fraction F	0.00±0.00	0.00±0.00	0.02±0.00	0.15±0.00	0.11±0.00	0.10±0.00	0.09±0.00	0.08±0.00	0.08±0.00
Fraction G	0.00±0.00	0.00±0.00	0.02±0.00	0.16±0.00	0.13±0.00	0.11±0.00	0.06±0.01	0.04±0.01	0.05±0.01

*Denotes significant difference from OD value of *Listeria monocytogenes* control (p<0.05)

Table C3. Comparison of mean *Escherichia coli* O157:H7 OD in peptone for treatments containing 10% jalapeño fraction A through G extract over time.

	Time (h)				
	0	3	6	12	24
<i>Escherichia coli</i> O157:H7 (no treatment)	0.00±0.00	0.02±0.00	0.03±0.00	0.04±0.00	0.04±0.00
Fraction A	0.00±0.00	0.04±0.01	0.06±0.01*	0.07±0.01*	0.08±0.01*
Fraction B	0.00±0.00	0.03±0.00	0.04±0.00	0.04±0.00	0.05±0.00
Fraction C	0.00±0.00	0.03±0.00	0.04±0.00	0.05±0.00	0.05±0.00
Fraction D	0.00±0.00	0.01±0.01	0.02±0.01	0.04±0.01	0.05±0.02
Fraction E	0.01±0.00*	0.01±0.00	0.01±0.00	0.01±0.00*	0.01±0.00
Fraction F	0.01±0.00*	0.01±0.00*	0.01±0.00*	0.01±0.00*	0.01±0.00*
Fraction G	0.00±0.00	0.03±0.01	0.04±0.00	0.05±0.00	0.05±0.00

*Denotes significant difference from OD value of *Escherichia coli* O157:H7 control (p<0.05)

Table C4. Comparison of mean *Escherichia coli* O157:H7 OD in tryptic soy broth for treatments containing 10% jalapeño Fraction A through G extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>Escherichia coli</i> O157:H7 (no treatment)	0.00±0.00	0.16±0.00	0.34±0.00	0.42±0.01	0.73±0.01	0.92±0.01	0.83±0.01	0.84±0.01	0.86±0.01
Fraction A	0.00±0.00	0.16±0.01	0.31±0.01	0.42±0.01	0.74±0.03	0.85±0.01	0.84±0.01	0.85±0.01	0.87±0.01
Fraction B	0.00±0.00	0.16±0.00	0.32±0.00	0.43±0.01	0.75±0.01	0.84±0.00	0.84±0.00	0.85±0.00	0.85±0.01
Fraction C	0.00±0.00	0.15±0.00	0.29±0.00	0.42±0.02	0.74±0.02	0.86±0.02	0.86±0.02	0.87±0.02	0.87±0.02
Fraction D	0.00±0.00	0.14±0.01*	0.27±0.04*	0.40±0.04	0.69±0.06	0.83±0.02	0.83±0.02	0.83±0.02	0.83±0.01
Fraction E	0.00±0.00	0.14±0.00*	0.26±0.01*	0.39±0.04	0.74±0.04	0.80±0.02*	0.81±0.02	0.80±0.02*	0.79±0.03*
Fraction F	0.00±0.00	0.13±0.01*	0.25±0.02*	0.36±0.10	0.69±0.05	0.77±0.04*	0.78±0.03*	0.77±0.03*	0.77±0.04*
Fraction G	0.00±0.00	0.15±0.00	0.32±0.0	0.46±0.04	0.77±0.02	0.83±0.00	0.83±0.01	0.82±0.01	0.81±0.04

*Denotes significant difference from OD value of *Escherichia coli* O157:H7 control (p<0.05)

Table C5. Comparison of mean *Salmonella enterica* OD in peptone for treatments containing 10% jalapeño Fraction A through G extract over time.

	Time (h)				
	0	3	6	12	24
<i>Salmonella enteric</i> (no treatment)	0.00±0.00	0.03±0.00	0.05±0.00	0.08±0.00	0.11±0.00
Fraction A	0.00±0.00	0.07±0.02*	0.14±0.03*	0.19±0.03*	0.19±0.02*
Fraction B	0.00±0.00	0.04±0.00	0.08±0.01	0.08±0.01	0.07±0.01
Fraction C	0.00±0.00	0.03±0.00	0.09±0.00	0.10±0.00	0.09±0.01
Fraction D	0.01±0.00*	0.02±0.00	0.06±0.02	0.08±0.03	0.08±0.03
Fraction E	0.01±0.00*	0.01±0.00	0.01±0.00	0.01±0.00*	0.01±0.00*
Fraction F	0.01±0.00*	0.01±0.00	0.01±0.00	0.01±0.00*	0.01±0.00*
Fraction G	0.00±0.00	0.01±0.01	0.04±0.02	0.06±0.04	0.07±0.04

*Denotes significant difference from OD value of *Salmonella enterica* control (p<0.05)

Table C6. Comparison of mean *Salmonella enterica* OD in tryptic soy broth for treatments containing 10% jalapeño Fraction A through G extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>Salmonella enterica</i> (no treatment)	0.00±0.00	0.21±0.00	0.42±0.00	0.65±0.00	0.92±0.00	0.98±0.01	0.98±0.00	0.99±0.00	1.00±0.00
Fraction A	0.00±0.00	0.21±0.00	0.42±0.00	0.64±0.02	0.92±0.01	0.95±0.01*	0.95±0.00*	0.96±0.01	0.97±0.03
Fraction B	0.00±0.00	0.20±0.00	0.42±0.01	0.65±0.01	0.91±0.01	0.95±0.02*	0.94±0.01*	0.95±0.01*	0.96±0.01*
Fraction C	0.00±0.00	0.20±0.00*	0.42±0.00	0.65±0.00	0.91±0.00	0.95±0.01*	0.93±0.00*	0.95±0.00*	0.97±0.01*
Fraction D	0.00±0.00	0.20±0.00*	0.42±0.01	0.64±0.02	0.93±0.01	0.98±0.01	0.97±0.01	0.98±0.01	1.00±0.01
Fraction E	0.00±0.00	0.21±0.01	0.42±0.01	0.62±0.01*	0.89±0.01*	0.96±0.01	0.94±0.02*	0.95±0.02*	0.97±0.03
Fraction F	0.00±0.00	0.20±0.00*	0.40±0.01*	0.58±0.02*	0.88±0.01*	0.96±0.01*	0.93±0.01*	0.94±0.01*	0.96±0.01*
Fraction G	0.00±0.00	0.21±0.00	0.42±0.01	0.65±0.01	0.93±0.01	0.99±0.00	0.96±0.01	0.97±0.01	0.98±0.02

*Denotes significant difference from OD value of *Salmonella enterica* control (p=0.05)

Table C7. Comparison of mean OD values of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* after 24 h growth in peptone for treatments containing 10% jalapeño subfraction E0 through E4 extract over time.

	<i>Listeria monocytogenes</i>		<i>Escherichia coli</i> O157:H7		<i>Salmonella enterica</i>	
	OD	Difference from control (%)	OD	Difference from control (%)	OD	Difference from control (%)
Control treatment	0.08±0.01	N.A.	0.04±0.00	N.A.	0.07±0.01	N.A.
Fraction E0	0.09±0.00	+12.5%	0.04±0.00	±00.0%	0.07±0.01	±00.0%
Fraction E1	0.01±0.00*	-87.5%	0.05±0.00*	+25.0%	0.07±0.01	±00.0%
Fraction E2	0.05±0.00*	-37.5%	0.04±0.00	±00.0%	0.07±0.01	±00.0%
Fraction E2.5	0.04±0.00*	-50.0%	0.04±0.00	±00.0%	0.07±0.01	±00.0%
Fraction E3	0.10±0.01	+25.0%	0.04±0.00	±00.0%	0.07±0.01	±00.0%
Fraction E4	0.08±0.02	±00.0%	0.04±0.00	±00.0%	0.07±0.01	±00.0%

*Denotes significant difference from OD value of control (p=0.05)

Table C8. Comparison of mean *Listeria monocytogenes* OD values in tryptic soy broth for treatments containing 10% jalapeño subfractions E0 through E4 extract over time.

	Time (h)									
	0	3	6	12	24	36	48	60	72	
<i>Listeria monocytogenes</i> control	0.00±0.00	0.01±0.00	0.11±0.00	0.34±0.00	0.34±0.01	0.30±0.00	0.24±0.00	0.17±0.00	0.13±0.00	
Fraction E0	0.00±0.00	0.01±0.00	0.10±0.00	0.31±0.01*	0.33±0.00	0.30±0.00	0.25±0.00	0.18±0.00	0.14±0.00	
Fraction E1	0.00±0.00	0.01±0.00	0.11±0.00	0.24±0.01*	0.27±0.00*	0.24±0.00*	0.21±0.00*	0.17±0.00	0.14±0.00	
Fraction E2	0.00±0.00	0.01±0.00	0.10±0.00	0.33±0.01*	0.34±0.00	0.30±0.00	0.25±0.00	0.17±0.00	0.13±0.00	
Fraction E2.5	0.00±0.00	0.01±0.00	0.11±0.00	0.33±0.00*	0.34±0.00	0.30±0.00	0.25±0.00	0.19±0.00	0.14±0.00	
Fraction E3	0.00±0.00	0.01±0.00	0.10±0.01	0.34±0.00	0.35±0.00	0.31±0.01	0.25±0.00	0.17±0.00	0.13±0.00	
Fraction E4	0.00±0.00	0.01±0.00	0.11±0.01	0.33±0.01	0.35±0.00	0.30±0.00	0.25±0.00	0.17±0.00	0.13±0.00	

*Denotes OD values that are significantly smaller than the OD value of *Listeria monocytogenes* control (p<0.05)

Table C9. Comparison of mean *Escherichia coli* O157:H7 OD values in tryptic soy broth for treatments containing 10% jalapeño subfractions E0 through E4 extract over time.

	Time (h)						
	0	3	6	12	24	36	48
<i>E. coli</i> O157:H7 control	0.00±0.00	0.20±0.00	0.29±0.01	0.69±0.01	0.84±0.00	0.81±0.00	0.80±0.01
Fraction E0	0.00±0.00	0.19±0.00*	0.27±0.01	0.70±0.01	0.87±0.00	0.86±0.00	0.84±0.00
Fraction E1	0.00±0.00	0.20±0.00	0.26±0.00*	0.68±0.02	0.87±0.01	0.86±0.01	0.85±0.01
Fraction E2	0.00±0.00	0.19±0.01	0.27±0.02	0.70±0.01	0.87±0.00	0.85±0.00	0.83±0.00
Fraction E2.5	0.00±0.00	0.19±0.01	0.28±0.01	0.71±0.01	0.87±0.00	0.85±0.00	0.82±0.00
Fraction E3	0.00±0.00	0.18±0.00*	0.29±0.01	0.71±0.01	0.86±0.00	0.84±0.00	0.82±0.00
Fraction E4	0.00±0.00	0.19±0.01	0.29±0.01	0.70±0.01	0.86±0.01	0.84±0.01	0.82±0.01

*Denotes OD values that are significantly smaller than the OD value of *E. coli* O157:H7 control (p<0.05)

Table C10. Comparison of mean OD values of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* after 24 h growth in peptone for treatments containing 10% jalapeño subfraction F1 through F7 extract over time.

	<i>Listeria monocytogenes</i>		<i>Escherichia coli</i> O157:H7		<i>Salmonella enterica</i>	
	OD	Difference from control (%)	OD	Difference from control (%)	OD	Difference from control (%)
Control treatment	0.05±0.01	N.A.	0.03±0.00	N.A.	0.06±0.00	N.A.
Fraction F1	0.04±0.00	±00.0%	0.04±0.00	+33.3%	0.06±0.01	±00.0%
Fraction F2	0.05±0.01	±00.0%	0.03±0.00	±00.0%	0.08±0.00	+33.3%
Fraction F3	0.05±0.00	±00.0%	0.03±0.00	±00.0%	0.07±0.01	±00.0%
Fraction F4	0.05±0.00	±00.0%	0.03±0.00	±00.0%	0.08±0.00	+33.3%
Fraction F5	0.05±0.00	±00.0%	0.03±0.00	±00.0%	0.07±0.00	+16.7%
Fraction F6	0.04±0.00	±00.0%	0.03±0.00	±00.0%	0.08±0.00	+33.3%
Fraction F7	0.05±0.00	±00.0%	0.03±0.00	±00.0%	0.08±0.01	+33.3%

*Denotes significant difference from OD value of control (p=0.05)

Table C11. Comparison of mean *Listeria monocytogenes* OD values in tryptic soy broth for treatments containing 10% jalapeño subfractions F1 through F7 extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>Listeria monocytogenes</i> control	0.00±0.00	0.01±0.00	0.11±0.00	0.41±0.01	0.37±0.00	0.32±0.00	0.23±0.00	0.19±0.00	0.16±0.01
Fraction F1	0.00±0.00	0.01±0.00	0.11±0.00	0.43±0.01	0.34±0.01*	0.29±0.01	0.22±0.00*	0.16±0.00*	0.14±0.00*
Fraction F2	0.00±0.00	0.01±0.00	0.12±0.00	0.41±0.01	0.36±0.00*	0.31±0.00	0.23±0.00	0.17±0.00*	0.14±0.00*
Fraction F3	0.00±0.00	0.01±0.00	0.11±0.00	0.40±0.02	0.36±0.01	0.31±0.00	0.23±0.00	0.16±0.01*	0.14±0.01
Fraction F4	0.00±0.00	0.01±0.00	0.11±0.00	0.38±0.01*	0.36±0.01	0.31±0.00	0.22±0.00*	0.17±0.01*	0.14±0.01
Fraction F5	0.01±0.01	0.02±0.01	0.12±0.01	0.42±0.01	0.38±0.01	0.33±0.01	0.24±0.01	0.18±0.01	0.16±0.00
Fraction F6	0.00±0.00	0.02±0.00	0.12±0.00	0.44±0.01	0.37±0.00	0.31±0.00	0.23±0.00	0.18±0.00*	0.16±0.00
Fraction F7	0.00±0.00	0.0.1±0.00	0.11±0.00	0.42±0.01	0.37±0.00	0.32±0.00	0.24±0.01	0.19±0.01	0.18±0.01

*Denotes OD values that are significantly smaller than the OD value of *Listeria monocytogenes* control (p<0.05)

Table C12. Comparison of mean *Escherichia coli* O157:H7 OD values in tryptic soy broth for treatments containing 10% jalapeño subfractions F1 through F7 extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>E. coli</i> O157:H7 control	0.00±0.00	0.21±0.00	0.40±0.02	0.75±0.01	0.90±0.02	0.90±0.01	0.86±0.01	0.87±0.01	0.87±0.01
Fraction F1	0.00±0.00	0.20±0.00*	0.37±0.01	0.73±0.02	0.83±0.00*	0.88±0.01	0.88±0.01	0.86±0.01	0.86±0.01
Fraction F2	0.00±0.00	0.19±0.00*	0.37±0.01	0.74±0.00	0.87±0.00*	0.90±0.01	0.88±0.01	0.87±0.01	0.87±0.01
Fraction F3	0.00±0.00	0.20±0.00*	0.41±0.01	0.78±0.01	0.84±0.01*	0.88±0.01	0.88±0.01	0.88±0.01	0.87±0.01
Fraction F4	0.00±0.00	0.20±0.01	0.38±0.01	0.73±0.02	0.87±0.01	0.88±0.01	0.84±0.01*	0.85±0.01	0.85±0.01
Fraction F5	0.01±0.01	0.20±0.00*	0.39±0.00	0.74±0.00	0.88±0.01	0.89±0.00	0.85±0.01*	0.86±0.01	0.85±0.01
Fraction F6	0.00±0.00	0.20±0.01	0.41±0.02	0.76±0.02	0.85±0.01*	0.86±0.00*	0.85±0.01*	0.84±0.03	0.82±0.04
Fraction F7	0.00±0.00	0.20±0.00*	0.38±0.01	0.73±0.01	0.86±0.01*	0.87±0.01*	0.84±0.01*	0.85±0.01	0.85±0.01

*Denotes OD values that are significantly smaller than the OD value of *E. coli* O157:H7 control (p<0.05)

Table C13. Comparison of mean *Salmonella enterica* OD values in tryptic soy broth for treatments containing 10% jalapeño subfractions F1 through F7 extract over time.

	Time (h)								
	0	3	6	12	24	36	48	60	72
<i>S. enterica</i> control	0.01±0.01	0.28±0.00	0.49±0.00	0.71±0.01	0.92±0.01	0.93±0.01	0.89±0.01	0.88±0.01	0.86±0.01
Fraction F1	0.01±0.01	0.25±0.00*	0.48±0.01	0.65±0.01*	0.91±0.01	0.93±0.01	0.90±0.01	0.90±0.01	0.89±0.01
Fraction F2	0.01±0.01	0.25±0.00*	0.50±0.02	0.65±0.01*	0.90±0.01	0.93±0.01	0.90±0.01	0.90±0.01	0.90±0.01
Fraction F3	0.00±0.00	0.24±0.00*	0.50±0.01	0.67±0.01*	0.89±0.01*	0.92±0.01	0.89±0.02	0.88±0.02	0.87±0.03
Fraction F4	0.00±0.00	0.25±0.00*	0.46±0.01*	0.69±0.01	0.92±0.01	0.93±0.01	0.91±0.01	0.90±0.01	0.89±0.01
Fraction F5	0.01±0.01	0.25±0.00*	0.52±0.00	0.66±0.00*	0.91±0.01	0.95±0.01	0.92±0.01	0.91±0.01	0.91±0.01
Fraction F6	0.00±0.00	0.24±0.01*	0.52±0.00	0.67±0.01*	0.89±0.01	0.94±0.00	0.91±0.01	0.91±0.01	0.91±0.01
Fraction F7	0.01±0.01	0.27±0.01	0.48±0.00*	0.69±0.02	0.91±0.01	0.92±0.01	0.88±0.01	0.86±0.01	0.84±0.01

*Denotes OD values that are significantly smaller than the OD value of *S. enterica* control (p<0.05)

Appendix D.

Mean bacterial growth curve O.D. values generated using Bioscreen C by pathogenic bacteria in association with jalapeño extract fractions in 0.1% peptone or tryptic soy broth (n=3).

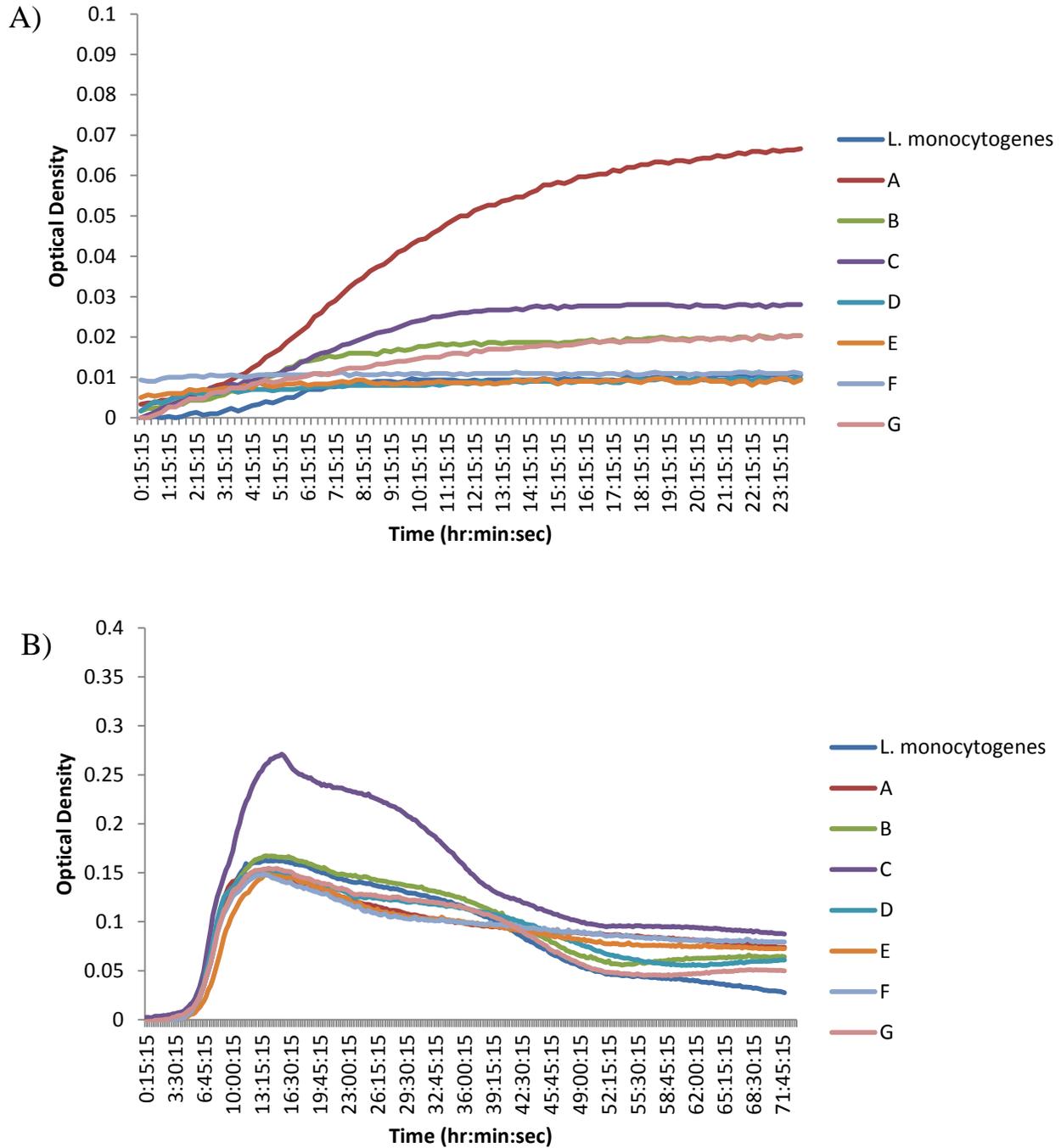


Figure D1. Growth curve OD values generated by Bioscreen C of *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G.

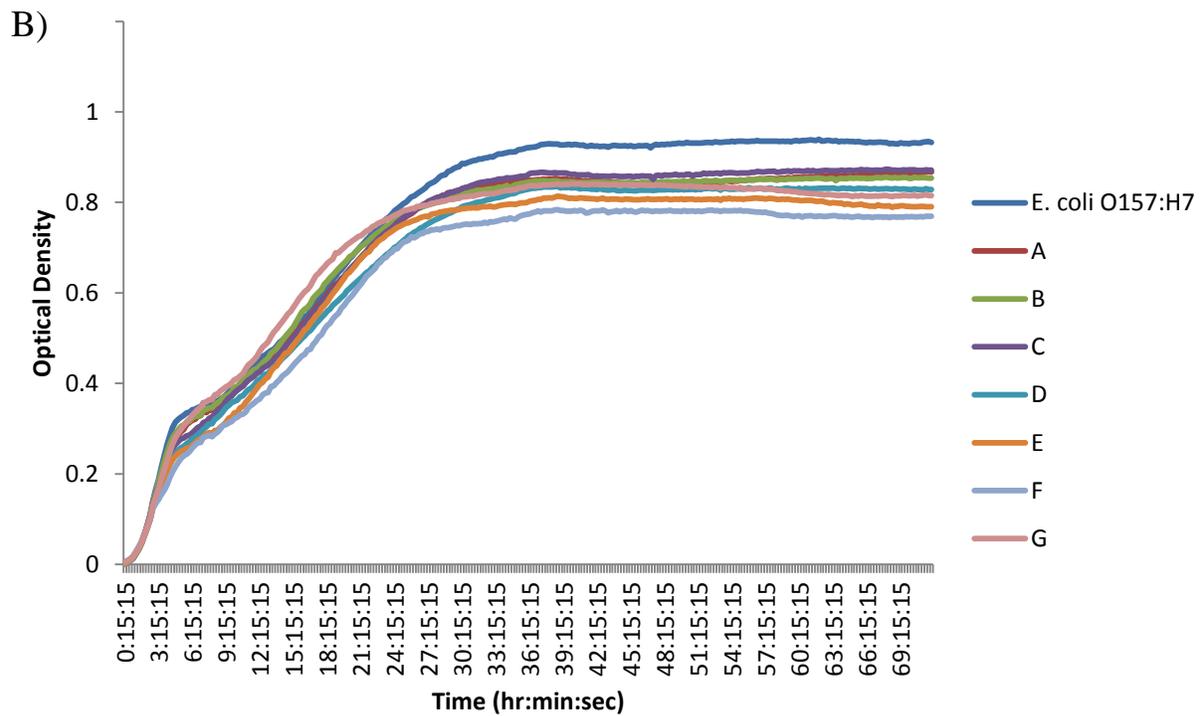
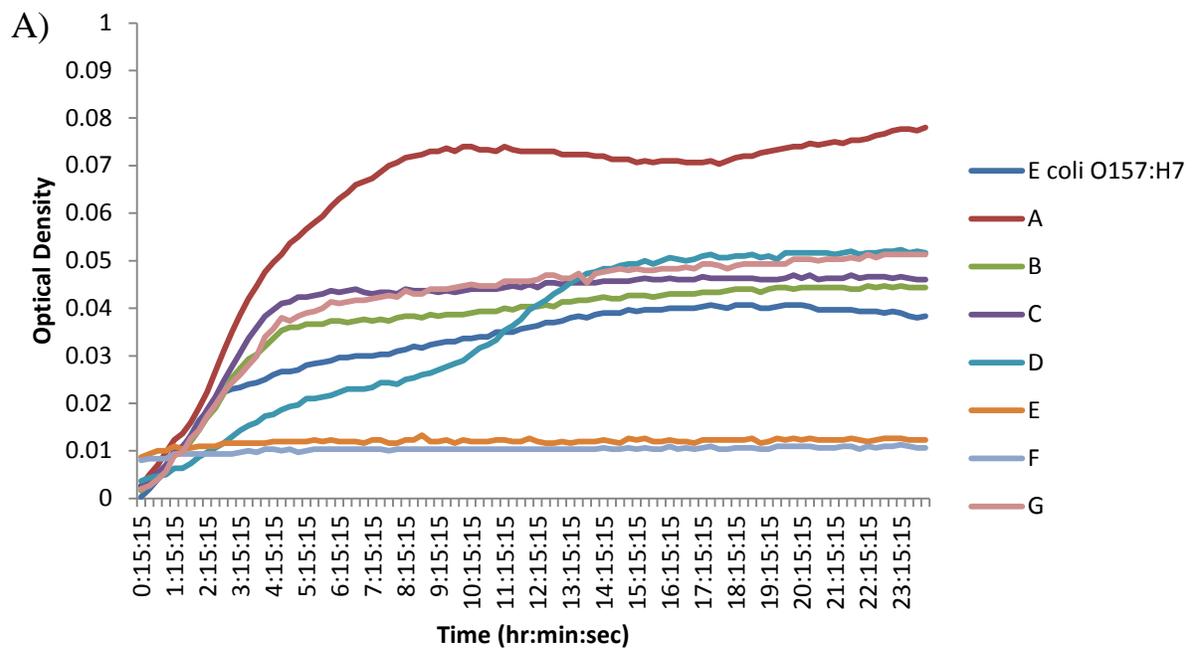


Figure D2. Growth curve OD values generated by Bioscreen C of *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G.

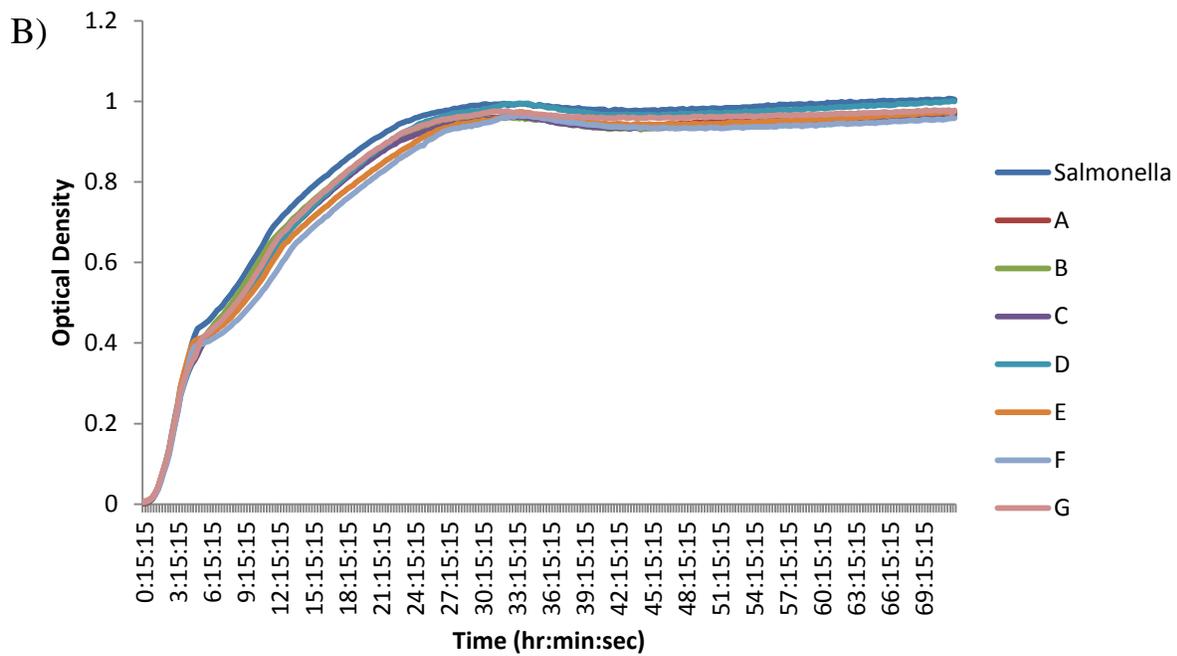
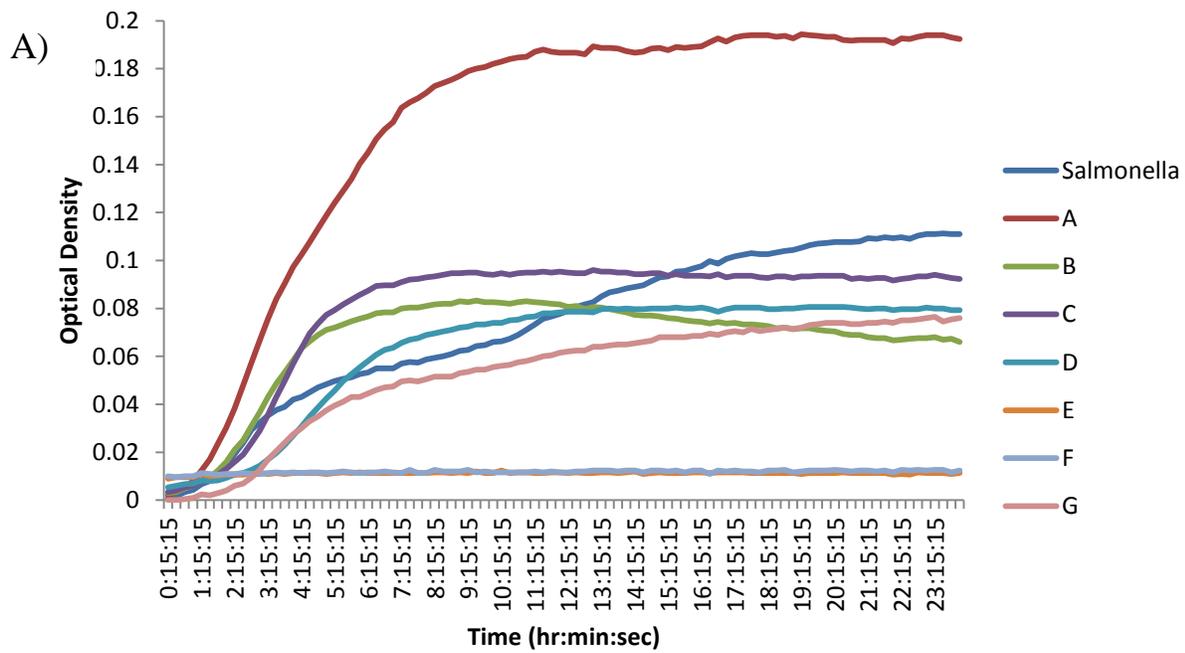


Figure D3. Growth curve OD values generated by Bioscreen C of *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions A through G.

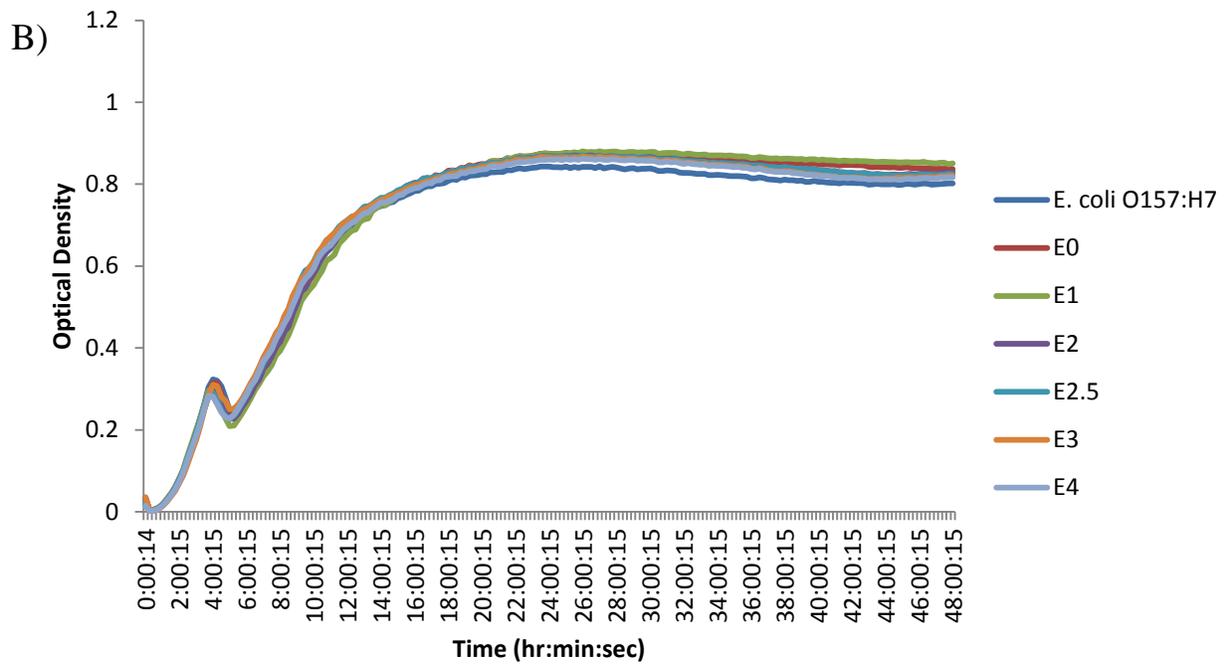
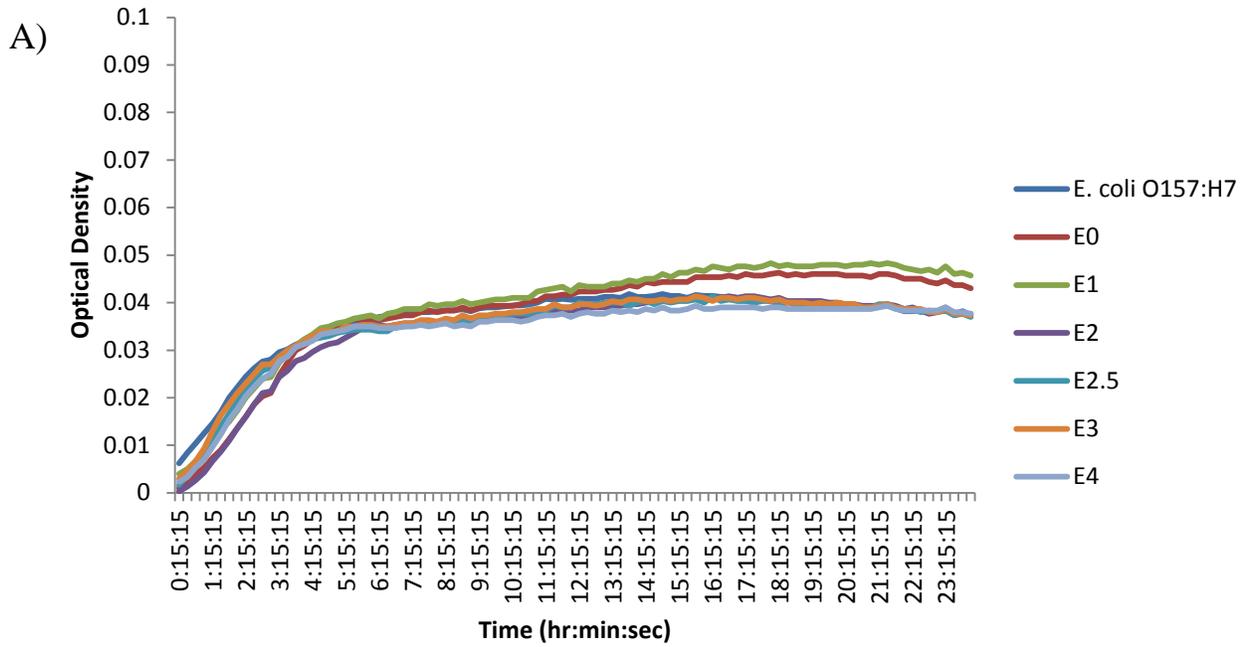


Figure D4. Growth curve OD values generated by Bioscreen C of *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4.

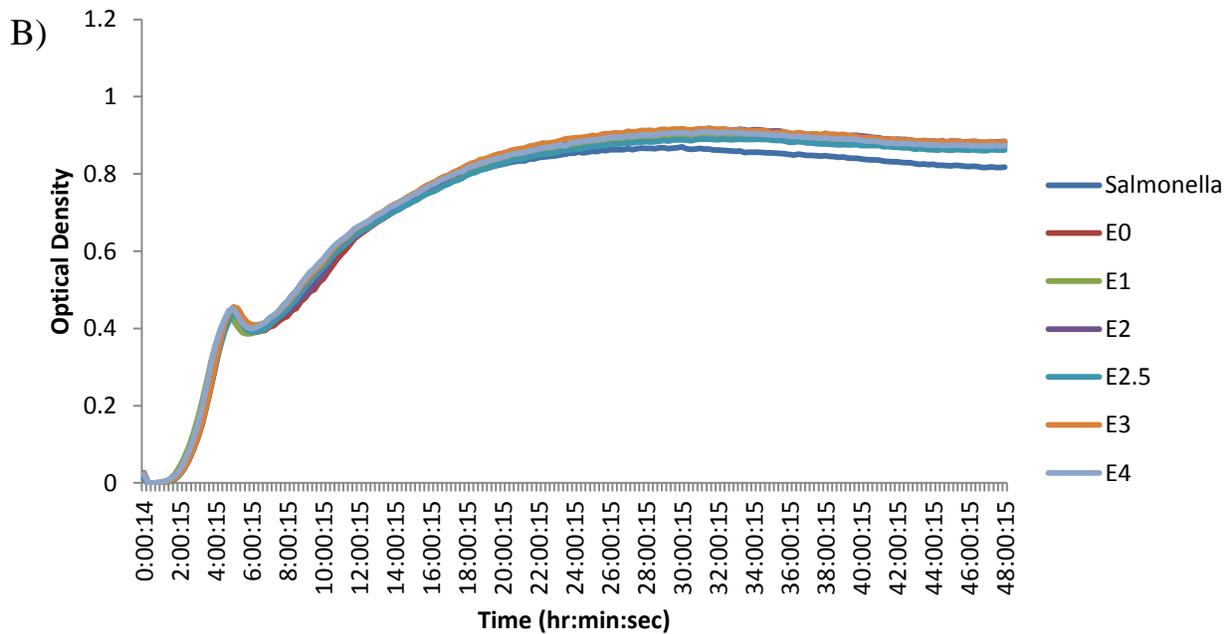
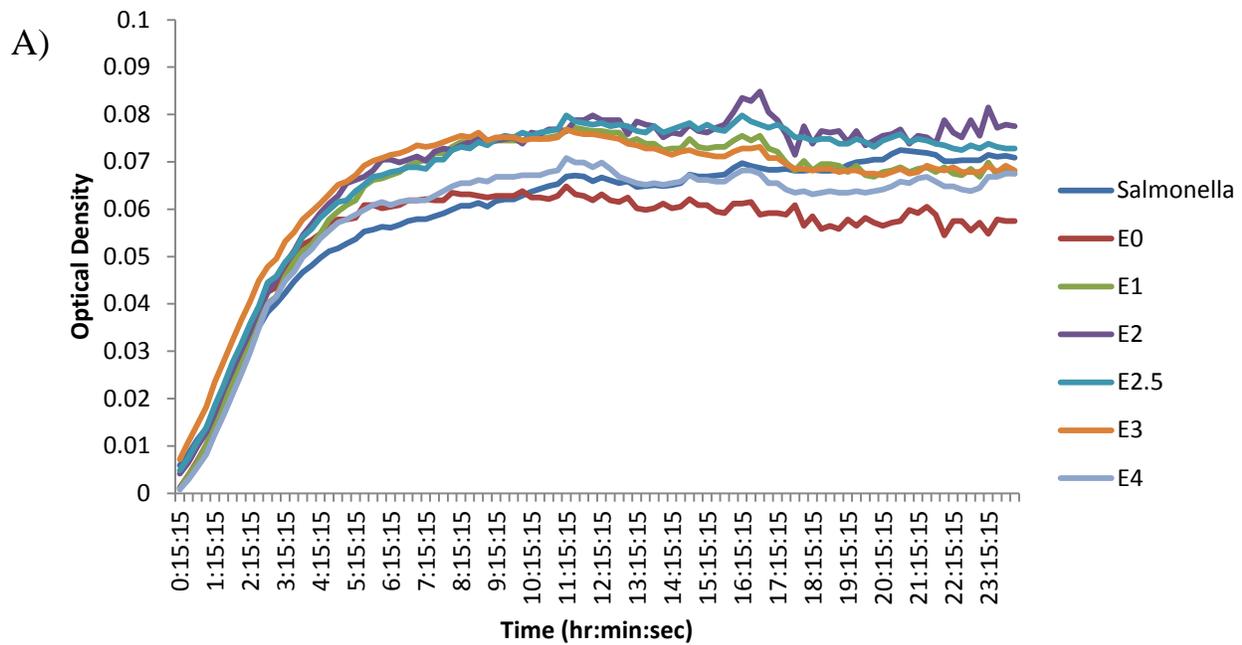


Figure D5. Growth curve OD values generated by Bioscreen C of *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions E0 through E4.

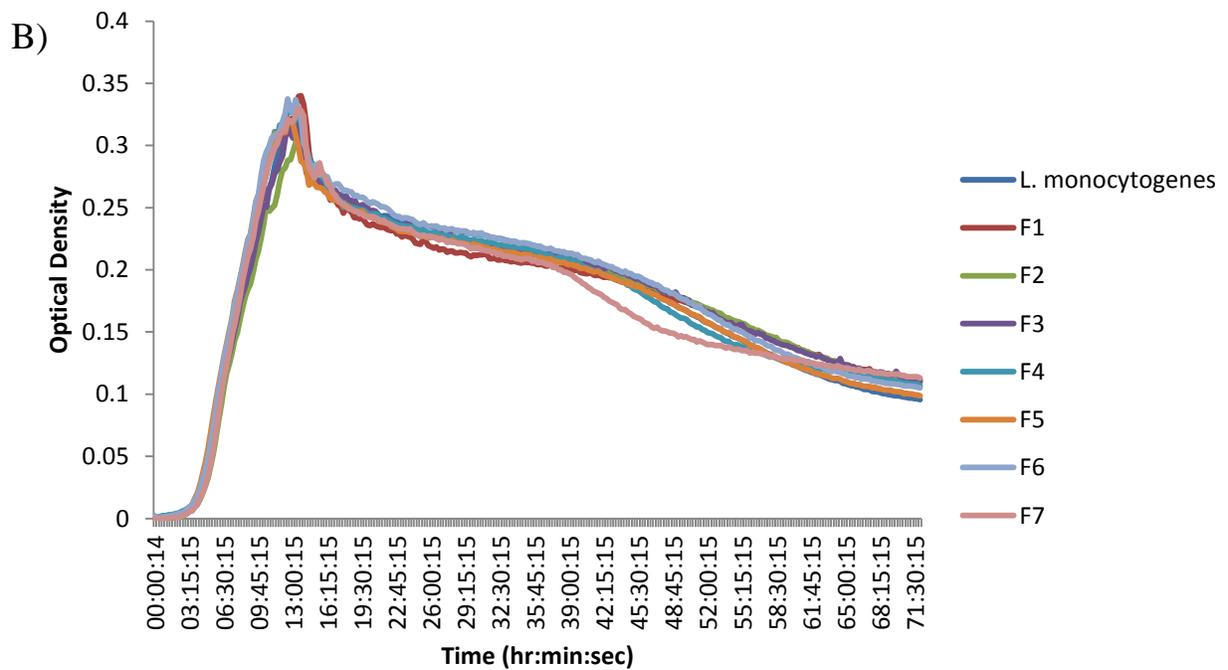
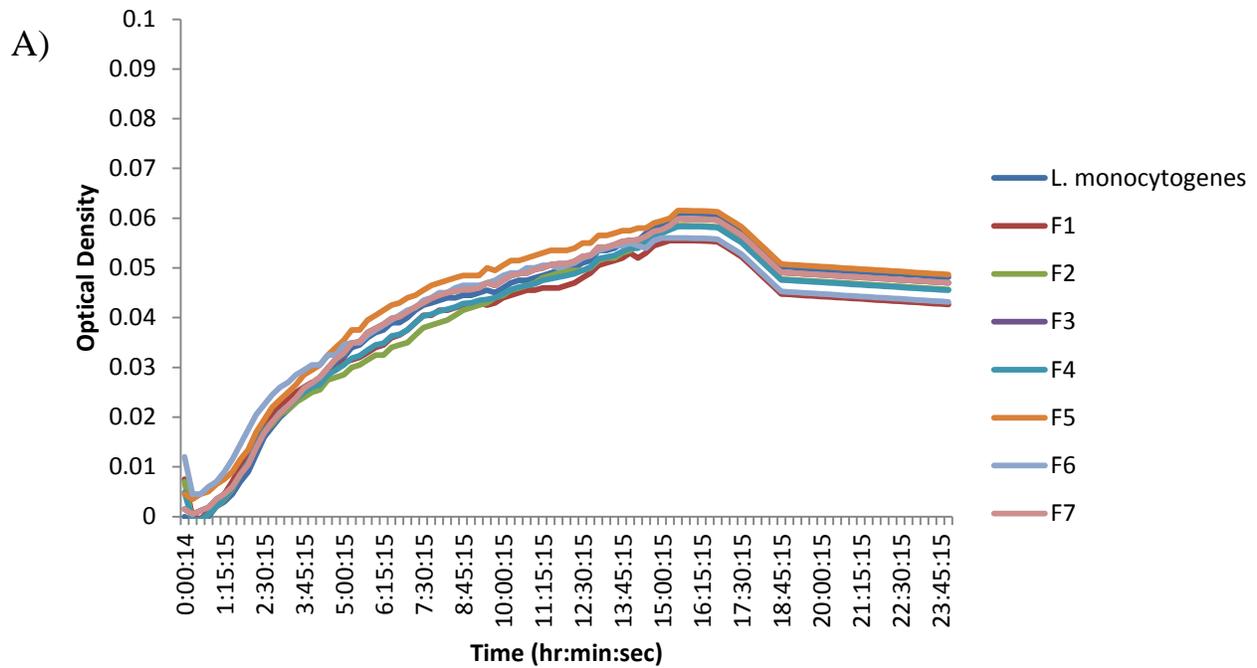


Figure D6. Growth curve OD values generated by Bioscreen C of *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7.

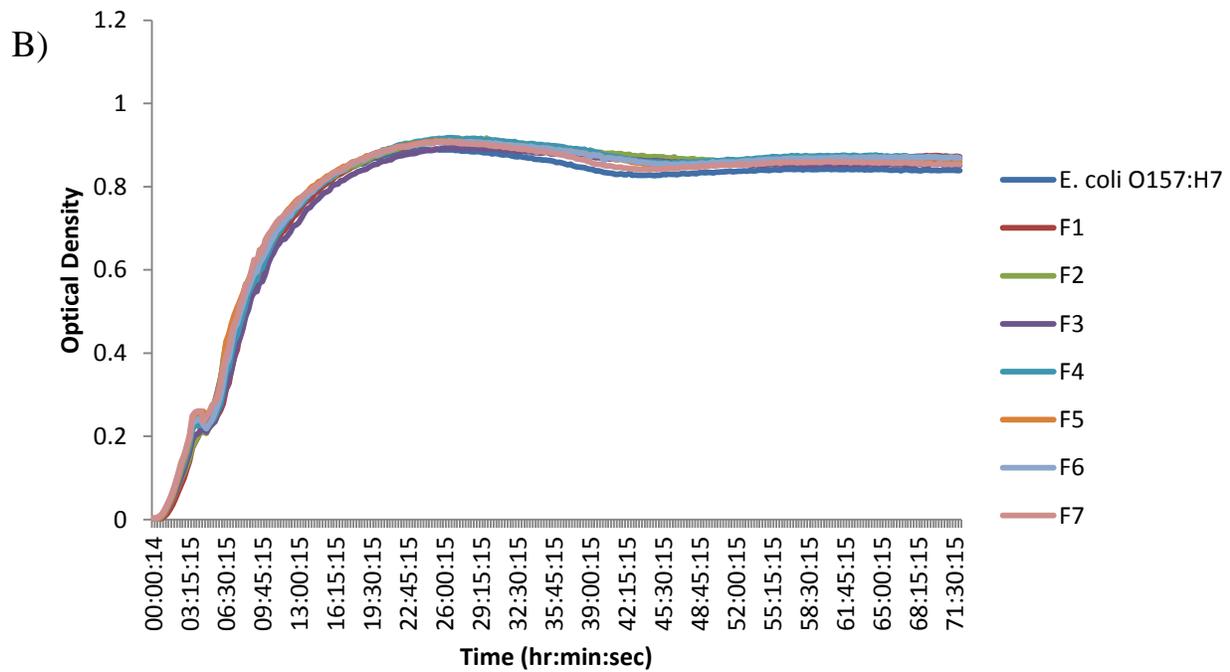
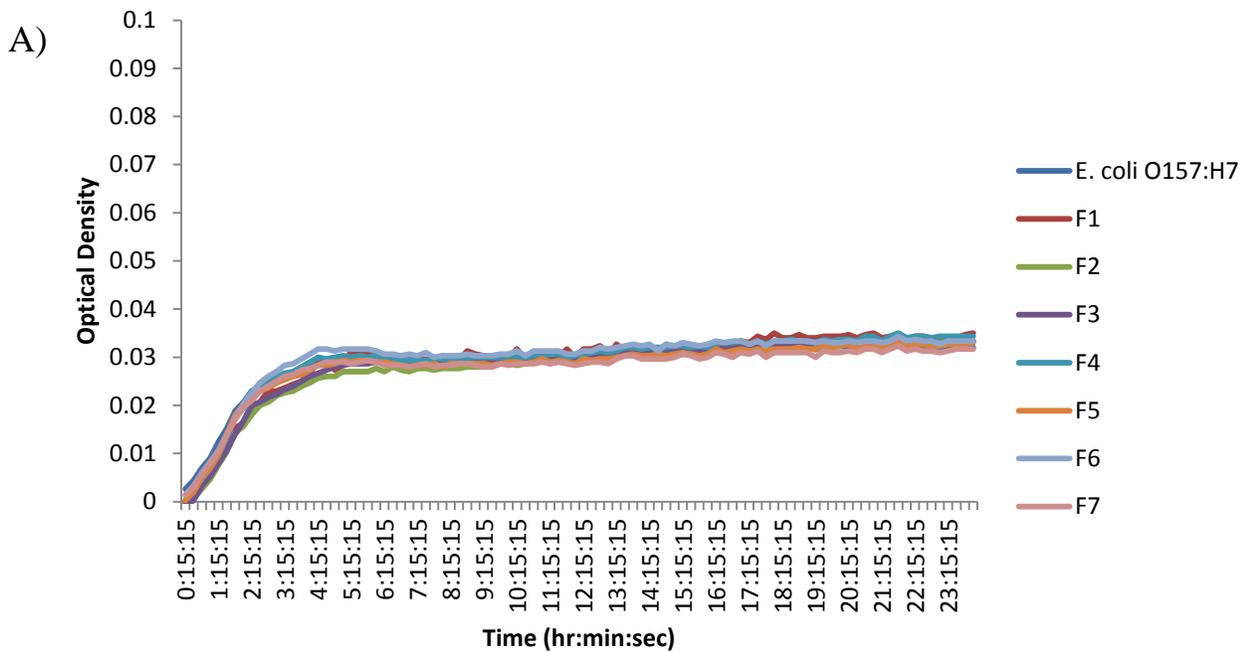


Figure D7. Growth curve OD values generated by Bioscreen C of *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7.

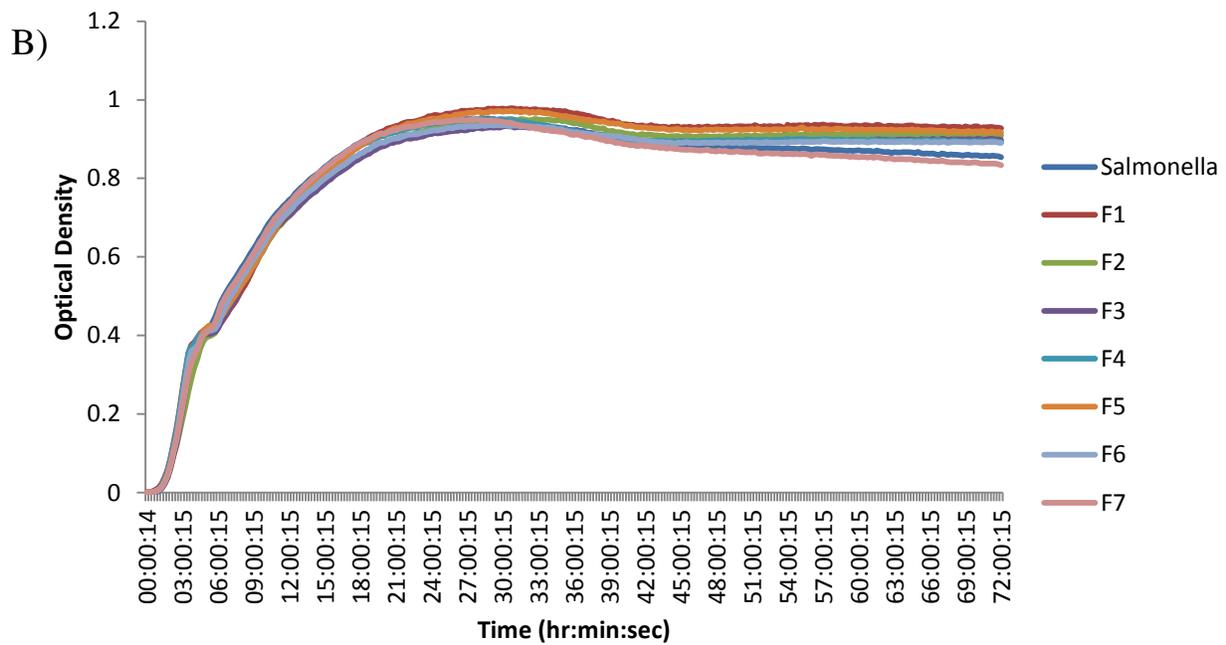
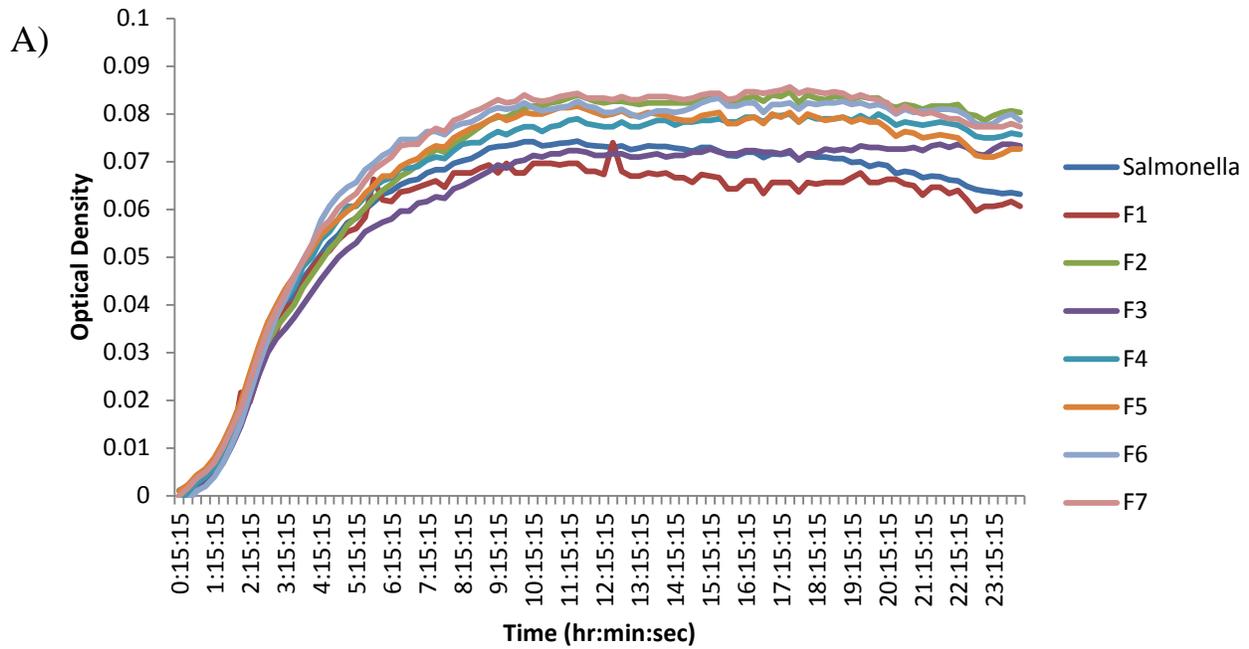


Figure D8. Growth curve OD values generated by Bioscreen C of *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7.

Appendix E.

HPLC chromatogram and peak collection assignment of jalapeño extract Fraction F.

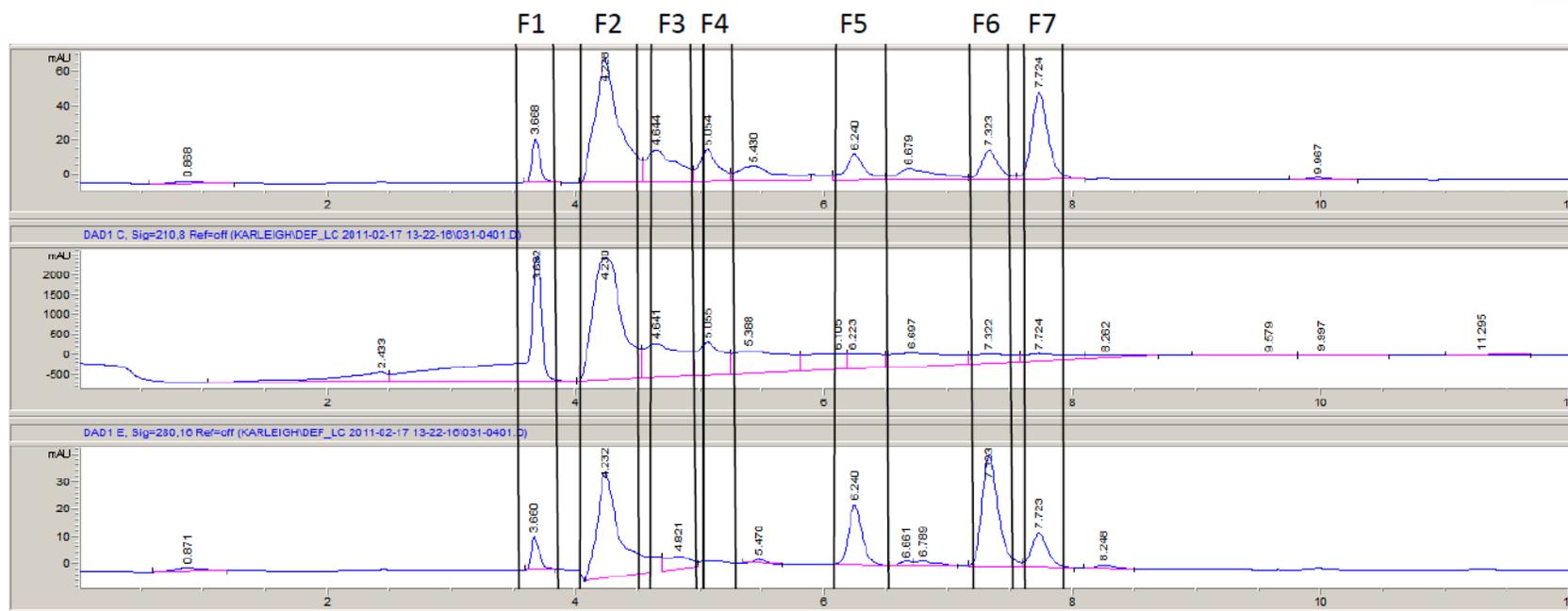


Figure E1. Reverse phase HPLC chromatogram of jalapeño extract Fraction F with vertical indicators of fraction collected. Fractions were collected based on a peak by peak basis and assigned numerical labels based on time of elution.

Appendix F.

Area under the growth curve analysis for pathogenic bacteria in association with jalapeño extract Fraction F subfractions.

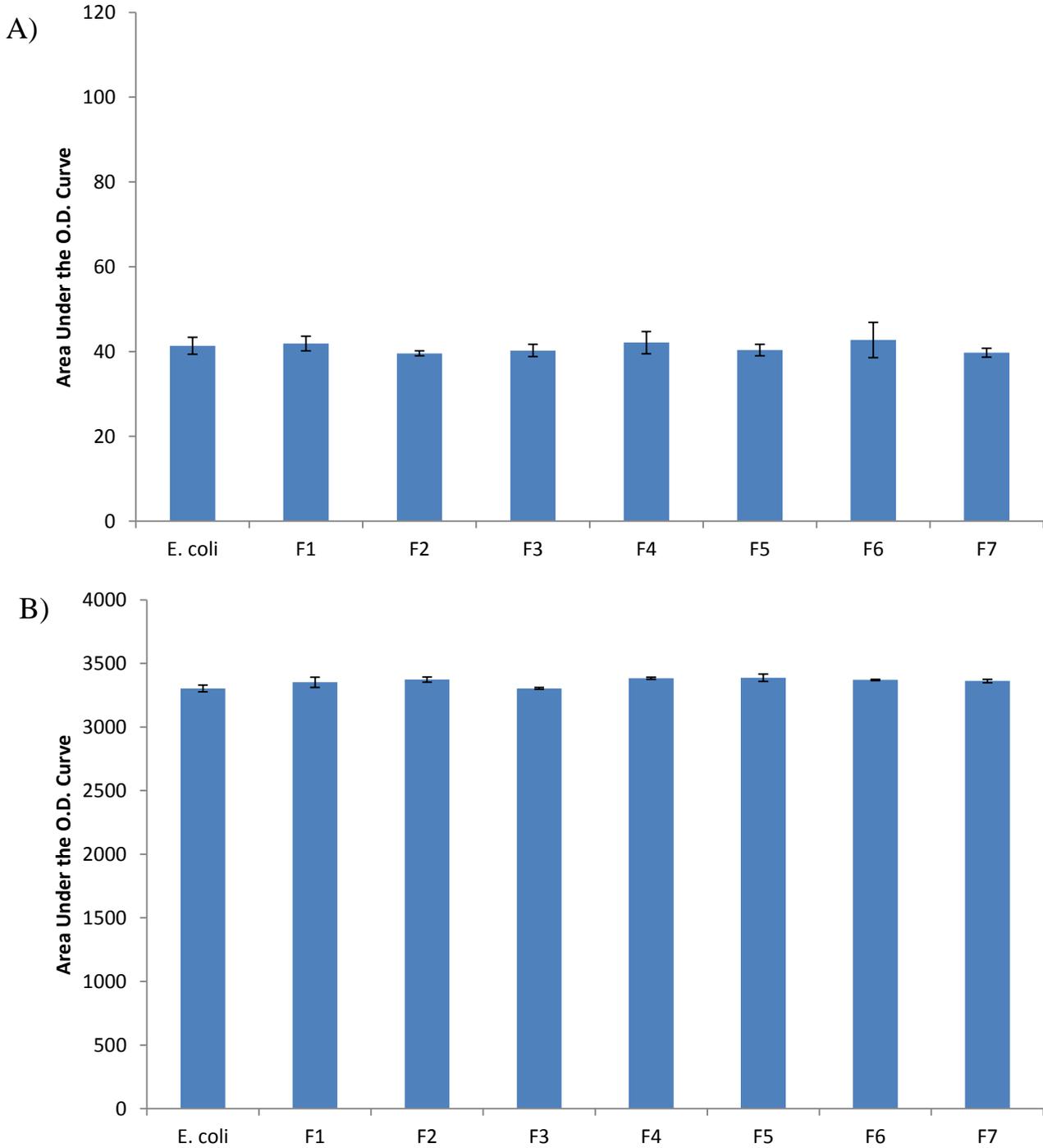


Figure F1. Area under the growth curve comparison for *E. coli* O157:H7 grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7. Error bars represent standard error of the mean. No significant difference from the control was observed.

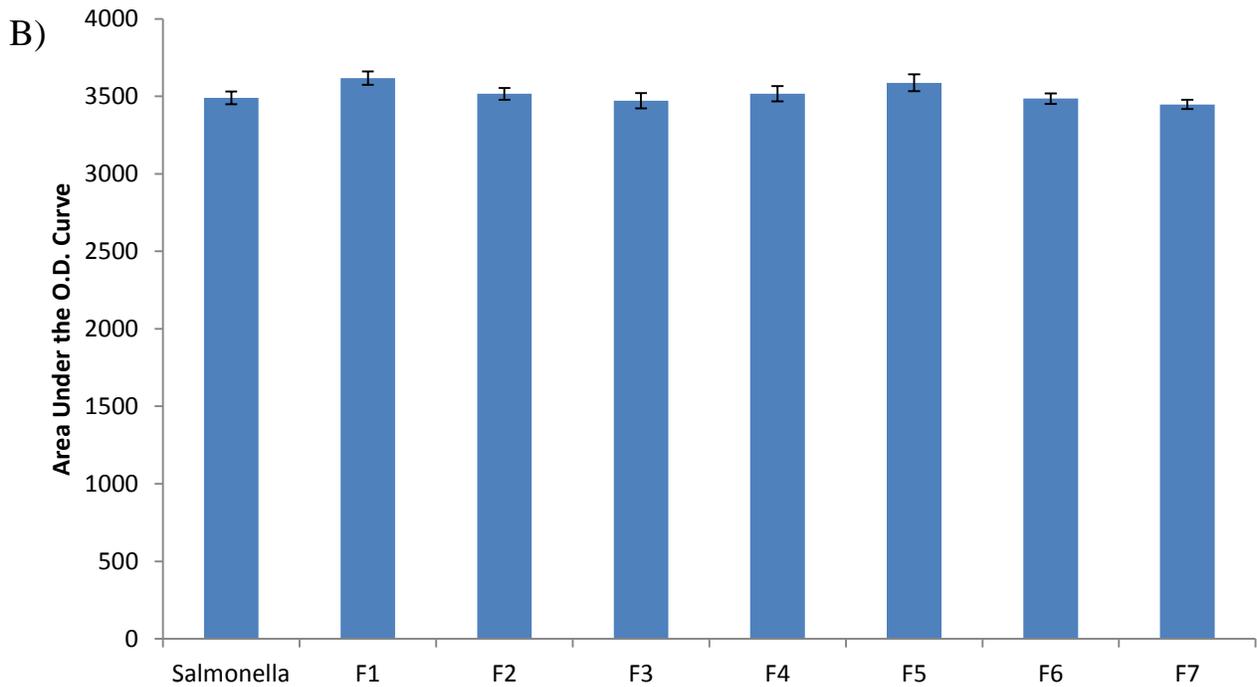
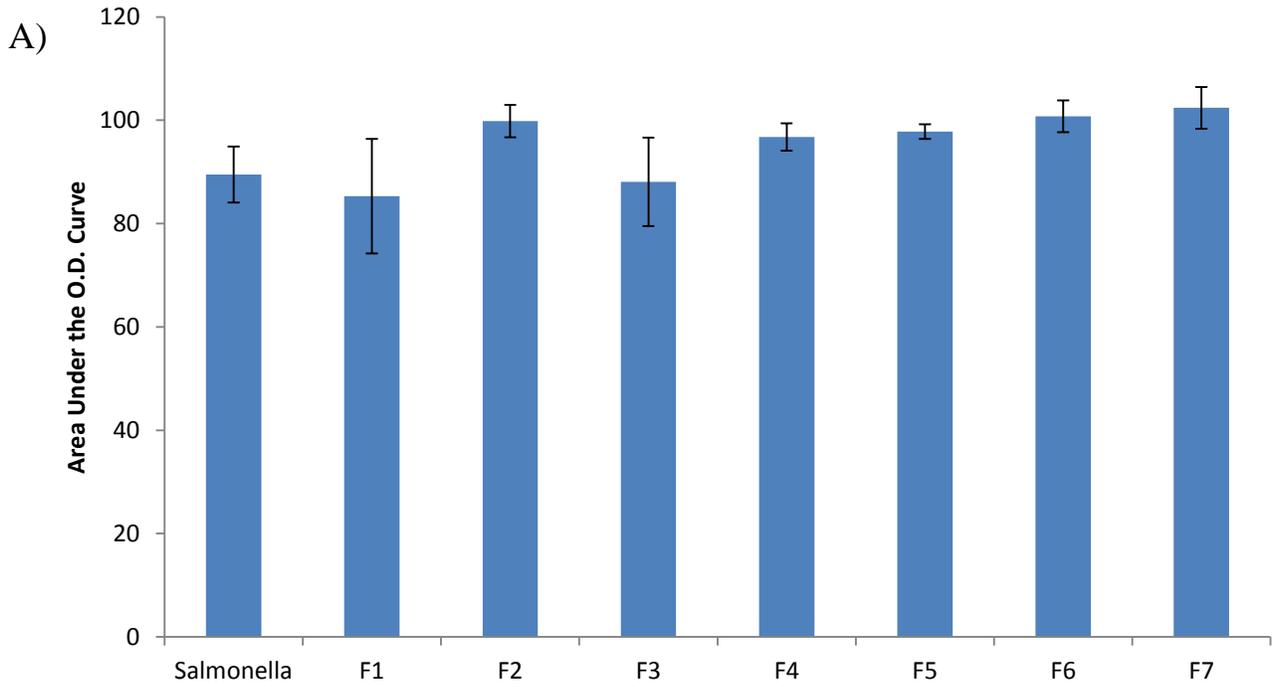


Figure F2. Area under the growth curve comparison for *S. enterica* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7. Error bars represent standard error of the mean. No significant difference from the control was observed.

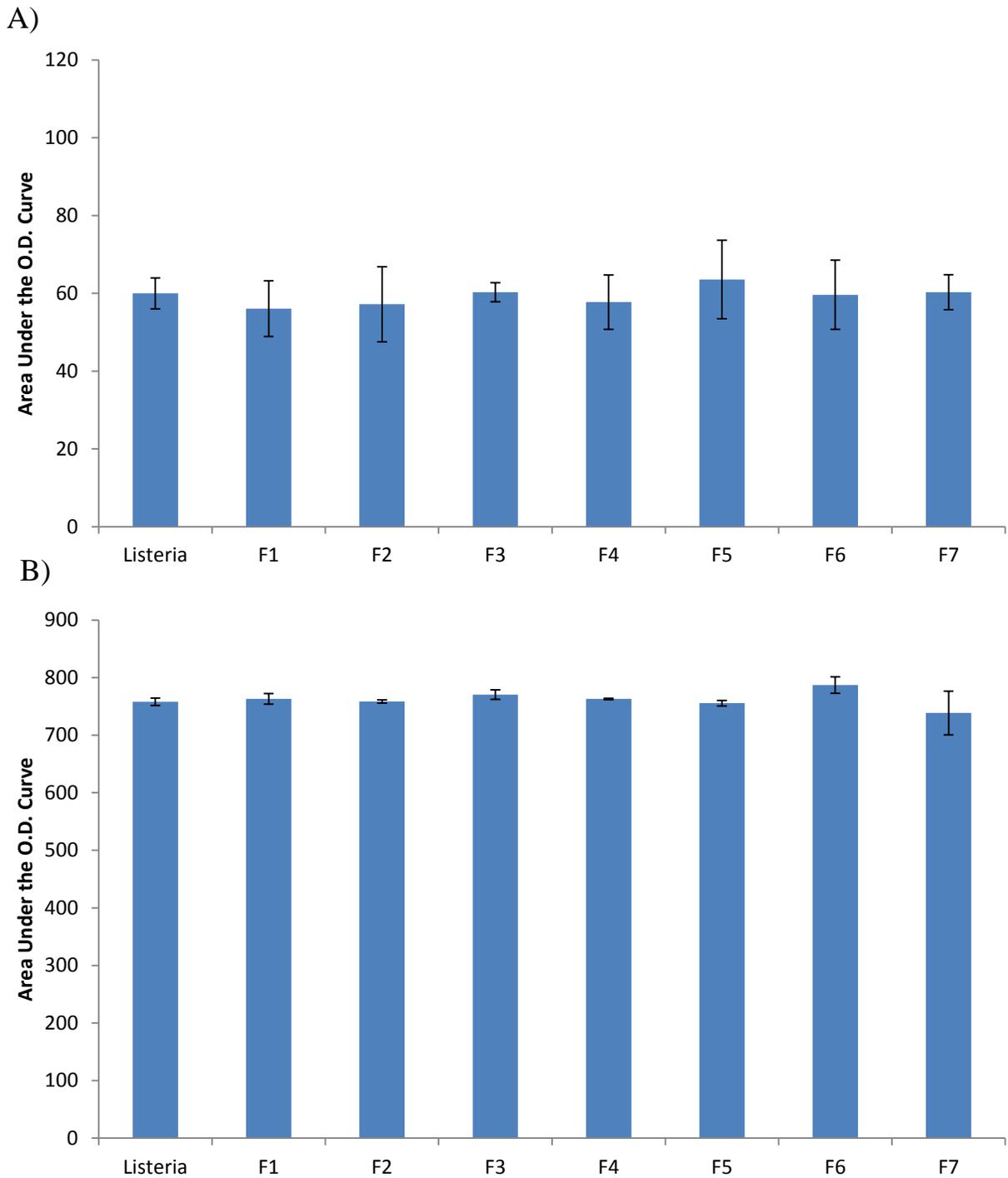


Figure F3. Area under the growth curve comparison for *L. monocytogenes* grown in A) 0.1% peptone and B) TSB in association with jalapeño extract Fractions F1 through F7. Error bars represent standard error of the mean. No significant difference from the control was observed.

Appendix G.

Mass spectrometry analysis of jalapeño extract Fraction E1 using a broad ion scanning technique.

Introduction

Samples of jalapeño extraction Fraction E1 were sent to the Virginia Tech Mass Spec Incubator for mass spectrometry (MS) analysis. Under the direction of Dr. Richard Helm, a number of analyses were conducted in order to hone in on the masses of the ions present in the sample. Samples were analyzed using ion trap electrospray, as well as matrix-assisted laser desorption/ionization MS. Although results from the broad scan MS contained a high level of background noise, some valuable information was collected.

Results

Below are the results of all the broad scan MS analysis run which were not included in Chapter 5 of this dissertation (Figures G1-G4). It should be noted that an ion with a mass of 1123 amu was detected using both MALDI (Figure G1) and electrospray (Figure G2). Two ions from the electrospray MS were selected to analyze with MS/MS due to their large peaks: the ion with a mass of 799 amu and the ion with a mass of 815 amu. The ion with a mass of 799 amu broke into daughter ions of 653, 509, and 347 amu (Figure G3). The ion with a mass of 815 amu broke into daughter ions with masses of 669, 509, and 347 amu (Figure G4).

In the literature, Capsianoside IX was analyzed with fast atom bombardment (FAB)-MS to reveal an m/z of $1123[M + Na]^+$, with daughter ions of 961, 815, 669, and $507[M + Na]^+$ (Leet et al., 2006). Most of these values can be found in our data in either the electrospray MS analysis

for the electrospray MS sample (Figure G2), or the analysis of the ion with a mass of 815 amu (Figure G4). Our results do not correlate exactly, as we determined there was an ion with a mass of 509 amu, while the analysis of Capsianoside IX determined an ion with a mass of 507 amu. We can infer, however, that our sample contains a compound that is very similar to Capsianoside IX, if not exact.

In addition, the analysis of the ion with a mass of 799 and 815 amu revealed that their daughter ions are also very similar in mass. A comparison in the differences in mass between peaks for each analysis also shows that both ions are losing the same mass between two of their peaks. This information can be used to infer that the ion with a mass of 799 amu is related to the ion with a mass of 815 amu in structure.

Overall, this analysis provided support that jalapeño extract Fraction E1 contained a compound that has an MS profile that matches that of Capsianoside IX almost exactly. In addition, another compound is present in our sample that has an MS profile related to the aforementioned compound, also likely a capsianoside. In order to determine the exact identification of the ion with a mass of 1123 amu, a daughter ion MS analysis could be conducted and results compared.

References

Lee, J.-H., N. Kiyota, T. Ikeda, and T. Nohara. 2006. Acyclic diterpene glycosides, capsianosides VIII, IX, X, XIII, XV and XVI from the fruits of paprika *Capsicum annuum* L. var. *groszum* Bailey and jalapeño *Capsicum annuum* L. var. *annuum*. Chem. Pharm. Bull. 54: 1365-1369.

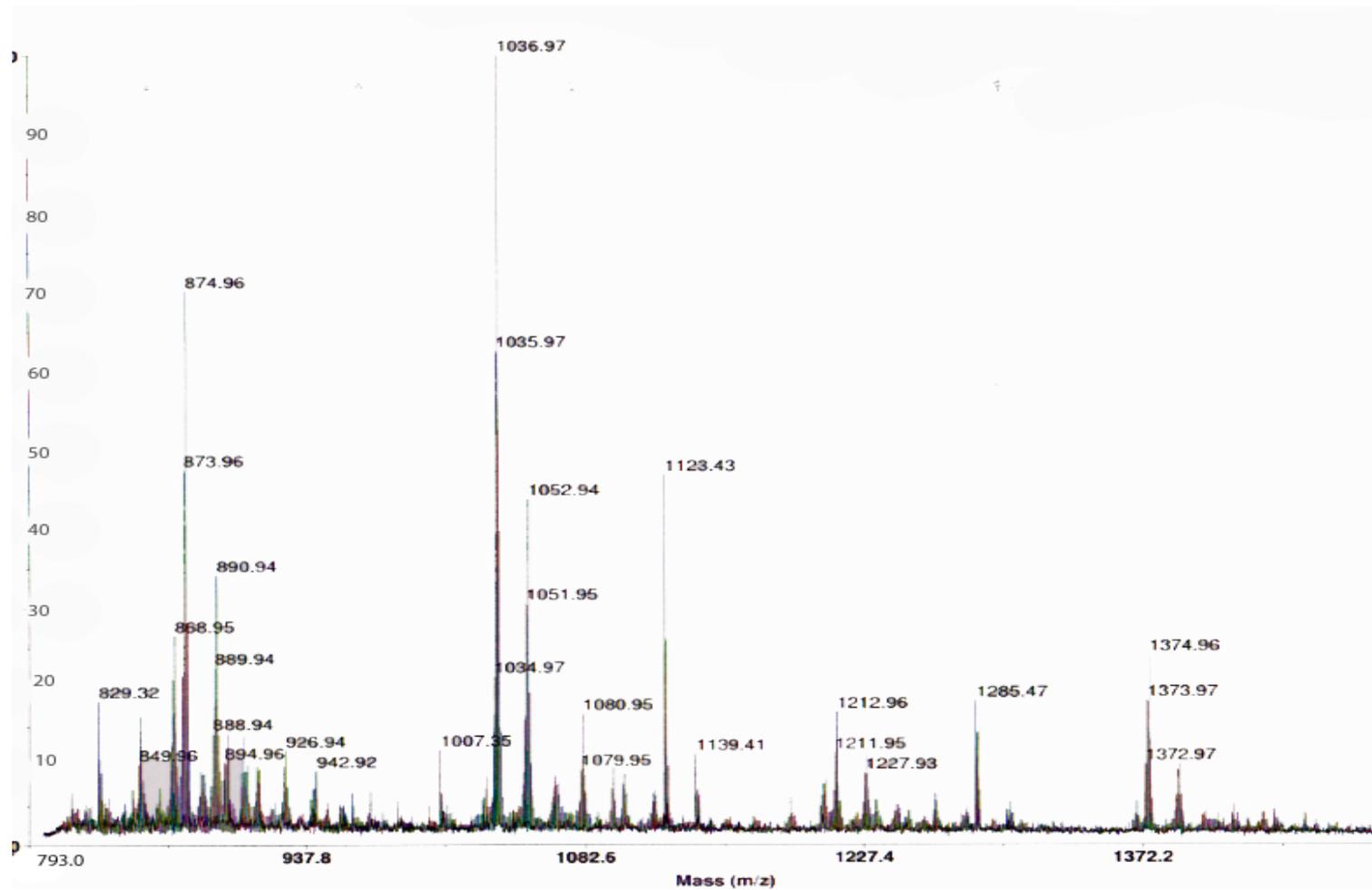


Figure G1. Broad scan MALDI-MS analysis of jalapeño extract Fraction E1.

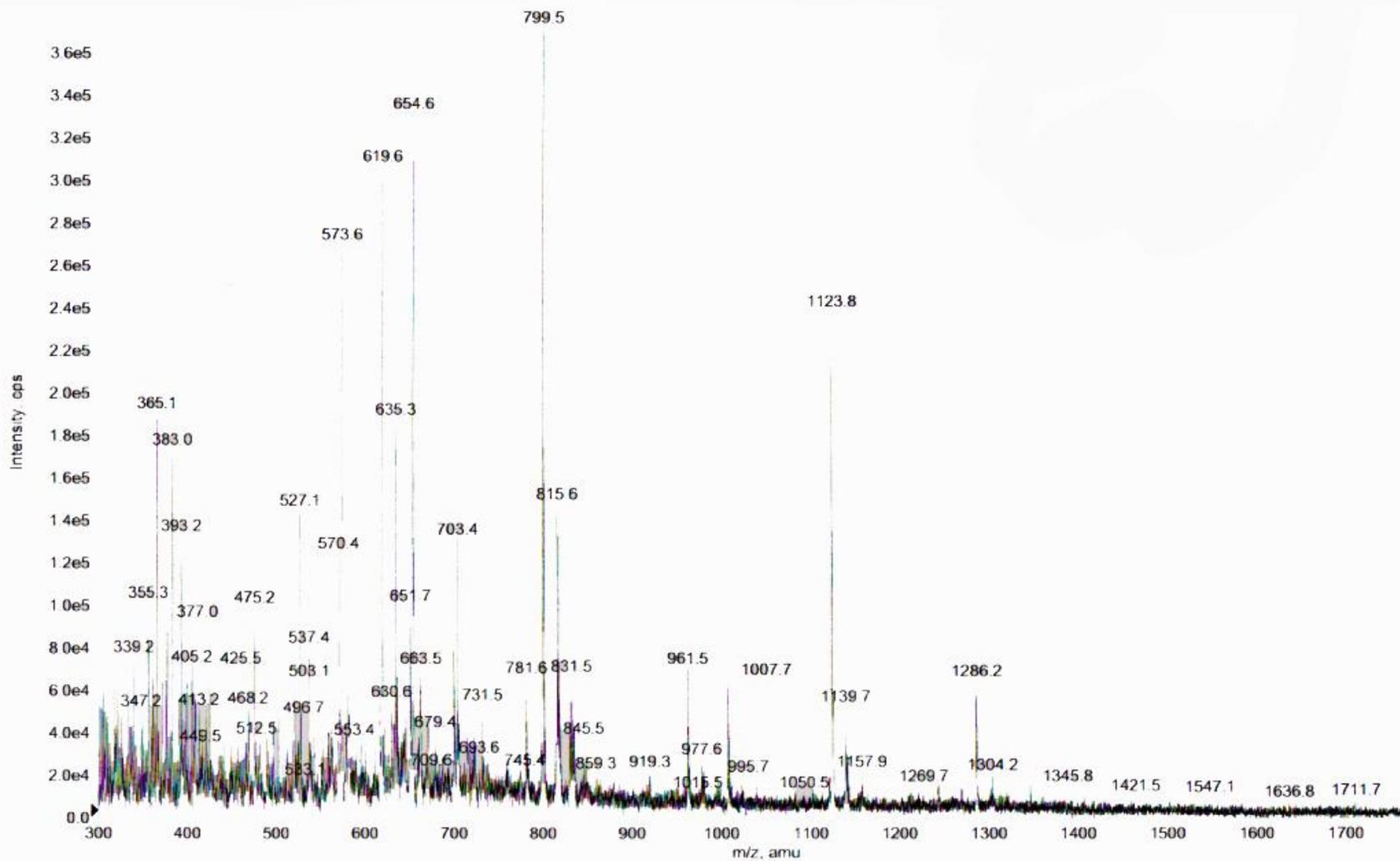


Figure G2. Broad scan electrospray MS analysis of jalapeño extract Fraction E1.

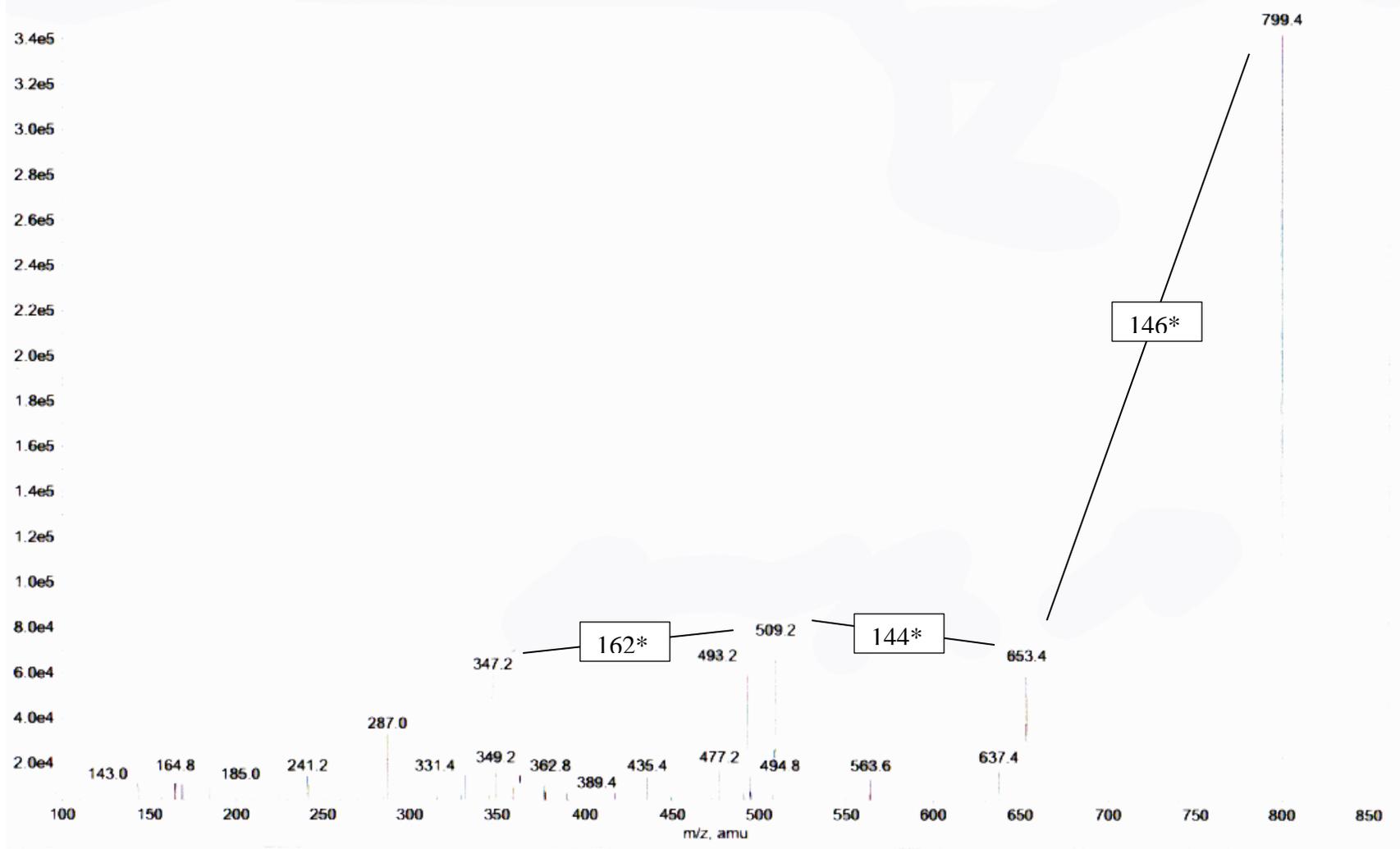


Figure G3. Electrospray MS/MS analysis of the ion with a mass of 799 amu from the broad scan electroscan analysis of jalapeño extraction Fraction E1. *Indicates the mass difference between prominent peaks.

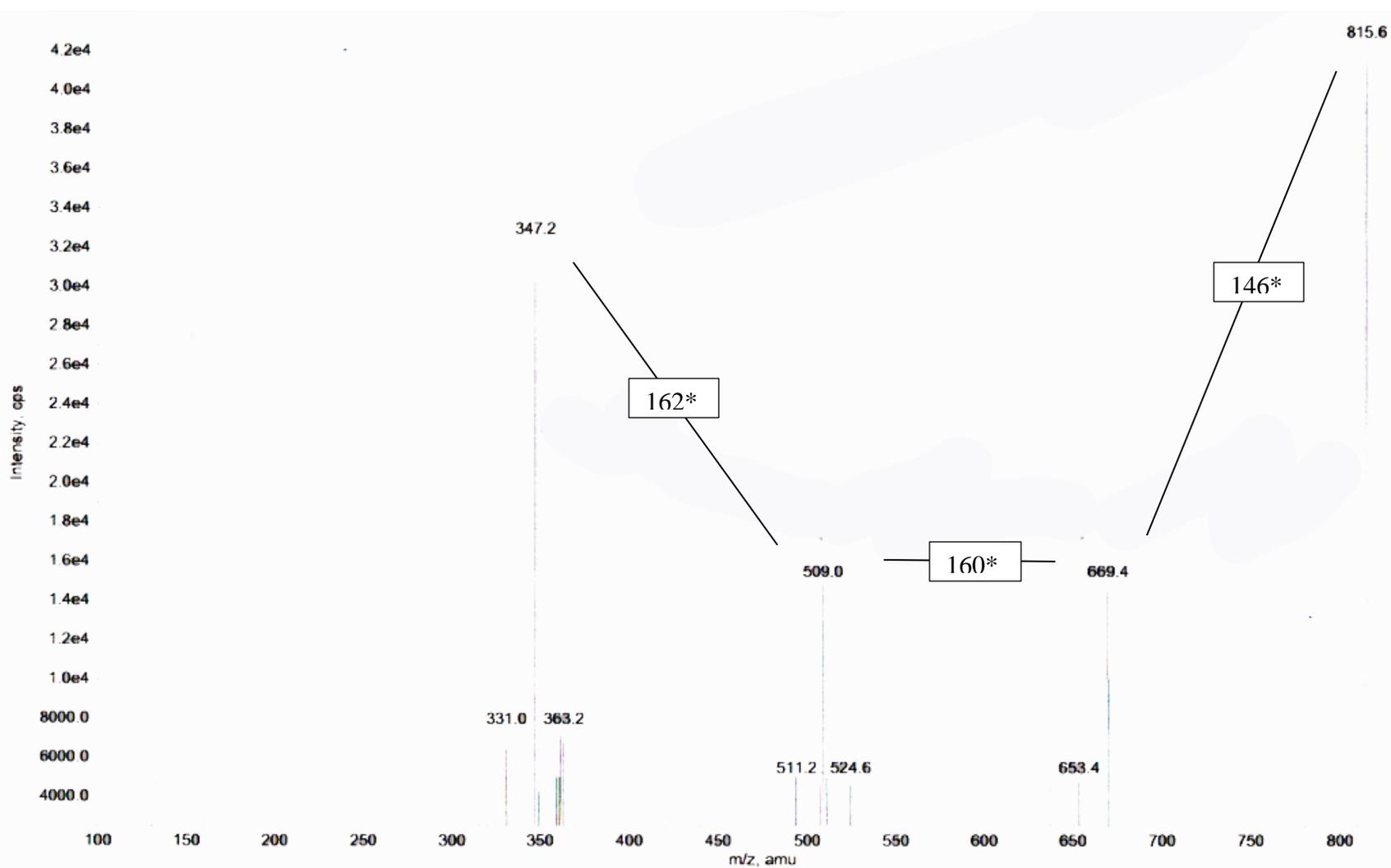


Figure G4. Electrospray MS/MS analysis of the ion with a mass of 815 amu from the broad scan electroscan analysis of jalapeño extraction Fraction E1. *Indicates the mass difference between prominent peaks.