ANTIMICROBIAL EFFECTS OF A MODEL SALSA AND ITS COMPONENTS ON THREE PATHOGENIC BACTERIA

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of MICHAEL LEONARD SCHMITT find it satisfactory and recommend that it be accepted.

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Chair

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ANTIMICROBIAL EFFECTS OF A MODEL SALSA AND ITS COMPONENTS

ON THREE PATHOGENIC BACTERIA

Abstract

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The antimicrobial properties of onion, garlic, chilies, citric acid, and acetic acid against *E. coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* were determined in a tomato base and a model salsa at 4˚C and 22˚C. Onion, garlic, and chilies did not significantly affect the inactivation of the pathogenic bacteria when compared to the tomato base at both temperatures. Citric acid (>0.5%) and acetic acid (>0.25%) did inactivate the pathogenic bacteria significantly when compared to the tomato base at both temperatures. Products incubated at refrigeration temperatures required longer holding times to achieve the same reduction of pathogen population as compared to products at room temperature. Blending of onion, garlic, and chilies into the model salsa did not significantly affect the inactivation of the three pathogens when compared to the tomato base at both temperatures. The addition of citric acid (>1.0%) and acetic acid (>0.5%) significantly affected the inactivation of the pathogens when compared to the model salsa. As the concentration of citric and acetic acids in the products was increased, the time required to reduce the pathogen population to below detection levels decreased. Citric acid and acetic acid demonstrated more effective antimicrobial properties in the complex systems. Approximately twice as much citric acid was needed to achieve comparable inactivation of pathogens when compared to acetic acid.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Dedication

This thesis is dedicated to those with patience.

Thank you to my parents, Dick Dougherty, my committee, and those who supported me

Thank you for not losing your patience.

Introduction

Maintaining a safe food supply has become an ever-changing endeavor as new information on pathogenic bacteria is discovered. Consumer demands for minimally processed foods means that the food industry can no longer rely on traditional methods of thermal processing to create microbiologically safe foods. Minimally processed foods such as fresh fruits and vegetables have been shown to harbor pathogenic bacteria (*19, 39, 45, 99*). Thermal processing of fresh fruits and vegetables is often not acceptable, so other methods of controlling pathogenic bacteria must be considered. Methods such as irradiation, high-pressure processing, low-temperature storage, chemical preservatives, modification of atmosphere, control of water activity, compartmentalization, or combinations thereof may be considered (*2, 50*). The use of antimicrobial compounds found within the foods themselves may be another way of controlling microorganisms. Garlic and onion have demonstrated antibiotic and antibacterial effects (*79*). The essential oils of cinnamon, thyme, basil, cumin, sage, rosemary, caraway, and clove have also demonstrated antibacterial properties (*2, 33, 72*). These antibacterial properties are the result of several compounds such as: α-pinene, β-pinene, camphene, limonene, α-terpinene, terpinolene, myrecene, p-cymene, caryophyllene, eugenol, thymol, methyl chavicol, carvone, dihydrocarvone, thujone, cumin aldehyde, linalool, and geraniol. Organic acids can also inhibit the growth of bacteria (*2, 50*).

Though research has studied the effects of these naturally occurring antimicrobial compounds on pathogenic bacteria, the experiments were done in relatively simple systems (e.g. antimicrobial compound and nutrient broth). The objective of this study was to evaluate a complex food system that contained ingredients that had exhibited bactericidal effects, and

determine which components, if any, played a significant role in the control of pathogenic bacteria in that system. The food system used was a model salsa which contained tomatoes, onion, garlic, salt, chilies, black pepper, oregano, cumin, and an organic acid. Previous studies have determined that most of these ingredients contain compounds that are bactericidal (*33, 72, 79*). However, few studies, such as the one by Iturriaga *et al.* (*49*), have determined the effects of the combined ingredients on pathogens in food systems.

This study concentrated on the enumeration of pathogenic bacteria in the model salsa with varying amendments at 4˚ and 22˚C over time. It also determined which ingredients played the greatest role in controlling the pathogenic bacteria by mixing individual ingredients in a tomato base and enumerating the pathogenic bacteria at 4˚ and 22˚C over time.

Review of Literature

Raw or minimally processed fruits and vegetables are part of the diet of people around the world. Advances in agronomic, processing, preservation, distribution, and marketing technologies allow the produce industry to provide fruits and vegetables to virtually anyone at any time of the year. With these new technologies come the increased risk of human illness associated with fresh fruits and vegetables. For example, Prazak *et al.* (*73, 74*) found that 20 of 21 isolates of *Listeria monocytogenes* from cabbage, environmental, and water samples were resistant to two or more antimicrobial agents (antibiotic medicine). Naturally occurring *Listeria* spp. are not commonly found in nature. Thus, multiresistant *Listeria monocytogenes* was contaminating the cabbage farm produce and was being distributed into the local Texas food supply.

Pathogenic bacteria can contaminate fresh fruits and vegetable through a wide range of mechanisms. Contamination can occur in the fields or orchards while growing, during harvesting, postharvest handling, processing or distribution. The contamination may come from people/workers, animals, feces, sewage, water, or soil (*19*).

Of particular concern are: a) *Listeria* due to the ubiquitous nature of the bacteria in the environment, and the severe effects of its growth on a fetus, b) pathogenic *E. coli* with the severity of illness associated with infections, and c) *Salmonella*, which is the second greatest cause of bacterial-induced diarrhea in the United States.

Listeria

The genus *Listeria* is taxonomically included in the Clostridium-Lactobacillus-Bacillus branch of the gram positive phylogeny, which also includes the staphylococci and streptococci (*16*). Members of this genus are gram positive, mesophilic, facultatively anaerobic,

nonsporeforming, nonacid fast, gram-positive bacteria that appear microscopically as nonbranching, regular, short (0.4 to 0.5 μm in diameter by 0.5 to 2.0 μm in length) rods with rounded ends. They have peritrichous flagella and grow well in complex media at a wide range of temperature (0 to 42˚ C) and pH (from ≤5.5 to 9.5) and in the presence of high concentrations of sodium chloride (up to 12%). L. monocytogenes can grow at a pH of 4.4 (*26*). *Listeria* spp. are catalase positive and oxidase negative, hydrolyze esculin, and ferment glucose without the production of gas. They are methyl red and Voges-Proskauer positive, do not produce indole or H_2S, and do not hydrolyze urea(*16, 27, 31, 48, 60, 67, 70*). This organism can withstand repeated freezing and thawing (*31*).

The genus *Listeria* has been divided into seven species with two genomically distinct groups (*16*). One group contains *L. murrayi* and *L. grayi*, which are rarely isolated and are considered to be nonpathogenic. The other five species are closely related and include three hemolytic species (*L. monocytogenes*, *L. seeligeri*, and *L. ivanovii*), and two nonhemolytic species (*L. innocua*, and *L. welshimeri*). Of these, only *L. monocytogenes* and, rarely, *L. ivanovii* are human pathogens. *L. ivanovii* is mostly pathogenic to animals (*16*).

Not all strains of *L. monocytogenes* are pathogenic. Rough variants of this bacterium have been shown to have reduced virulence (*46*). Some serotypes of *L. monocytogenes* seem to have enhanced virulence. It is suggested that serotype 4b strains may have an enhanced ability to cause human disease over serotypes 1/2a or 1/2b (*18, 23, 31, 53*). Identification of strain 4b as the most virulent type of *L. monocytogenes* may be due to many poorly understood factors, including prevalence in foods (50%-70%), unique surface antigens, and the organism's ecology and physiology. Identification of these factors are important to maintaining safe foods (*53*).

Listeria spp. are widely distributed in the environment and have been isolated from soil (believed to be the natural reservoir) (*16*), plants, decaying vegetation, silage, water, and sewage. *Listeria monocytogenes* has been associated with many raw or processed foods (usually in low numbers) (*16, 19*) including dairy products, meat, vegetables, and seafood, as well as from the food processing environment (*35*).

Many wild and domestic animal species are intestinal carriers of *Listeriae*. These include sheep, cattle, pigs, poultry, birds, and fish (*70*). *L. monocytogenes* is often found in the digestive tracts and feces of animals, probably after ingestion from contaminated feed (*16*). The organism can also be found in asymptomatic human carriers (*92*).

Human infection with *L. monocytogenes* often causes influenza-like symptoms which are self-limiting and resolve themselves after several days. If unrecognized and untreated in pregnant women, infection with these bacteria may lead (within days or weeks) to amnionitis and infection of the fetus. This may result in stillbirth, abortion, or premature birth of an infected infant (*16*). The infective dose of *L. monocytogenes* is unknown, but it is believed that the dose varies with the strain and susceptibility of the victim (*10, 64*). It is assumed that healthy adults can consume <1000 CFU with no ill effects, but susceptible persons may not be able to tolerate such a dose of this pathogen. A major outbreak associated with frankfurters found the levels of *L. monocytogenes* to be 0.3 CFU/g. Canadian compliance criteria have three categories based on risk, and differentiates foods intended for immunocompetent and immunocompromised individuals(*26*).

It has been shown that after ingestion of contaminated food, invasive infection occurs in some people. The infection depends on several factors including: host susceptibility, gastric acidity, inoculum size, and virulence factor[s]. *L. monocytogenes* can then penetrate the

epithelial barrier of the intestinal tract. The relatively small size of the bacterium allows invasion and growth within hepatic and splenic macrophages (*16*).

L. monocytogenes can infect the central nervous system (CNS) (and can cause encephalitis), and the female reproductive tract (causing spontaneous abortions). In nonpregnant humans, *L. monocytogenes* mainly causes meningitis, encephalitis, multiple adenitis, or septicemia. Elderly patients or immunocompromised individuals are at particular risk. Infection of the CNS with *L. monocytogenes* requires prompt recognition and therapy and often leads to high mortality (20 to 50%) or neurological sequelae (secondary consequence or result) among survivors (*16*).

Because of the ubiquitous distribution of *L. monocytogenes* in the environment, special processing considerations must be made when processing raw or minimally processed foods. The processing of raw milk into several varieties of cheeses requires the cheese to be aged for at least 60 days at a temperature of not less than 4.4˚C (*77*). This requirement is based on FDA rules and regulations. Regular pasteurization of milk will normally kill *Listeria* cells in milk though cells found within polymorphonuclear leucocytes have a higher chance of survival at marginal pasteurization times and temperatures (*92*). Heat resistance of *L. monocytogenes* can depend on the type of food in which the bacteria are found. Doyle *et al.* (*30*) reported that heat shock can increase the heat tolerance of *L. monocytogenes* and that solutes in food systems (salt, glucose, curing salts) can increase the thermal resistance of this bacteria. Acid shocked *L. monocytogenes* were more heat sensitive when heated in cabbage juice (pH 4.6 vs. 5.6). The lowering of the pH in an environment can cause *L. monocytogenes* to be killed at a faster rate at the same temperature.

Most organisms of the family *Enterobacteriaceae* are gram negative, rod shaped, are more motile by peritrichous flagella or non-motile; do not form spores; grow on peptone or meat extract media without the addition of supplements; grow well on MacConkey agar; grow both aerobically and anaerobically; ferment (not oxidize) D-glucose, often with gas production; are catalase positive and oxidase negative; reduce nitrate to nitrite; and have a 39% to 59% cytosine and guanine content of DNA (*16*). Enterobacteriaceae are widely distributed on plants and in soil, water, and in the intestines of humans (*16*) (*2*). Strains of this family have been associated with abscesses, pneumonia, meningitis, septicemia, and infections of wounds, the urinary tract, and the intestine. Of those genera which cause these human infections, only four have been documented as including enteric pathogens: *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia*.

Escherichia coli

Studies of *Escherichia coli* have been ongoing since 1885 when it was isolated from children's feces. Since then, the scientific attention given to *E. coli* has probably made it the best understood free living organism (*2*). This organism is of the Enterobacteriaceae family.

E. coli is almost universally found within the gut of humans and warm blooded animals (*2, 16, 37, 92*). It is a predominant facultative anaerobe, but is only a small component of the total microflora. Generally, most strains of this bacteria are harmless to the carrier, but can be an opportunistic pathogen and cause human disease.

E. coli is a gram negative, facultative anaerobe. It is capable of growing in a minimal water activity range of .93-.95, a pH range as low as 3.6 and a maximum pH of 9.5. *E. coli* can grow in environments of up to 8% salt by weight, with a minimum growth temperature ranging from 33-37˚F (0.6-3˚C) and a maximum temperature of 113˚F (45˚C) (*27*).

The pathogenic strains of *E. coli* are divided according to clinical symptoms and mechanisms of pathogenesis. These groups classically have been enterpathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohemorrhagic (EHEC) (*16*). Recently, new groups have been added: the enteroaggregative (EaggEC) (*50*), and diffusely adherent (DAEC) groups (*35, 36*). Strain O157:H7 of *E. coli* is an EHEC (*48*) (*36*), but one of several that produces Shiga-like toxins (STEC), also called verotoxins. Other STEC serogroups include O26, O111, O103, O128, O91, O113, O2, O9, O145, OX3, O1, O8, O22, 121, O146, O5, O18, O117, AND O118. These groups have been isolated from bovine stools, sheep at slaughter in Germany, goats at slaughter in the Czech Republic, pigs, cats, and dogs, but not from poultry. STEC serogroups have also been isolated from ground beef, raw milk in Austria, and from milk filters in Italy (*52*).

E. coli O157:H7 is the most recognized serotype of *E. coli* in the United States due to outbreaks in the last 20 years and is the only serotype that can be easily isolated and identified in the clinical microbiology laboratory (*16*). *E. coli* O157:H7 has been shown to be a foodborne pathogen and has been associated with ground beef, venison jerky, raw vegetables (particularly lettuce and sprouts), apple juice or cider, and in water (swimming and drinking water). Studies have shown ground beef to be contaminated with *E. coli* O157:H7 with rates as high as 23 to 25% (*1, 81*). Vegetables and fruits can also become contaminated with *E. coli* O157:H7 while growing in the field or orchard. Contamination can also occur during harvesting, postharvest handling, processing, and distribution (*19*). *E. coli* O157:H7 is capable of surviving in water and cattle feces for many weeks (*100*). In most outbreaks associated with fresh produce, *E. coli* O157:H7 has not been isolated from the implicated food. This may be due to low numbers of *E. coli* cells in these foods, and the cells may be in a viable but nonculturable (VBNC) state (*52*).

Infective dose of this bacteria is unknown, but epidemiological data shows that is may be as low as 10 organisms depending on the immune strength of the victim (*11, 64*).

Children, the elderly, or immunocompromised patients are the most vulnerable members of the population to become infected and develop hemorrhagic colitis (HC), which may lead to hemolytic uremic syndrome. HC is a less severe form of infection by *E. coli* O157:H7 than HU*S.* HC symptoms start with the sudden onset of severe abdominal cramps, grossly bloody stools, little or no fever. These symptoms may last from 4 to 10 days. In most cases, there is no long-term impairment of the patient. In 2-7% of these patients (and up to 30% in some outbreaks), HC will progress to HU*S.* The leading cause of death of children with HUS is acute renal failure, and thrombocytopenia is the leading cause of death for adults in this case. Neural complications may include seizures, stroke, cerebral edema, and coma (*36, 52*). Healthy adults who become infected with *E. coli* O157:H7 may develop thrombic thrombocytopenic purpura (TTP) which can lead to damaged kidneys and central nervous system. TTP is a decrease in the number of blood platelets and hemorrhaging into epidermal tissues.

Thermal inactiviation studies using *E. coli* O157:H7 have shown that the organism is not heat resistant, and adequate heating of the food product will lead to thermal inactiviation of the organism (155˚F [68.3˚C] for several seconds) . Though the organism may not be heat tolerant, studies have shown *E. coli* O157:H7 to be acid tolerant. Strains of *E. coli* O157:H7 have been shown capable of surviving pH 2.5 for more than 2 hours, and can survive the cheddar cheese manufacturing process and for more than 60 days of curing (the minimum curing period required by the FDA [21CFR133]) at a salt content of 2.75-3.76% in the moisture phase and pH values from 4.95 to 5.2 (*76*). A study by Maher *et al.* (*64*) described recovering viable E *coli* O157:H7

from a smear-ripened cheese after 90 days of ripening. These studies indicate that FDA regulations may be need to be updated to reflect current findings.

Salmonella

Salmonella, like *E. coli*, is a genus of the Enterobacteriaceae family. It is comprised of gram-negative, facultative anaerobic bacteria. *Salmonella* can grow to a minimum water activity of .92 and in environments of a pH range from 4.0 up to a maximum pH of 9.0. The temperature range of growth for *Salmonella* is from 41˚ to 117˚ F (5˚ to 47˚ C).

Salmonellosis has been associated with a wide variety of foods, including raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and salad dressing, cake mixes, cream filled desserts and toppings, dried gelatin, peanut butter, cocoa, chocolate, tomatoes, raw bean sprouts, mung bean seeds, and melons (*19, 35*). Outbreaks have been reported in several states over the last 5 years that have isolated *Salmonella* strains from cantaloupe, unpasteurized orange juice, unpasteurized milk, toasted oats cereal, and shell eggs (*7-9, 12*).

The genus *Salmonella* contains over 2300 different serotypes or 'strains'. Some serotypes were based initially on the location at which the serotype was originally isolated (for example, *S. dublin* and *S. heidelberg*). Some serotypes were named after the disease and the type of animal the bacteria affected. For example, *S. typhii* causes typhoid in man while *S. typhimurium* causes typhoid in mice. Currently, serotypes of *Salmonella* are defined on the basis of the bacteria's antigenic structure, and occasionally by specific biochemical reactions. Serotypes are given by formulas that are based on the O- (lipopolysaccharide), H- (flagellar), and Vi- (capsule) antigens. Common names are given to those serotypes of *Salmonella* that are the

most important in research. For example, *S. typhimurium* is also known as *Salmonella* 1,4,5,12:i:1,2 (*19, 35, 36, 44*).

Salmonella strains cause a wide variety of human enteric disease that ranges from mild symptoms with short duration (nausea and abdominal cramping) to severe gastroenteritis with or without bacteremia to typhoid fever, a potentially life-threatening illness. *Salmonella* food poisoning is characterized by an incubation period of 8 to 72 hours, and the illness is usually self limiting, lasting 4 to 7 days with most people recovering without medical intervention. This type of food poisoning is rarely fatal (less than 1%). The dominant serotype causing food poisoning has changed over recent decade from *S. agona*, *S. hadar*, and *S. typhimurium* to the current *S. enteritidis*. (*35*).

There are other foodborne pathogenic bacteria such as *Yersinia enterocolitica*, *Shigella* spp., *Vibrio* spp., *Aeromonas* and *Plesimonas* spp., *Clostridium botulinum*, *Bacillus cereus*, and toxigenic molds, and foodborne viruses and parasites. Though these other microorganisms and parasites may cause illness and/or death in humans, *E. coli* O157:H7, *Salmonella*, and *L. monocyto*genes cause a higher proportion of the current total foodborne diseases than the rest.

Control of Pathogens in Foods

The control of microorganisms in foods is accomplished through heat processing (pasteurization or cooking of foods), irradiation, high-pressure processing, low-temperature storage, chemical preservatives, modification of atmosphere, control of water activity, or compartmentalization (*2, 50*). In addition to these methods, one can also allow the natural antimicrobial agents found within foods to control the growth of pathogenic bacteria.

Garlic and onion have demonstrated antibiotic and antibacterial effects (*79*). Louis Pasteur demonstrated the antibacterial effect of garlic and onion over 100 years ago with his

publication *Mémoire sur la fermentation appelée lactique* in 1858. Raw juices from the onion and garlic have been shown to be highly effective against *E. coli*, *Pseudomanas*, *Salmonella*, *Candida, Klebsiella, Micrococcus, Bacillus subtilis*, and *Staphylococcus aureus*. Concentrations as low as 8.4 ppm of volatile allyl isothiocyanate (AIT) can result in more than a 7-$\log_{10}$ reduction in *E. coli* population on alfalfa sprouts (*69*), and concentrations as low as 0.1% by weight in low-fat cheese reduced the population of *Salmonella* and *L. monocytogenes* by 5 $\log_{10}$ (*87*). AIT used in the studies were from industrial sources such as Sigma chemical co. (*69*). Studies have also demonstrated that entertoxic *E. coli* strains and other pathogenic intestinal bacteria are more susceptible to inhibition by crushed garlic than the natural microflora found within the gut. Garlic can contain from 2 to 7 mg/g of AIT in fresh weight of crushed garlic, and onion contains from 1 to 5 mg/g of AIT fresh weight. AIT is not detected in whole garlic cloves or whole onion (*58*).

The essential oils of certain spices have been shown to have antibacterial properties. A study by Farag *et al.* (*33*) demonstrated that the essential oils of sage, rosemary, cumin, caraway, clove, and thyme were used to inhibit 8 types of bacteria and a yeast. Concentrations as low as 0.25 mg/ml was enough to prevent microbial growth using filter paper disc agar diffusion method. Other herbs and spices have also shown antimicrobial properties such as cinnamon, oregano, and hops. As a consequence, herbs and spices may contribute to the microbial stability of the foods in which they are used (*2, 15*).

Table salt (NaCl) has also been used as an antimicrobial agent in many foods such as nuoc mam and sauerkraut. The salt content may contribute to the inhibition of growth of bacteria through the creation of a toxic environment in which a bacteria cannot survive due to

osmotic differences on either side of the cell membrane (*2, 50*). Higher concentrations of salt in solution causes low water activity (A_w) which can inhibit bacterial growth.

Bacteria maintain an intracellular pH of near neutrality (pH 7.0) to prevent conformational changes of cell structural proteins, enzymes, nucleic acids, and phospholipids (*28, 75*). Maintenance of a neutral pH is necessary for the normal functioning of these components. To maintain an intracellular pH of 7.0, cells tend to move the protons of acids (H+) to the outside of the lipid bilayer membrane of the cell. Protons are transported out through the membrane bound protein-based proton pumps, depleting energy reserves. Depletion of the energy reserves of bacteria diverts energy from growth-related functions that results in the production of an injured but viable cell that may multiply if the environment becomes favorable. Exposure to pH levels below a critical threshold can render intracellular proteins irreversibly denatured, and cell death (inability to multiply) will occur (*66*).

Organic acids can inhibit the growth of bacteria and kill bacteria through several methods. Examples of organic acids are acetic, lactic, and citric acids. The pK_a of these acids are 4.75, 3.1 and 3.1 respectively (*15*). First, organic acids can produce a high concentration of H+ outside the cell and expose the cell membrane, periplasmic space, and outer surfaces of the cytoplasmic, or inner, membrane to the detrimental effects of low pH. The low pH creates a change in the proteins of the cell membrane, and interfere with membrane permeability (*84*). This interference reduces adenosine triphosphate (ATP) production by uncoupling the electron transport system or by inhibiting active transport of nutrients into the cell. Undissociated organic acid molecules can pass though the cell membrane from a low acid environment to the neutral to high pH of the cytoplasm of a bacterium. At high pH, the equilibrium shifts to a higher ratio of dissociated molecule, and the ionized molecule may lower the pH and break the proton motive

force. The cell then will maintain its internal pH by expending energy and removing the protons, but this action will divert energy from growth-related functions (*2*).

Acetic acid is lipophilic and permeates the lipid bilayer of cells, which facilitates the diffusion of acetic acid into the cytoplasm. Once in the cytoplasm, the undissociated molecule will dissociate, creating H+ and anions. H+ can be neutralized somewhat by the buffering capacity of cytoplasmic materials, but once the buffering capacity is exceeded, the bacteria must transport H+ outside the cell membrane or conformational changes to internal cell structural proteins, enzymes, nucleic acids, and phospholipids may occur. Growth may slow or stop due to these conformational changes, and may resume if the conformational changes are not permanent, and the pH of the environment is changed back to a favorable environment. Sheu and Freese (*85*) observed that acetic acid inhibits oxygen uptake and reduces ATP production of *B. subtilis* by 76% to 77%. Acetic acid also interferes with the proton motive force of the cell (*85*).

Though the aforementioned products may exhibit the ability to inhibit the growth of bacteria both *in vivo* and sometimes *in situ*, it is important to note that these antimicrobial properties were observed in controlled and usually simple (e.g. antimicrobial compound and nutrient broth) systems. Further study is needed to determine the effects of these antimicrobials in complex systems at concentrations that would be acceptable to the human palate, such as a salsa with acetic acid, cumin, salt, onion, and garlic components or other products with may use one or more of these antimicrobials as ingredients (pickled asparagus).

Recent Research

Tauxe (*91*) discussed the evolution of pathogenic bacteria in the food supply. Well-established pathogens become controlled or are eliminated while new pathogens emerge. The new pathogens emerge because the increasing body of knowledge on well-established pathogens leads to control or elimination of those pathogens, which leads to reallocation of resources to those recently identified, which leads to increasing the body of knowledge of the newly identified pathogens, and the cycle continues. This cycling of identification of pathogens, learning of the pathogen, and the control of the pathogen requires a shift in the way foodborne disease prevention is executed. A new paradigm for the integration of research data, food-control monitoring, epidemiological investigations, and disease surveillance was presented by Schlundt (*83*). Identification of new sources of pathogens becomes more important as public health improves. In the past, separating human sewage and animal manure from human food and water supplies were sufficient to maintain a safe food supply. Now, public health depends on taking measures one step further and maintain the safety of the feed and water supplies of the animals themselves so contamination of the food supply further up the chain is minimized.

It has been shown that since the early 1990s there has been a dramatic increase in resistance to antimicrobial drugs in several types of foodborne bacteria (*43, 54, 56, 93, 97*). These bacteria include *Salmonella enterica*, *Campylobacter* spp., and *E. coli* O157:H7. Ward *et al.* (*102*) has shown that increased antibiotic and antimicrobial resistance can even be caused by nutraceuticals. Increases in multi-drug resistant strains of foodborne pathogenic bacteria requires control of these organisms before outbreaks occur.

Many types of pathogenic bacteria can be found on fresh produce. *L. monocytogenes* has been found on cabbage, cucumbers, potatoes, radishes, and field cress (*45, 73, 94*). *Salmonella* has been found in lettuce, cauliflower, sprouts, mustard cress, eggplant, endive, and spinach (*19, 94*). *E. coli* O157:H7 has been found in sprouts, cabbage, lettuce, and cilantro (*19, 40, 94*). Even herbs and spices, shown to have antimicrobial properties (*72, 79*), can contain fecal coliform bacteria, indicators of possible pathogenic coliform bacteria contamination (*40*). Contamination of cabbage by *L. monocytogenes* may even occur during postharvest processing, though a study by Prazak et. al. shows that the cabbage may have arrived contaminated (*73*). It is then shown in another article by Prazak *et al.* (*74*) that 95% of the *Listeria monocytogenes* found on the cabbage and in the packing sheds were resistant to two or more antimicrobial medications. These medications included penicillin, streptomycin, tetracycline, and erythromycin, among others. Chen *et al.* (*24*) described the transfer rates between hands and other common surfaces involved in food preparation, which may explain why fresh fruits and vegetables may be contaminated in the field or in the home. Wachtel and Charkowski were able to contaminate a large volume of dry lettuce with only one inoculated dry lettuce piece, and colonies could grow on lettuce held at room temperature (*98*).

Fresh fruits and vegetables can be contaminated with bacteria through irrigation water (*19*), and the irrigation method plays a role in percentage of plants that become contaminated. Solomon *et al.* (*88*) showed spray irrigation contaminated a larger percentage of lettuce heads than surface irrigation. Wachtel *et al.* (*99*) demonstrated the association of *E. coli* O157:H7 with leaf lettuce using a hydroponic system as well as a soil system. Soil, irrigation water and process water all contributed to the contamination of melon rinds (*39*). Once an irrigation source has

been contaminated with a pathogenic bacteria, that bacteria may adhere and linger in that system (*13*).

Contaminated fresh fruits and vegetables may or may not provide conditions that allow the growth of pathogenic bacteria. Knowing growth or death rates of pathogens on fresh fruits and vegetables is important, since the infectious dose of pathogenic bacterium can be as low as 10 cells (*10, 11*). Control of pathogens can be achieved through processing, but fresh or minimally processed fruits and vegetables may lack a lethal treatment during production. It has been shown that *Shigella sonnei* and *S. flexneri* can maintain their numbers on mixed lettuce under modified atmosphere packaging for seven days but will die off in chopped bell peppers and grated carrots under the same modified atmospheric conditions (*14*). Palmai and Buchanan (*68*) and Stewart *et al.* (*89*) have shown *Listeria monocytogenes* to grow during germination of alfalfa sprouts. *Salmonella* and *E. coli* O157:H7 can survive but not grow on fresh and frozen strawberries (*57*). *E. coli* O157:H7 can grow in leaf lettuce structures, and contamination of the lettuce can occur through plant roots (*99*). Once *E. coli* O157:H7 is absorbed into plant tissue, destruction of viable bacterium almost impossible by chlorine treatment, since the tissues of the plant protect the bacterium from contact with chlorine was shown with in lettuce tissues (*90*).

Once fresh fruits and vegetables are contaminated with pathogens, it becomes important to understand how to disinfect the contaminated object. Ukuku and Sapers (*95*) demonstrated that *Salmonella stanley* can survive 5 days on cantaloupe surfaces. Water washes did not significantly reduce the bacterial population, but a chlorine or hydrogen peroxide solution wash were effective and reduced the population by 3 logs. *Salmonella* can grow on fresh-cut pieces of cantaloupe. Chlorous acid at 268 ppm and lactic acid at 2% were effective in reducing *Salmonella typhimurium* and *L. monocytogenes* in mung bean sprouts (*61*). Reduction of

Salmonella on alfalfa sprouts with the use of electrolyzed oxidizing water has been shown by Kim *et al.* (*55*). Sonication in combination with the electrolyzed oxidizing water reduced the population greater than electrolyzed oxidizing water alone. Hot water immersion of apple surfaces reduced the population of *E. coli* O157:H7 by 5 logs without significant increase of the internal temperature of the apple itself (*34*).

Recent journal articles report experiments using new methods of controlling pathogens on fresh produce. Bari *et al.* (*17*) found in their study that calcinated calcium could be useful in controlling pathogenic microorganisms in fresh produce. Wang *et al*. (*101*) demonstrated 1 to 3 log reduction of pathogens on broc*coli*, cauliflower, and radishes using cetylpyridiudinium chloride. Sodium hypochlorite (NaOCl), Tween 80, acetic acid, sodium phosphate (Na_3PO_4), and hydrogen peroxide (H_2O_2) were used as individual treatments on strawberry fruit to determine the survival of *E. coli* O157:H7. Yu *et al.* (*103*) proved that of all these chemical agents, hydrogen peroxide was most effective at killing the pathogen on strawberries by 2.2 log CFU/g. The bactericidal activity of allyl and methyl isothiocyanate (purified from Japanese green mustard [wasabi]) was noted by Lin and others (*62*)and was used to reduce *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* populations on tomato skin and stem scars, apple stem scars, and lettuce. Pathogen reduction ranged from 3 to 8 logs. A review of the identification, characterization, and a call for toxicity data on bacteriocins for use in food preservation was published by Cleveland *et al.* (*25*). Annamalai *et al.* (*6*) determined that a synthetic peptide (PR-26) could be used to control pathogens, but applications in appropriate foods needed to be validated.

Ross and colleagues discussed fermentation as a preservation technique from the past, in the present and in the future (*78*). Previous generations have used lactic acid bacteria (LAB) to

produce inhospitable environments which spoilage and pathogenic bacteria could not survive. Presently, work is being completed to ascertain what the antagonistic primary and secondary metabolites of the lactic acid bacteria are. Nisin has been identified as a bacteriocin and is currently used as an effective biopreservative in some dairy products. Future identification of bacteriocins and efficacy studies are necessary to extend the shelf life and safety of foods. Building on the use of bacteria producing compounds inhibitive to other microorganisms, Amezquia and Brashears (*5*) found that lactic acid bacteria (LAB) could reduce the *Listeria monocytogenes* load of some ready-to-eat meats from 2.6 to 4.7 $\log_{10}$ cycles. Scheunzel and Harrison undertook the task of identifying microbial antagonists found naturally on fresh, minimally processed vegetables (*82*). They determined that of the 1,180 isolates, 37 (3.22%) were found to have various degrees of inhibitory activity against at least one test pathogen. The isolates with the most extensive inhibitive properties came from finished lettuce piece shreds. All isolates with inhibitory activity were able to multiply at 4 and 10˚C. Liao and Fett also discovered strains of native microflora that were antagonistic to human pathogens, and discussed the use of these antagonistic microflora as potential biopreservatives for fresh produce.

Naturally occurring antimicrobial compounds are also found in spices and herbs used throughout the world. Oregano and thyme were shown by many recent studies to have inhibitory effects on the growth of pathogenic bacteria and some fungi (*32, 38, 51, 80*), and various extracts from herbs and spices from around the world have been evaluated for their antimicrobial properties. Delaquis *et al.* (*29*) mixed several of these extracts from dill, cilantro, coriander, and eucalyptus plants and determined that additive, synergistic, or antagonistic effects occurred. Hseih *et al.* (*47*) performed a similar experiment with Asian plant extracts from corni fructus, cinnamon, and Chinese chive. Edible plant extracts of commonly consumed plants in Asia were

also determined to have antibacterial properties, as shown by Alzoreky and Nakahara (*4*). These plants included wormwood, green tea, cassia, caraway, fennel, star anise, sweet basil, radish, thyme, ginger, and Christ's thorn.

While some experimenters were determining the antimicrobial effects of herb, spice, and plant extracts in nutrient broth and on agar plates, other scientists used the extracts in food products themselves. Gill *et al.* (*42*) showed that though cilantro oil by itself may have antibacterial properties, there is no significant bactericidal activity against *L monocytogenes* on vacuum packed ham. It was concluded that the fat content of the ham played a role in the reduced efficacy of the cilantro oil due to the association of the oil with the fat. Allyl isothiocyanate (from mustard and horseradish plants) was shown to be a potential alternative to chlorine for the purpose of killing *E. coli* O157:H7 cells and perhaps other pathogens on alfalfa seeds (*69*). Hsieh *et al.* (*47*) also used plant extracts to show their antimicrobial effects in food products such as dumplings, guava juice, and green and black tea. Smith-Palmer *et al.* (*87*) discussed the use of bay, clove, cinnamon, and thyme essential oil in soft cheese. Fat content of the cheese was shown to be an important factor in determining the effectiveness of the plant essential oil (*87*).

Singh *et al.* (*86*) were creative and described a synergistic inhibition of *L. monocytogenes* by nisin and garlic extract in broth and in hummus. Adler and Beuchat (*3*) mixed butter and garlic to show that garlic butter at 20% garlic to butter wt/wt had bactericidal effects on *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes*. These three pathogens did not grow in unsalted butter. Canillac and Mourey (*22*) also took a unique route in finding naturally occurring antibacterial compounds and looked at sprucewood (Picea spp) for the compounds. It was

discovered that the essential oil from this wood was inhibitory towards seven strains of *L. monocytogenes*, *Staphlococcus aureus*, and six coliform bacteria.

Though the essential oils of herbs, spices, and plants may have antimicrobial properties, the intact herb, spice, or plant may not have antimicrobial properties. Work by Brandl and Mandrell (*20*) discusses how *Salmonella* can actually grow on cilantro plants, though cilantro essential oil has been shown to have antimicrobial properties (*29, 42*).

Organic acids, such as lactic, citric and acetic acid, can be used to preserve foods from spoilage and pathogenic bacteria. Lactic and acetic acids are created during the fermentation of milk in kefir. Garrote *et al.* (*41*) determined that the lactic and acetic acids were responsible for the bacteriostatic effect that was observed in *E. coli* bacteria. In this study, it was also determined that the organic acids were the primary agents responsible for the inhibitory effect of *E. coli* bacteria. Milk acidified with HCl did not exhibit the same inhibitory effects as the milk acidified with acetic and/or lactic acid. It was concluded that lactic and acetic acids were necessary to produce the inhibitory effect observed with kefir production. Although lactic acid was important for inhibition, the presence of acetic acid improved the inhibitory effect.

Acetic acid was the factor having the most influence in a growth-no growth interface in a model created by McKellar and Lu (*65*). While the study observed growth factors such as temperature, pH, acetic acid, and/or salt, it was shown that as little as 0.5% acetic acid in the tryptic soy broth (TSB) environment was enough to inhibit *E. coli* O157:H7 growth. McKellar and Lu created a growth model based on 1,820 treatment combinations. During the validation of the growth model, acetic acid had the greatest influence on the growth/ no growth boundary of data sets. Increasing salt increased the minimum temperature at which growth was observed.

The model was accurate enough to predict the growth or no growth of 25 of 26 conditions specified by the authors.

Le Marc *et al.* (*59*) confirmed that the addition of a weak acid (lactic, acetic, or proprionic) to a system was important as a food preservation technique. While modeling the growth kinetics of *Listeria* as a function of temperature, pH, and organic acid concentration, minimal inhibitory concentration (MIC) of undissociated acid was determined. For lactic acid it was 8.0 mM, acetic acid was 20.3 mM, and proprionic acid was 8.8 mM. All modeling done for this experiment was based on extensive use of advanced mathematical equations and advanced statistical analysis.

Lactic acid and hydrogen peroxide washes have been shown to be more effective at reducing pathogenic populations than just lactic acid or hydrogen peroxide alone on lettuce, apples, oranges, and tomatoes (*63, 96*). The combination of lactic acid (1.5%) and hydrogen peroxide (0.1%) at 22˚C was highly effective in killing large populations of *E. coli* O157:H7 (>6.0 log CFU) while neither lactic acid nor hydrogen peroxide alone at the same concentration and temperatures could reduce the pathogen population by 5.0 log CFU. The combination of lactic acid and hydrogen peroxide produced a hurdle and synergistic effect against the growth of the pathogen.

Materials and Methods

Bacterial strains: Three strains of *E. coli* O157:H7 (ATCC 35150, 43889, and 43890), three strains of *L. monocytogenes* (ATCC 19114, 7644, and 19113), and three strains of *Salmonella typhimurium* (DT104 Killercow, ATCC 19585 and ATCC 363755) were obtained from the Food Microbiology Culture Collection at Washington State University (from Dr. Dong-Hyun Kang, Pullman, WA). All cultures are maintained on tryptic soy agar (Difco Laboratories, Detroit, MI) slants and subcultured monthly.

Cell suspension and inoculation: Mixtures of the three strains each of *E. coli* O157: H7, of *Listeria monocytogenes*, and of *Salmonella typhimurium* were used as inocula. Each strain of each species was grown separately in tryptic soy broth (Difco) at 37˚ C for 24 hours, and the mixtures of the three strains of each species was combined into sterile, plastic 50 ml centrifuge tubes (Corning Inc., Corning, NY; 1 species per tube). The tubes were closed and then mixed using a vortex device for 15 seconds. The resulting cocktail of bacterial strains were serially diluted using sterile peptone water (0.2%) (Difco) to achieve ~ 10^4 CFU/ ml of each species in the sample. The inoculum was added aseptically to the samples, and then mixed completely with shaking for 30 sec for uniform distribution.

Sample treatment: The model salsa was created by referencing salsas from a bulletin written by Val Hillers and Richard Dougherty (Pacific Northwest Cooperative Extension Publication PNW0395). All formulations were converted from volumes to weights, and percentage of each ingredient was determined on a weight basis. The model salsa is listed below:

Table 1 Standard salsa formulation.

Standard Salsa Ing.	Percentage
Tomato	75.00%
Lemon Juice/ Vinegar	11.00%
Onion	8.00%
Chilies	4.35%
Garlic	0.75%
Salt	0.60%
Black Pepper	0.10%
Oregano	0.10%
Cumin	0.10%
	100.00%

All ingredients were homogenized in a consumer type blender for at least 1 minute on highest speed. The salsa was equilibrated to a pH 4.6 using a saturated NaOH solution, and a flat surface combination probe (Model 430, Corning Inc.), and a solids content of 5% using a handheld refractometer (Model 2912, Atago Co, Tokyo, Japan). Tomato paste was added as necessary to maintain a 5% solids content. The salsa was transferred to Kimax narrow mouth glass dilution bottles (model 14915-160, Kimble/Kontes, Vineland, NJ) and processed in an autoclave for 20 minutes, steam only, and no pressure within the chamber to simulate a heat process for the salsa and reduce the natural bacterial load of the ingredients. All model salsa was stored at –30˚ F (Room G20-AB FSHN, Washington State University, Pullman, WA) until used. Samples were equilibrated to holding temperature (4˚C or 22˚C, depending on treatment) before inoculation.

Roma tomatoes, white onion, Anaheim chilies, and common garlic were used for the fresh ingredients in this experiment. Citric acid was substituted for lemon juice, and the glacial acetic acid (for vinegar) was used to achieve the varying amendments used for this experiment.

Morton salt (iodized), McCormick fine ground pepper, ground oregano, and ground cumin were used as the spices. All ingredients used for this experiment, except the acids, are commonly available for public consumption. The acids added to the specific amendments were diluted with deionized sterile water to achieve the 11% by weight as specified in the model salsa. Deionized sterile water was added as the control for this experiment.

The tomato base used in this experiment was created from Roma tomatoes, homogenized in a consumer-available blender for at least 1 minute on highest speed. The tomato base was equilibrated to a pH 4.6 using NaOH solution, and a flat surface combination probe (Model 430, Corning Inc.), and a solids content of 5% using a handheld refractometer (Model 2912, Atago Co, Tokyo, Japan). Tomato paste was added as necessary to maintain a 5% solids content. Like the model salsa, the tomato base was transferred to Kimax narrow mouth glass dilution bottles (model 14915-160, Kimble/Kontes, Vineland, NJ) and processed in an autoclave for 20 minutes, steam only, and no pressure within the chamber. All model salsa was stored at –30˚ F (G20-AB FSHN, Washington State University, Pullman, WA) until used. Samples were equilibrated to holding temperature (4˚C or 22˚C, depending on amendment) before inoculation.

Bacterial enumeration: Inoculated samples were serially diluted 10-fold with 9 ml sterile buffered peptone water (0.2%). Pathogenic bacteria were then enumerated by spread plating 0.1 ml onto selective media. Sorbitol MacConkey agar (SMAC; Difco), Oxford agar base (OAB; Difco), and xylose lysine desoxycholate (XLD) agar (Difco) were used as the selective media for the enumeration of *E. coli* O157: H7, *L. monocytogenes*, and *Salmonella typhimurium,* respectively. The spread plates were incubated at 37˚ C for 24 hours. Each amendment was duplicated per experiment, with two enumerations per amendment. All experiments were

repeated three times. Statistical analysis of the data were calculated with Minitab statistical software (State College, PA). Comparison of sample means between control and amended samples at comparable time intervals was made to determine if significant differences could be observed. All observations were considered significant at $\alpha=0.05$.

Enumeration of samples held at refrigeration temperature (4˚C) were conducted at 4˚C to ensure that temperature fluctuations would not affect the final results of this experiment.

Results

Tomato base at 22˚C: (Figures 1, 2, and 3) Roma tomato base, and the Roma tomato base with onion, garlic, Anaheim chilies, or citric acid amendment of 0.50% did not reduce the populations of the pathogenic bacteria to below detectable limits within the 168 hour incubation period. *E. coli* and *Salmonella* populations were reduced to below detectable limits after 96 hours of incubation with 1.50% CA amended salsa and after 120 hours with *Listeria* populations with the same treatment. After 72 hours of incubation with the 0.75% AA *amendment*, *E. coli* and *Salmonella* populations were below the detectable limit. After a 96 hour incubation, *Listeria* populations were reduced to below 1.3 CFU/ml. The 0.25% AA amendment reduced *Salmonella* populations to below detectable limits after 144 hours, but did not do the same for *E. coli* and *Listeria* within the experimental time period.

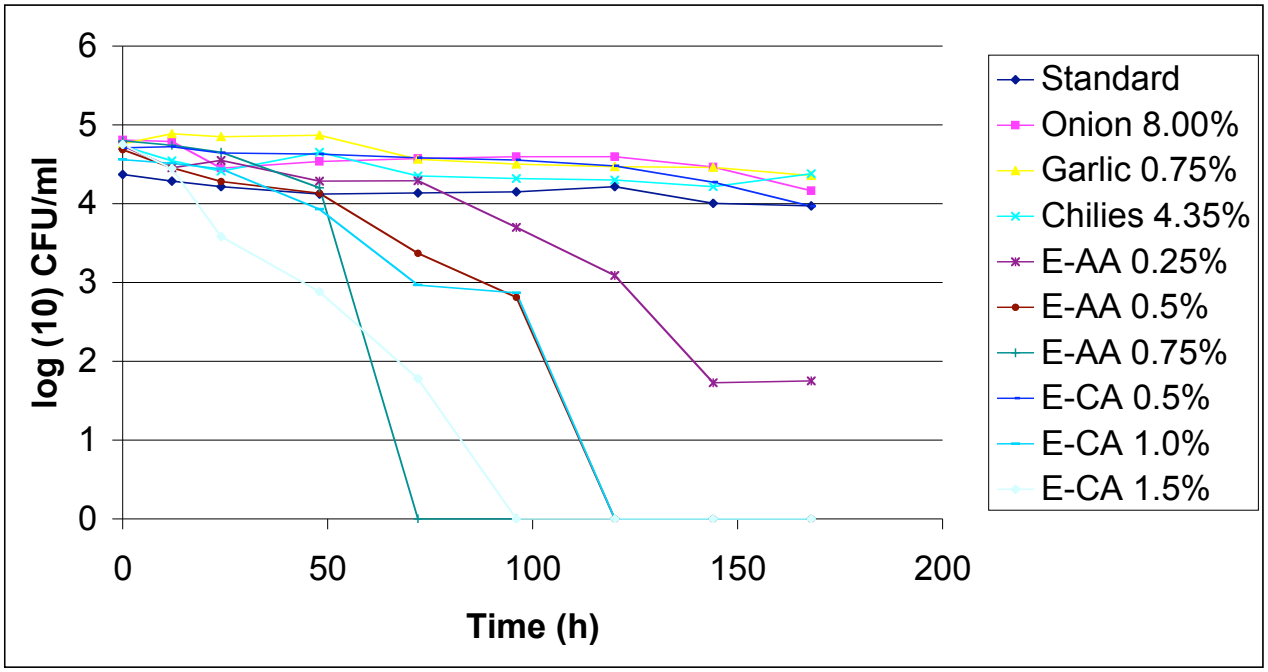

Figure 1. Growth of *E. coli* O157:H7 at 22˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, and 0.5% CA did not show any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids yielded a decreased time required before the *E. coli* O157:H7 population was below detectable limits.

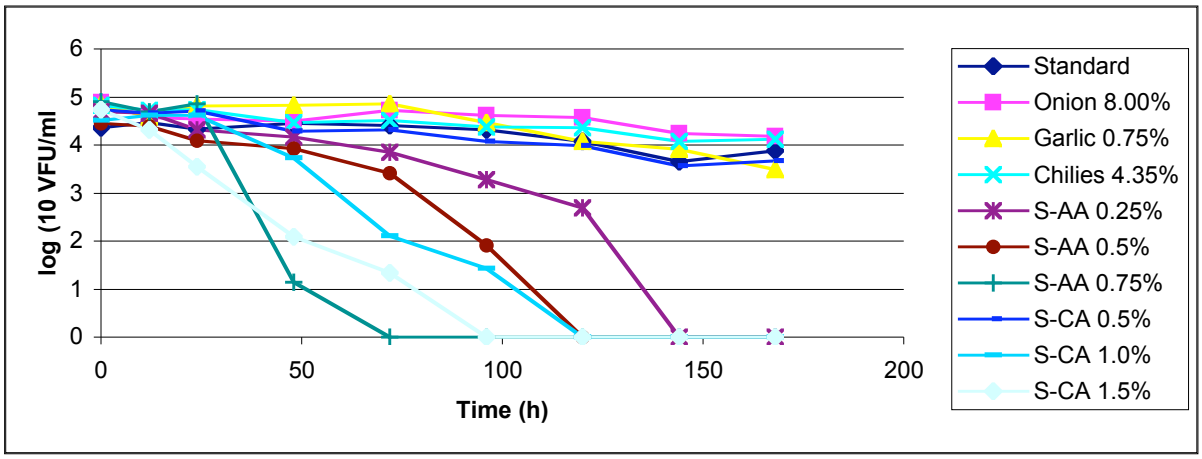

Figure 2. Growth of *Salmonella typhimurium* at 22˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, and 0.5% CA did not show any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids yielded a decreased time required before the S. *typhimurium* population was below detectable limits.

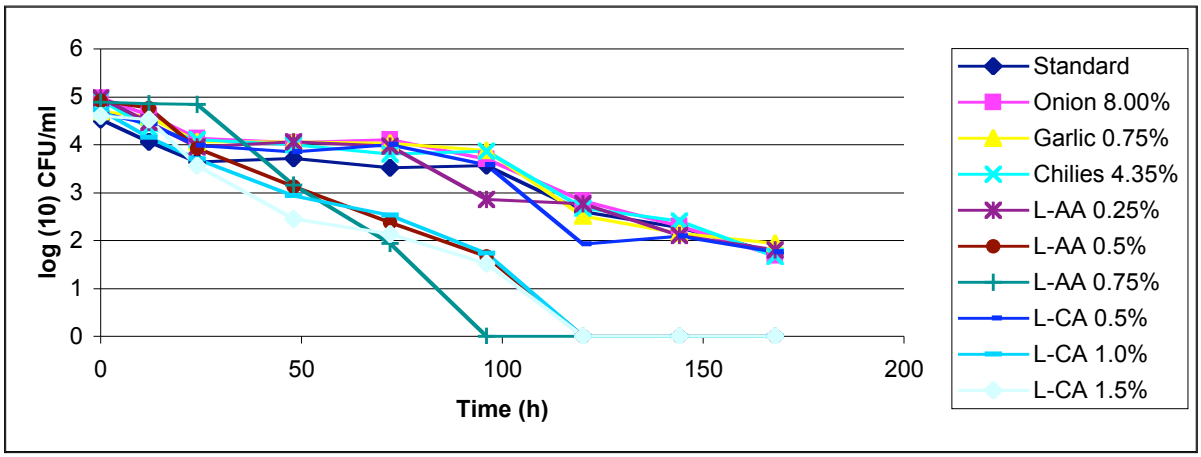

Figure 3. Growth of *Listeria* at 22˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, and 0.5% CA did not show any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids led to decreased time required before the *L. monocytogenes* population was below detectable limits. *L. monocytogenes* population reduction is greater in the tomato base, tomato base amended with onion, garlic, chilies, and 0.5% CA than compared to reduction of pathogenic bacteria in similar samples inoculated with *E. coli* O157:H7 and *Salmonella typhimurium*.

Tomato base at refrigeration temperature: (Figures 4, 5, and 6) No *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* were detected after 14 days in the tomato base amended with AA at 0.75%. *E. coli* and *Salmonella* populations were below detection levels with amendment at 1.50% of CA after 14 days, and *Listeria* populations were below detection levels after 21 days. Amendment with AA at 0.25% showed reduction of *E. coli* O157:H7 and *Salmonella* to the lowest detection limit by 21 days, but *Listeria* showed 3.4 log reduction after 28 days. CA amendment at 0.5% did not reduce the population of any of the pathogens to below detection limits.

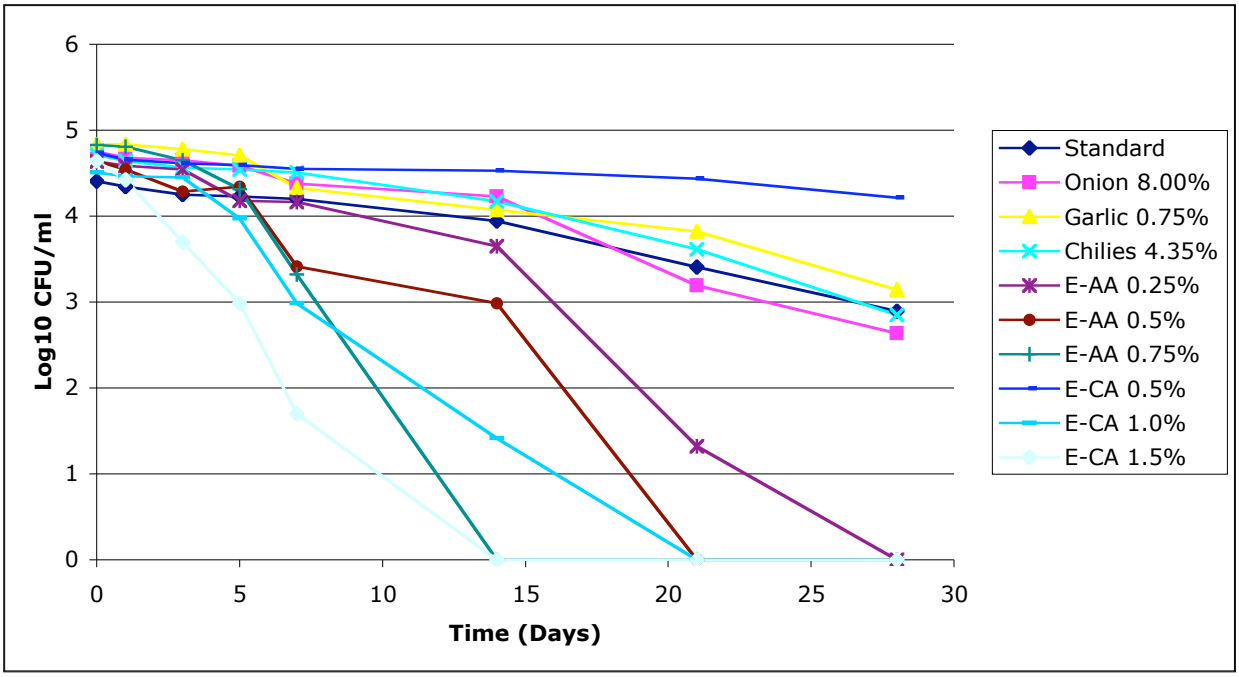

Figure 4. Growth of *E. coli* O157:H7 at 4˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, and 0.5% CA did not show any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids led to decreased time required before the *E. coli* O157:H7 population was below detectable limits.

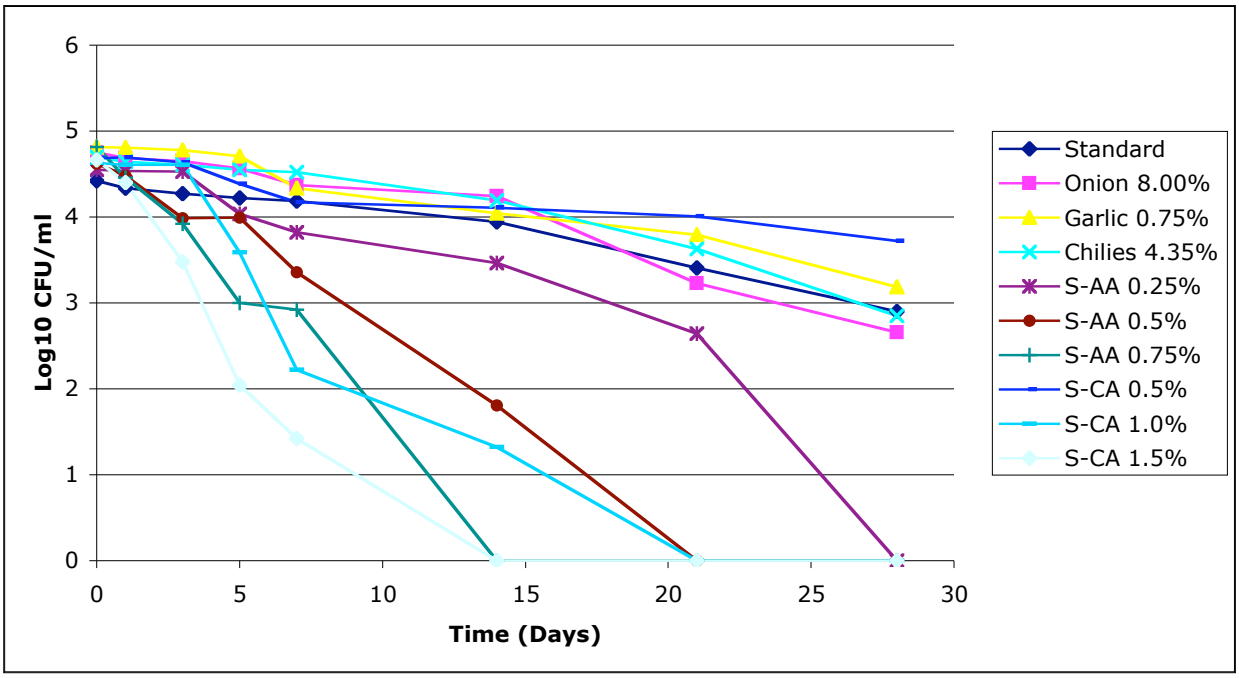

Figure 5. Growth of *Salmonella typhimurium* at 4˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, and 0.5% CA did not exhibit any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids led to decreased time required before the *S. typhimurium* population was below detectable limits.

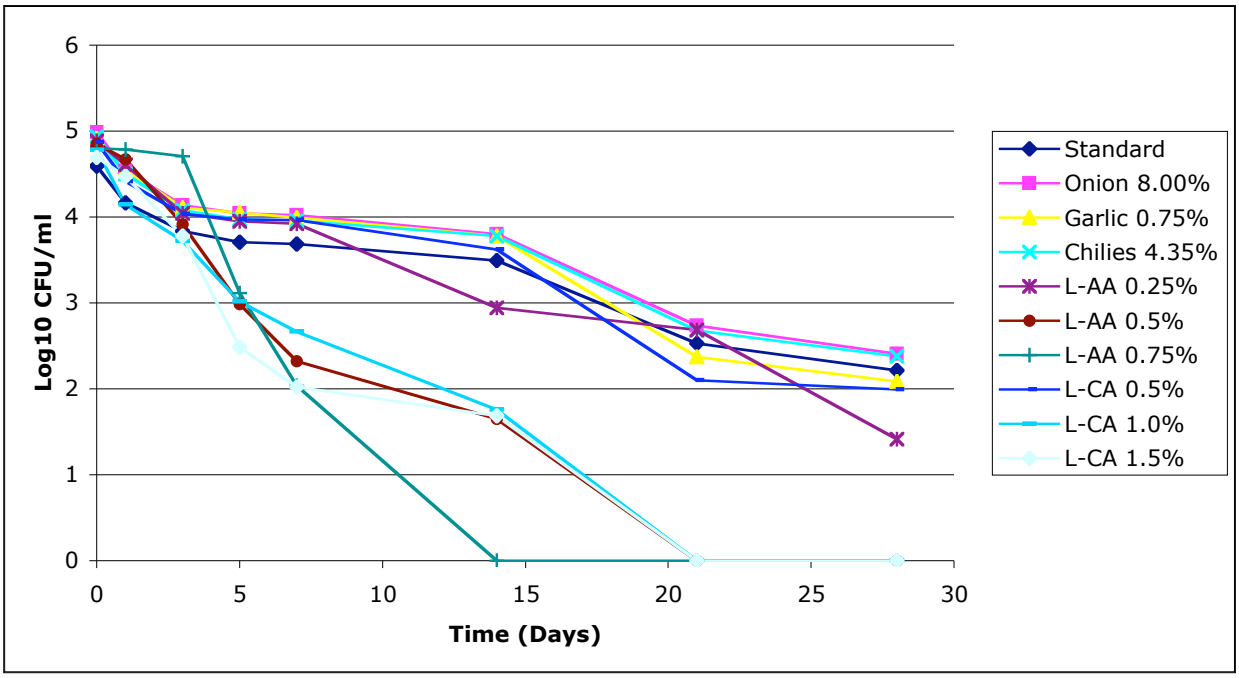

Figure 6. Growth of *Listeria monocytogenes* at 4˚C in tomato base with and without added ingredients. Tomato base amended with onion, garlic, chilies, 0.25% AA and 0.5% CA did not show any significant reduction in pathogens than from the control sample. Increasing amounts of organic acids led to decreased time required before the *L. monocytogenes* population was below detectable limits. *L. monocytogenes* population reduction is greater in the tomato base, tomato base amended with onion, garlic, chilies, and 0.5% CA than compared to reduction of pathogenic bacteria in similar samples inoculated with *E. coli* O157:H7 and *Salmonella typhimurium*.

Salsa base at 22˚C: The experiments with the model salsa held at 22˚ C were conducted for a maximum of 168 hours (7 days). Enumeration of the pathogenic bacteria occurred at 0, 12, 24, 48, 72, 96, 120, 144, and 168 hours after inoculation. (Figures 7, 8, and 9)

E. coli and *Salmonella* showed only a slight reduction in numbers in the model salsa over the course of the 168 hour incubation period while *Listeria* had a nearly 3 log reduction. *Listeria* was not detected after 48 hours of incubation at the highest acetic acid (AA) and citric acid (CA) concentrations (1.25% and 2.00% respectively), was not detected at the lowest concentration of acetic acid (0.50%) after 96 hours, and was not detected at the lowest concentration of CA (0.75%) after 144 hours. *Salmonella* was not detected after 48 hours with the CA amendment of 2.00% and was not detected after 24 hours with 1.25% AA. Amendment with AA at 0.50% reduced the number of viable *Salmonella* bacteria to below detectable limits after 120 hours, and CA amendment at 0.75% did not reduce *Salmonella* enumeration significantly. *E. coli* was reduced to below detectable limits after 24 hours with 1.25% AA and after 48 hours with the 2.0% CA amendment. The 0.50% AA amendment reduced *E. coli* numbers to below detectable limits after 120 hours, but the 0.75% CA amendment did not reduce the population to below detectable limit; it yielded an average of 2.4 log reduction over the 168 hour incubation period.

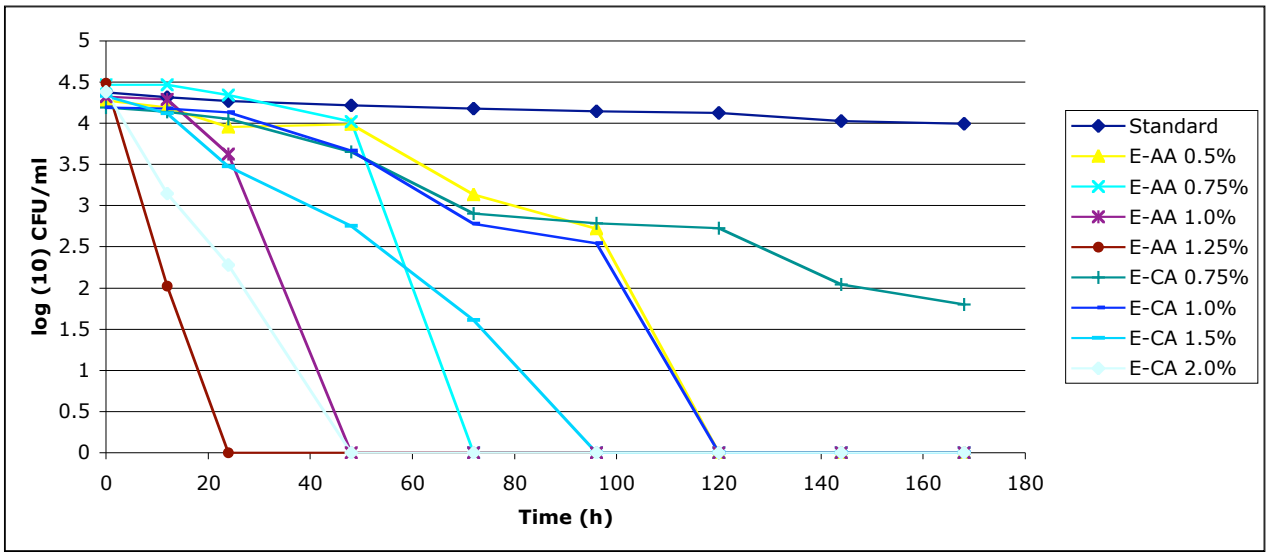

Figure 7. Growth of *E. coli* O157:H7 at 22˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *E. coli* O157:H7 significantly when compared to samples amended with the organic acids. The amended sample with 0.75% CA did not show a significant reduction of pathogenic bacteria compared to the control though reduction of the *E. coli* O157:H7 population did occur at a greater rate.

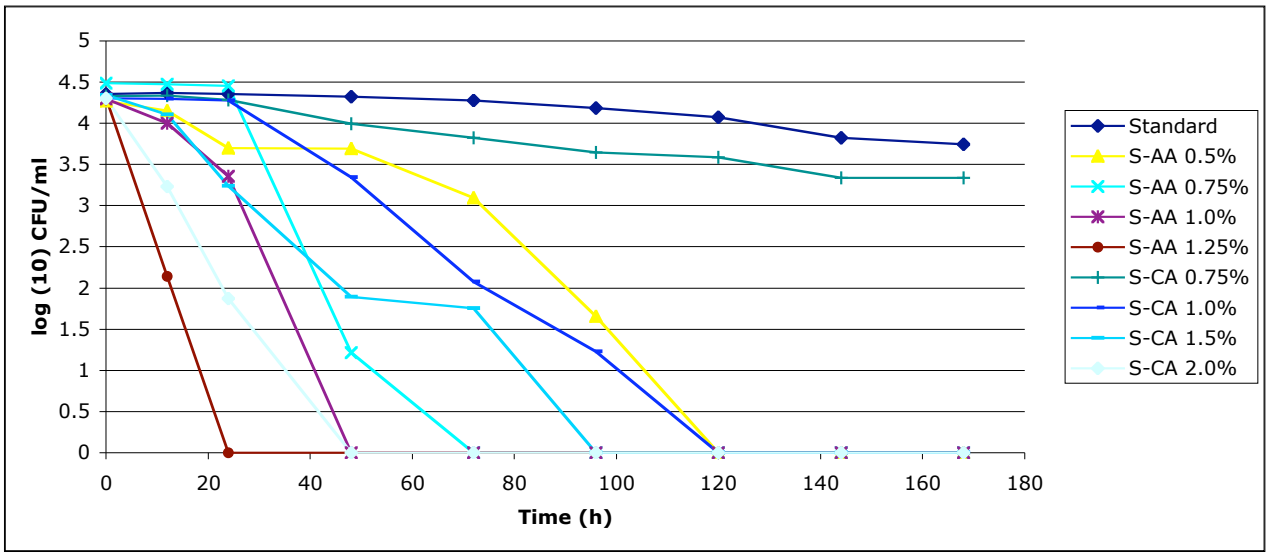

Figure 8. Growth of *Salmonella typhimurium* at 22˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *S. typhimurium* significantly when compared to samples amended with the organic acids. The amended sample with 0.75% CA did not show a significant reduction of pathogenic bacteria compared to the control though reduction of the *S. typhimurium* population did occur at a greater rate.

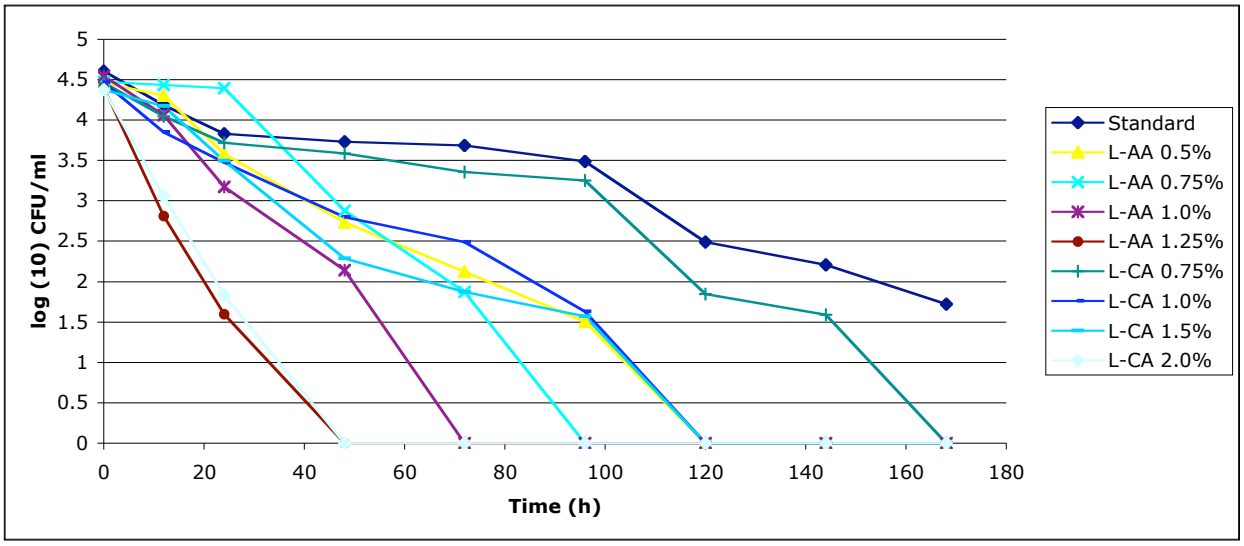

Figure 9. Growth of *Listeria monocytogenes* at 22˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *L. monocytogenes* significantly when compared to samples amended with the organic acids. The amended sample with 0.75% CA did not show a significant reduction of pathogenic bacteria compared to the control though reduction of the *L. monocytogenes* population did occur at a greater rate. The reduction of the *L. monocytogenes* population in the model salsa without amendments was greater than the reduction of the population of *E. coli* and *Salmonella* in similar samples.

Salsa base at 4˚C: (Figures 10, 11, and 12) The experiments with the model salsa held at refrigeration temperature (4˚C) were conducted for a maximum of 28 days. Enumeration of the pathogenic bacteria occurred at 0, 1, 3, 5, 7, 14, 21, and 28 days after inoculation.

Salmonella was not detected after 7 days with the 2.00% amendment of CA; the same amendment required 14 days of incubation to reduce the population of *E. coli* and *Listeria* to below detection limits. *E. coli*, *Salmonella*, and *Listeria* were not detected after 14 days with AA amendment at 1.25%. Amendment with AA at 0.50% resulted in *E. coli* and *Listeria* populations below detectable limits after 28 days, but *Salmonella* populations were below after 21 days. The same results occurred with CA at 0.75%.

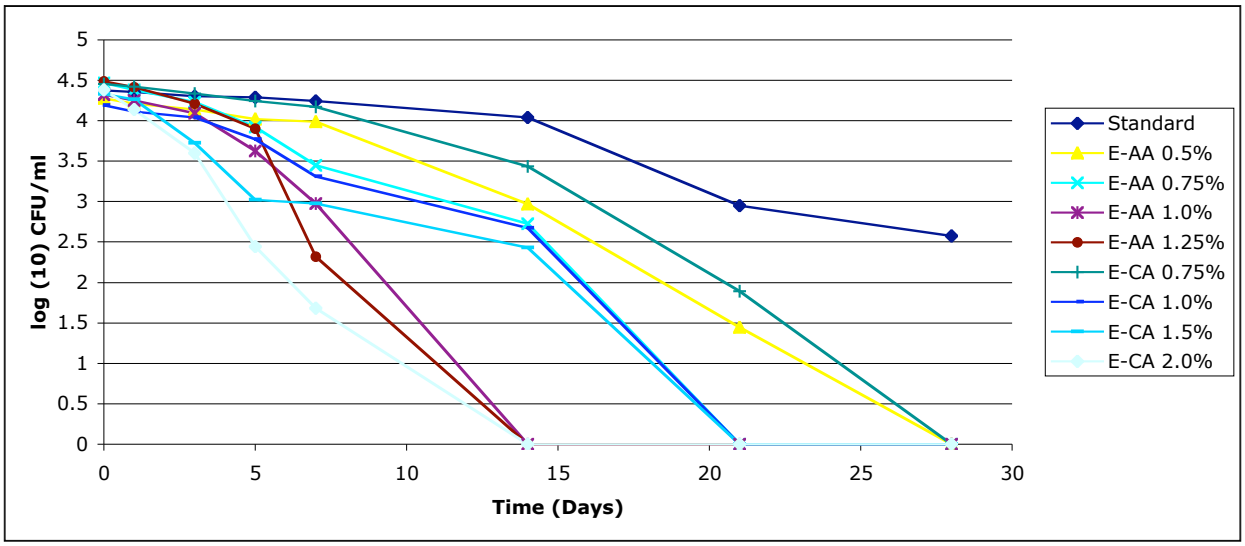

Figure 10. Growth of *E. coli* O157:H7 at 4˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *E. coli* O157:H7 significantly when compared to samples amended with the organic acids.

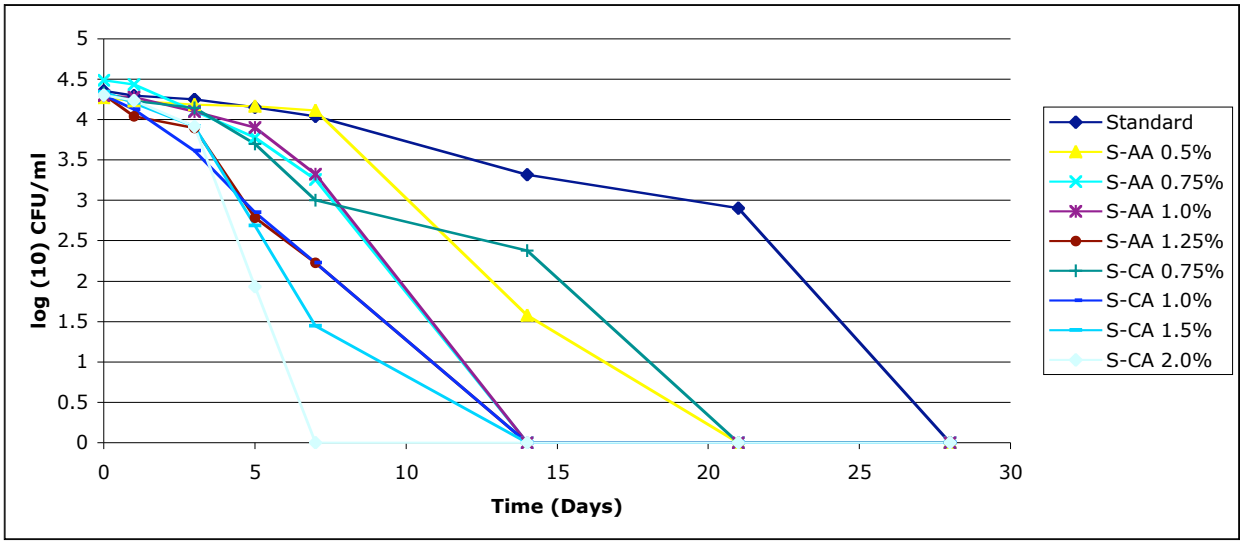

Figure 11. Growth of *Salmonella typhimurium* at 4˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *S. typhimurium* significantly when compared to samples amended with the organic acids, though *S. typhimurium* was reduced to undetectable levels by the 28th day.

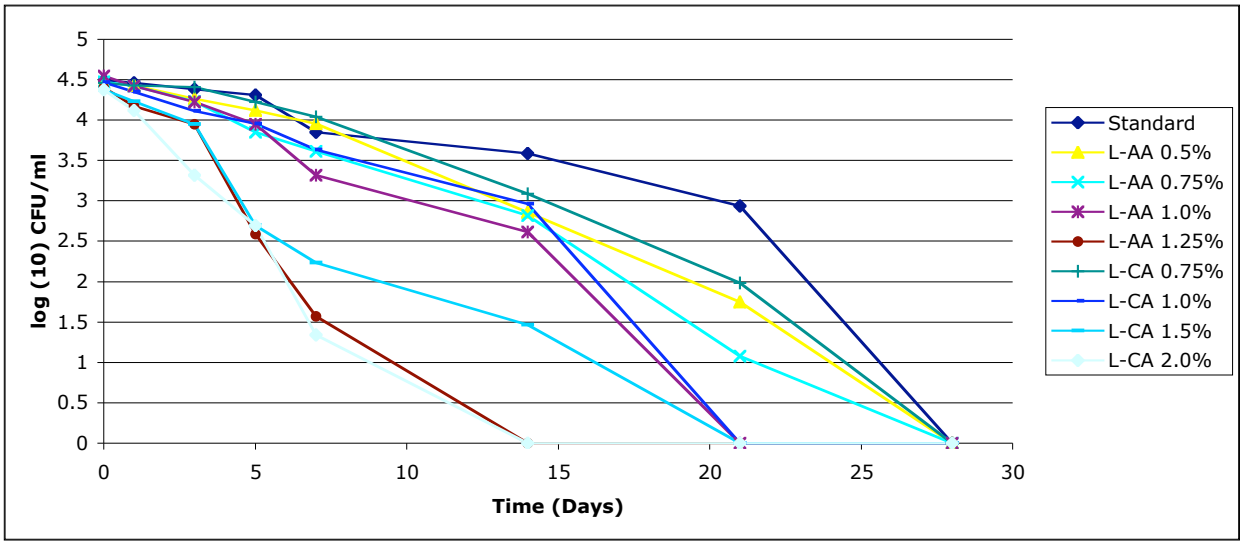

Figure 12. Growth of *Listeria monocytogenes* at 4˚C in model salsa with and without added ingredients. The control sample did not reduce the population of *L. monocytogenes* significantly when compared to samples amended with the organic acids, though *L. monocytogenes* was reduced to undetectable levels by the 28th day.

Discussion

The standard salsa, made of Roma tomato, white onion, Anaheim chilies, garlic, salt, black pepper, oregano, and cumin, did not demonstrate any significant (α>0.05) antimicrobial properties when *Listeria*, *Salmonella*, and *E. coli* O157:H7 were incubated at 22˚C for up to 168 hours, though reduction of pathogenic populations were greater in the salsa than in the tomato base. *E. coli* and *Salmonella* showed .5 to 1 log reduction of bacterial populations, but *Listeria* exhibited a nearly 2.5 log reduction in the model salsa without amendment. As the pH of the model salsa was brought to pH 4.6, it would be expected that the three pathogenic bacteria would suffer a decline in population over time, since the optimal range of growth for most of these bacteria is about pH 4.6. *Listeria* can grow from pH 4.4 at 30˚C to pH 9.5, *Salmonella* pH 4.4 to pH 9.0, and *E. coli* from pH 4.4 to pH 9.5 under ideal conditions (*16, 27, 67*). The pH of the standard and amended salsas were not in the range of growth normally associated with the pathogenic bacteria. Bacteria maintain an intracellular pH of near neutrality (pH 7.0) to prevent conformational changes of cell structural proteins, enzymes, nucleic acids, and phospholipids (*28, 75*). Maintenance of a neutral pH is necessary for the normal functioning of these components. To maintain an intracellular pH of 7.0, cells tend to move the protons of acids (H+) to the outside of the lipid bilayer membrane of the cell (*66*). Protons are transported out through the membrane bound proton pumps, depleting energy reserves. Depletion of the energy reserves of bacteria diverts energy from growth-related functions that results in the production of an injured but viable cell that may multiply if the environment becomes favorable. Exposure to pH levels below a critical threshold (individual to each protein) can render intracellular proteins irreversibly denatured, and cell death (inability to multiply) will occur (*66*).

The difference in reduction of populations between *Salmonella* and *E. coli* and *Listeria* can be explained due to the difference in cell membrane structure. Gram-positive cell walls have many layers of peptidoglycan, which form a thick, rigid structure, while the gram-negative cell walls are relatively thin. The gram-positive bacteria also have teichoic acids, which consist primarily of an alcohol and a phosphate. Teichoic acids may bind and regulate the movement of cations (positive ions such as [H+]) because of the negative charge from the phosphate. Gram-negative bacteria have a periplasm that contain a high concentration of degradative enzymes and transport proteins. Gram-negative cell walls to not have teichoic acids. *Salmonella* and *E. coli* are gram-negative bacteria, and *Listeria* is a gram-positive bacteria. Differences in susceptibility to antimicrobial agents has been noted by others. Farag *et al.* (*33*) observed a trend in which Gram-positive bacteria were more sensitive towards essential oils than Gram-negative ones.

Though the model salsa contained many antimicrobial agents mixed together into a homogenous product, the control salsa did not exhibit any significant difference in its ability to kill the pathogens than tomato alone. This suggests that the tomato in the salsa may contain compounds which may interfere with the efficacy of the natural antimicrobial compounds. Salt has been known to lend protection to bacteria until high levels are added (*21*). Particle size may have played a role in the extraction of the antimicrobial compounds from within the matrix of the vegetables and spices used in the salsa. It is possible that a homogenization of the salsa resulting in smaller particle size may lead to a greater extraction of the antimicrobial compounds and a greater reduction of pathogenic bacteria than what was observed in these experiments.

The model salsa, when amended with the organic acids (acetic and citric) showed a statistically significant difference in kill rates from the control product except in *Salmonella* and *Listeria* with CA at 0.75%. Efficacy of acetic acid was roughly double that of citric acid when

kill rates of the organic acids are compared. Total titratable acidity of both the model salsa and tomato base were 10.1% as citric acid, which is typical of tomato purée (*71*).

The organic acids exhibit antimicrobial effects on the pathogenic bacteria through two mechanisms: the lowering of the pH to below the growth range of the bacteria, and the metabolic inhibition by the undissociated molecules (*50, 67*). The undissociated molecules pass from the low acid environment on the outside of the cell, through the cell membrane, and into the neutral to high pH of the cytoplasm of a bacterium. At a higher pH, the equilibrium shifts and favors a disassociated molecule. The ionized molecule releases H^+ into the internal environment of the bacterium, lowering the internal pH. The cell will then maintain a desired pH by expending energy and removing the protons. This action diverts energy from growth-related functions (*2*), and will eventually kill the bacterium. At pH 3, acetic acid is 98.5% undissociated compared to 53.0% citric. At pH 4, acetic is 84.5% undissociated compared to citric at 18.9%; pH 5 has acetic acid at 34.9% vs 0.41% citric acid (*67*). The effects of acetic acid on the kill rate of bacteria is usually more effective than citric acid.

Table 2 Percentage of organic acids undissociated at various pH values:

	pH Value				
Acid	3	4	5	6	7
Acetic	98.5	84.5	34.9	5.1	0.54
Citric	53	18.9	0.41	0.006	0.001

Standard tomato base incubated at 22˚C did not show any statistical difference in kill rates on the pathogenic bacteria when compared to tomato base with onion, garlic, or chilies. This observation matches what the model salsa at 22˚C has shown; the antimicrobial properties of the onion, garlic, and chilies are interacting with the compounds of the tomato and are being inactivated.

Approximately twice the amount of added citric acid vs. acetic acid was needed to achieve the effect of added acetic acid. Acetic acid was more effective at killing pathogenic bacteria than citric acid at the same percentage levels.

Conducting the same experiments at 4˚C with the salsa and tomato base yielded much the same results when comparing salsa and tomato base held at 22˚C; approximately twice as much added citric acid was necessary to achieve the same results as amended salsa and tomato base with acetic acid.

One major difference between the salsa and tomato bases at 4˚C was the fact that there was a significant difference between the model salsa, and the tomato base with and without added onion, garlic, and chilies. The model salsa without amendment showed a significant difference from tomato base on survival of *Salmonella* and *Listeria*; the difference with *E. coli* and the same comparison was not statistically significant.

Comparing the results between the 22˚C experiments and the 4˚C experiments shows that the inactivation rates increase as storage temperatures are raised. This is due to the increased metabolism of the organism at the higher temperature. The more energy allowed for chemical reactions to occur (higher temperature), the faster the total energy of the bacterium is diminished as the bacterium pumps out protons from disassociated organic molecules. This observation follows observations made by others (*21*).

Conclusions

Though the model salsa at pH 4.6 contained many ingredients that contain naturally occurring antimicrobial components at levels usually encountered in salsas, the ingredients (excluding the citric and acetic acids) were ineffective in killing the pathogenic bacteria. Particle size may have been too large to allow for efficient extraction of the antimicrobial compounds from the various sources of these compounds. Acetic acid and citric acids were both effective at killing pathogenic bacteria.

In the tomato base at room temperature, *E. coli* and *Salmonella* populations were below the detectable limit after 72 hours of incubation with the 0.75% AA amendment, and after a 96-hour incubation, *Listeria* populations were reduced to below 1.3 CFU/ml. *E. coli* and *Salmonella* populations were reduced to below detectable limits after 96 hours of incubation at 1.50% CA and after 120 hours with *Listeria* populations with the same amendment.

At refrigeration temperatures, *Listeria* was not detected at the lowest concentration of acetic acid (0.50%) after 96 hours, and was not detected at the lowest concentration of CA (0.75%) after 144 hours. Amendment with AA at 0.50% reduced the number of viable *Salmonella* bacteria to below detectable limits after 120 hours, and CA amendment at 0.75% did not reduce *Salmonella* population significantly. The 0.50% AA amendment reduced *E. coli* numbers to below detectable limits after 120 hours, but the 0.75% CA amendment did not reduce the population to below detectable limit; it yielded an average of 2.4 log reduction over the 168 hour incubation period.

In the room temperature model salsa, *Listeria* was not detected at the lowest concentration of acetic acid (0.50%) after 96 hours, and was not detected at the lowest concentration of CA (0.75%) after 144 hours. Amendment with AA at 0.50% reduced the

number of viable *Salmonella* bacteria to below detectable limits after 120 hours, and CA amendment at 0.75% did not reduce *Salmonella* enumeration significantly. The 0.50% AA amendment reduced *E. coli* numbers to below detectable limits after 120 hours, but the 0.75% CA amendment did not reduce the population to below detectable limit; it yielded an average of 2.4 log reduction over the 168 hour incubation period.

At refrigeration temperatures, amendment with AA at 0.50% resulted in *E. coli* and *Listeria* populations below detectable limits after 28 days, but *Salmonella* populations were below after 21 days. The same results occurred with CA at 0.75%.

Acetic and citric acids were the components responsible for the majority of the reduction in population of the pathogenic bacteria, though there is a difference in efficacy amongst the pathogens used in this experiment. This work confirms that inactivation rates increase as storage temperatures are raised.

An observation on old salsa and old tomato base with organic acid amendments showed that there was a visual difference in the growth of molds in the products after the experiments were completed. Increased levels of the organic acids seemed to decrease the amount of mold growth on the products. Further study of the efficacy of the organic acids as anti-spoilage compounds is recommended.

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