Effect of cinnamic acid-cyclodextrin inclusion complexes on populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in fruit juices

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ABSTRACT

Cinnamic acid (CA) is a naturally occurring organic acid that is found in some fruits and a number of spices. CA has antimicrobial activity against certain spoilage microorganisms and pathogenic bacteria. However, the acid is poorly soluble in water. Cyclodextrin molecules have a hydrophobic cavity that allows them to serve as a host for insoluble molecules in aqueous matrices. This study was conducted to determine if the aqueous solubility of cinnamic acid could be improved via complexation with α- or β-cyclodextrins, and if these complexes could be used to control bacterial pathogens in juices. Based upon phase solubility analysis, α-cyclodextrin was chosen as the host molecule for the remainder of this study. In complex with α-cyclodextrin, the solubility of cinnamic acid increased from approximately 400 mg/L to 3800 mg/L. Prepared cinnamic acid complexed with α-cyclodextrin was aseptically added (400 mg/L and 1000 mg/L) to orange juice inoculated with a *Salmonella enterica* (7 log CFU/mL) and apple cider inoculated with *Escherichia coli* O157:H7 (7 log CFU/mL). Cider and orange juice samples were extracted on day 0 and at 24 h intervals for seven days and spread plated onto Tryptic Soy Agar. Cinnamic acid was effective for reducing populations of both bacterial pathogens in juice. Populations of *E. coli* O157:H7 in the apple cider were significantly reduced after 7 days at $25.6 \pm 0.42°$ C at concentrations of 400 mg/L (5-log CFU/mL reduction) and 1000 mg/L (6-log CFU/mL reduction) cyclodextrin-cinnamic acid. *S. enterica* counts were also reduced in orange juice at 4° C treated with 400 mg/L (2.7-log CFU/mL reduction) and 1000 mg/L (3.2-log CFU/mL reduction) complexed cinnamic acid. The much improved solubility of this compound provides food processors with greater flexibility in using cinnamic acid in their product formulations.

TABLE OF CONTENTS

TABLES and FIGURES

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INTRODUCTION

Outbreaks of foodborne illness due to the consumption of contaminated foods are a major concern for the food industry and for consumers. Non-pasteurized apple cider/juice and orange juice contaminated with *Escherichia coli* O157:H7 and *Salmonella enterica* respectively have lead to many dangerous outbreaks *(44)*. Apple and orange juice are high acid (pH < 4.6) *(28)* foods which were historically believed to inhibit survival of *E. coli* and *S. enterica.* However, these organisms are able to survive in such environments *(7)*.

The Food and Drug Administration (FDA) announced on January 18, 2001 that juice processors must use Hazard Analysis Critical Control Points (HACCP) for juice harvesting and processing; this was implemented to help ensure microbiological safety of the juice. Along with the HACCP plan, processors must use processes that yield a 5-log reduction of resistant pathogens found within the juice. FDA inspections guarantee that these guidelines are being obeyed; the HACCP regulation applies to both interstate and intrastate commerce *(33)*.

Refrigeration is an effective method of juice preservation to extend shelf-life. However, organisms such as *E. coli* O157:H7 and *Salmonella* have been shown to survive refrigeration. In addition, cold conditions can improve survival of these organisms in acidic environments *(18)*. Since non-pasteurized juice must be refrigerated this leads to potential pathogen outbreaks.

Traditional heat pasteurization inactivates vegetative pathogenic bacteria present, but negatively alters sensory characteristics and nutritional value of the juice *(32, 49)*.

Weak acids such as sorbic acid, benzoic acid, sulphite, acetic acid, and propionic acid, have been used as preservation methods. These additives are steadily becoming rejected by "health conscience" consumers *(3)*. Non-thermal methods have been studied as alternatives; however some are not economically feasible or consumer accepted *(5)*.

Consumers prefer fresh tasting, natural products *(20, 49, 55)*. To meet consumer demands, manufacturers produce fresh tasting juices with minimal heat treatment or synthetic additives. These practices may not be adequate to control spoilage and pathogenic microorganisms. The lack of a processing step that inactivates bacteria has potential to lead to outbreaks. Natural, plant-derived preservatives are ideal because consumers find natural ingredients appealing. The use of an inhibiting agent may help to reduce the need for heat treatments that sacrifice the juice flavor and nutritional value.

Cinnamic acid is a phenolic compound found naturally in many spices, cranberries, prunes, cinnamon, and cloves; it provides a natural protection against pathogenic organisms *(3, 8, 17)*. The mechanism employed by cinnamic acid is the same as other weak acids; it diffuses through microorganism membranes, dissociates, and causes the cell's internal pH to decrease which kills the cell *(3)*.

Cinnamic acid is bactericidal to pathogens such as *E. coli* at concentrations greater than approximately 400 mg/L *(30, 35, 46)*. Also, cinnamic acid is shown to have a synergistic bactericidal effect with other organic acids; this is ideal for juices which naturally contain weak acids such as citric and malic acid *(3)*.

Currently cinnamic acid use has been limited due to its poor intrinsic solubility. Recent cyclodextrin work may lead to an answer for this constraint. Cyclodextins are cyclic oligosaccharides that are capable of housing smaller molecules within its

hydrophopobic cavity creating an inclusion complex. Inclusion complex formation

increases both the solubility and stability of certain molecules; it has the potential to do

the same for cinnamic acid *(51)*.

LITERATURE REVIEW

Salmonella enterica

  Salmonella are small, Gram negative, facultatively anaerobic, non-spore forming,

motile rods. Their optimum growth temperature is 37 °C *(1)* and *Salmonella* are capable

of adapting to stressful environmental conditions, yet they are killed at milk

pasteurization temperatures and times. The optimum pH for growth is 6.5 to 7.5;

however, they are capable of survival in environments with pH ranging from 4.5 to 9.5.

It has been shown that strains preconditioned in acidic environments are capable of

survival at an even lower pH. Salmonellae are unable to tolerate high salt concentrations;

brine above 9% is bactericidal and water activities of <0.93 do not allow for *Salmonella*

growth. Salmonellae are capable of fermenting and produce gas by utilizing glucose and

other monosaccharides. The growth parameters for this organism show that *Salmonella*

species are hardy; this resilience helps them to be a leading cause of foodborne bacterial

illnesses in humans *(28)*.

  Most cases of human salmonellosis are caused by species *Salmonella enterica.*

Salmonella enterica is one of two *Salmonella* species and it has approximately two

thousand serovars. Many *S. enterica* serovars, such as Enteritidis, Typhimurium,

Newport, and Stanley have been linked to foodborne illness *(28)*. *Salmonella* food

poisoning results from the ingestion of foods containing a significant concentration of the bacteria *Salmonella (22)*. This organism is responsible for outbreaks that cause salmonellosis (food poisoning due to the consumption of food contaminated with *Salmonella)* which can lead to gastrointestinal infections and septicemia. These diseases cause significant morbidity and mortality.

Salmonella food poisoning symptoms typically develop in 8 to 72 hours and last 2 to 3 days. Symptoms include nausea, vomiting, abdominal pains, headache, chills, and diarrhea. Along with these symptoms, patients feel muscle weakness, faintness, moderate fever, drowsiness, and restlessness. Mortality rate depends on age and immune system stability, and averages at 4.1% *(22)*. Isolation of salmonellae from urine and blood in early stages of the infection, and from fecal matter in later stages (after onset of clinical symptoms), is needed to positively diagnose *Salmonella* as the causative agent *(28)*.

Reduction of the distinguishing, non-bloody diarrhea and abdominal pains occurs in about five days. If treatments are required, fluid and electrolyte replacement is conducted. Antibiotics lengthen the carrier state and therefore are not used *(28)*.

The primary reservoirs for *S. enterica* are poultry and eggs *(28)*. They are also commonly found in swine, cows, and mutton feces. Salmonellae are excreted in feces and easily transmitted by insects and other living organisms increasing the risk of food contamination. *Salmonella* cells are repeatedly found in human food and water supply. This is a result of their natural presence in the environment; intense farming practices can promote the spread of the organism among animals. The recycling of slaughterhouse by-products into animal feeds can also introduce the pathogen to livestock. The organism

can also be found in polluted water which can easily contaminate produce. Contaminated

water and food are then consumed and again shed in feces. The expansion of this cycle

through international shipment of contaminated products is largely responsible for the

worldwide distribution of salmonellosis. Production practices, produce washers,

handlers, distribution, and cross contamination also play a vital role in transmission of the

organism *(22, 28)*. Improper handling and preparation of foods in both homes and food

service businesses are major factors in outbreaks *(22)*.

The infectious dose (ID_{50}) depends on both the susceptibility of the individual and

the type of food contaminated. Newborns, infants, and immunocompromised individuals

are more susceptible to infections than healthy adults. The immune systems of these

individuals are not completely developed, are weak, or are delayed. The chemical

contents of food, such as high fat content, protects the infective agent from antimicrobials

or stomach acid thus reducing the dose necessary to cause illness *(28)*.

The isolation of *S. enterica* from fresh vegetables and fruits has been linked to

salmonellosis cases. Orange juice was first identified as a vehicle of transmission for

Salmonella in 1944 (though the first written documentation was in an "infectious sweet

cider" consumed in 1923) *(14, 36)*. A large outbreak of salmonellosis due to *S. enterica*

serovars, Hartford, Gaminara, and Rubislaw*,* was linked to contamination of orange juice

during the summer of 1995 at a theme park in Florida. These serovars were isolated from

patients, orange juice, and the processing environment. The Centers for Disease Control

and Prevention's (CDC) national *Salmonella* surveillance system records were reviewed

and an investigation was lead to determine the cause of the outbreak. Sixty-two patients

matched the case definition for *Salmonella* infections; the CDC indicates that 630 to 6300

cases may have occurred but were not identified. All patients drank orange juice in the theme park during their visit and reported diarrhea (3 or more lose stools in 24 hours), abdominal cramps, bloody stools, and vomiting that lasted an average of seven days. Ten out of twelve orange juice containers from the same company that supplied the park tested positive for *Salmonella* contamination *(36)*.

S. enterica has been the causative agent in many similar outbreaks due to the consumption of unpasteurized orange juice. Outbreaks in Ohio (1944) with 18 cases of illness and one death in New York with 67 cases (1989) from non-pasteurized orange juice are thought to have been caused by improper handling. The 1995 outbreak in Florida and another outbreak in 1999 (Canada/United States) are thought to have been caused by a contaminated processing environment and storage area. Amphibians were found in the facility (1995 outbreak) which may have been the source of *Salmonella* in both the processing and storage steps of juice production *(45)*.

After *Salmonella* is inoculated into juice there is a lag phase before cell death occurs. Organisms have a stress-response mechanism which allows them to survive the low pH (~3.8) of the juice and persist for many days *(45)*. *S. enterica* cells exposed to an acidic environment may develop an acid tolerance response (ATR). This allows for the survival of the organism under stressful acid conditions. The response involves the induction of 43 acid shock and outer membrane proteins, reduced growth rate, and pH homeostasis. Intake of only a few *Salmonella* cells may potentially be infective, so the survival of only a few cells in a product can be hazardous *(28)*.

Escherichia coli O157:H7

Escherichia coli are Gram negative, facultatively anaerobic, motile rods which produce acid and gas from lactose in 48 hours at 45.5 °C *(42)*. This organism has a minimum growth pH of 4.0 to 4.5, yet this is reliant on pH interaction with other factors such as the type of acid used (ex. organic acids) *(28)*. *E. coli* are pathogenically versatile and have the ability to cause diarrhea, dysentery, hemolytic uremic syndrome (HUS), bladder infections, septicemia, pneumonia, and meningitis.

Serological classification of *E. coli* is based on two types of surface structures; the lipopolysaccharide (LPS) antigen (O) and flagella antigen (H). The serogroup is identified by the O antigen and the serotype by the H antigen. Virotyping is characterized by patterns of attachment, toxin production, and invasiveness. There are at least six *E. coli* virotypes, including enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) *(42)*.

E. coli O157:H7 belongs to the virotype enterohemorrhagic *E. coli* (EHEC). In 1982, *E. coli* O157:H7 was identified as a human pathogen that causes bloody diarrhea and hemolytic uremic syndrome (HUS) *(19, 28, 49)*. This virotype has the ability to attach to and colonize the intestinal cells of cattle and other farm animals such as sheep, mutton, and swine *(19)*.

EHEC strains have Stx toxins, endotoxins, host derived cytokines (TNF-a), and intereukin-1β *(22)*. EHEC strains produce two types of Stx toxins, Stx1 and Stx2. Stx1 is very similar to the Shiga toxin (Stx) produced by *Shigella* species. Stx2 is related to Stx1 but contains differing antigens. Stx2 is the toxin more commonly associated with

the EHEC strains, such as *E. coli* O157:H7, that cause HUS. The s*tx* genes greatly increase virulence by inhibiting protein synthesis in endothelial cells *(22)*. Receptors for this toxin are present on kidney and intestinal cells, which may be the reason acute kidney failure and hemorrhages are typically associated with this bacterium. The s*tx* genes are located on a bacteriophage which gives the microorganism a great amount of versatility *(42)*.

Cattle are the primary reservoir for *E. coli* O157:H7. Environmental factors (feed and water source), farm management practices, and manure handling influence this pathogen's presence on farms. Fecal shedding of *E. coli* O157:H7 is a common means of water and produce contamination. *E. coli* O157:H7 is not pathogenic to weaned calves and adult cattle even though they carry the pathogen and shed it in feces. This makes infected livestock difficult to identify and control. The organism is commonly found in water troughs and animal feces where it can survive for weeks or months *(28)*. The long survival time makes transmission to other animals and contamination of produce through water and fecal matter more likely. This causes a food safety concern with produce grown near farms.

E. coli O157:H7 infections climax during the warmer months in the year (May through October). The infectious dose of this virotype is very low; fewer than 100 cells are needed to cause illness. Symptoms include non-bloody, bloody diarrhea, and abdominal cramps. *E. coli* O157:H7 also causes complications such as kidney failure in children and adults due to both HUS and thrombotic thrombocytopenic purpura (TTP) respectively *(28)*. After ingestion there is a 3 to 4 day incubation period which is followed by non-bloody diarrhea and severe abdominal cramps for 1 to 2 days. The

patient then gets bloody diarrhea that may last between 4 and 10 days. Symptoms last about a week but the illness of some individuals may develop into HUS. HUS is common among children while TTP is predominately found in adults. Similar to *S. enterica* the young, elderly, and immunocompromised are most susceptible to *E. coli* O157:H7 infections *(28)*. With infections such as this, the only treatment is to compensate for kidney failure (due to HUS and TTP), if needed, through dialysis *(42)*.

E. coli O157:H7 infection is typically self-limiting. Antibiotics are not used to treat infections. Even though antibiotics have been shown to reduce symptoms for other *E. coli* diseases (ETEC and EPEC), they have been shown to make EHEC diseases worse. As a side effect, the antimicrobial fluoroquinolones cause DNA damage. This damage causes stress which then stimulates the SOS response. The SOS response induces high levels of expression of the phages and the *stx* genes carried on them. Lytic growth of these phages and genes cause production of high amounts of Shiga toxin Stx *(42)*. In summary, antibiotics such as fluoroquinolones may cause proliferation of the phages and the *stx* genes they carry.

It is difficult to rid food of contamination therefore it is best to prevent foods from becoming contaminated; especially products that are consumed raw. Monitoring can be expensive, but rapid new tests (enzyme-linked immunosorbent and chip tests) may help with this problem. Also, thoroughly cooking and pasteurizing food products will kill the organism before consumption *(42)*.

E. coli O157:H7 outbreaks have been associated with apple cider and apple juice. There was an outbreak of hemorrhagic colitis and hemolytic syndrome in 1991 and two more in 1996 *(36)*. One of the outbreaks in 1996 was large consisting of multiple states.

It was linked to contaminated apple cider. This outbreak involved production of unpasteurized apple juice and apple juice blends from a large processing facility in California. This company was the largest juice processing facility in the United States, equipped with modern amenities and a wide-spread delivery system. Fresh, unpasteurized juices were distributed throughout the Western U.S and into Canada. The death of a 16-month-old Colorado child resulted from this outbreak *(36)*. Apple juice associated outbreaks have been traced back to the use of contaminated apples. Dropped apples are easily contaminated by manure, livestock feces, and/or from the soil. The 1991 outbreak investigation showed that the processor also raised cattle in a nearby field, which may have been the cause of the contamination *(28)*.

Recent outbreaks of *E. coli* O157:H7 have been associated with consumption of other acidified foods such as mayonnaise, yogurts, and mustards which were initially considered to be an abnormal occurrence. However, studies have confirmed that *E. coli* is tolerant to some organic acids and can survive in acidified refrigerated foods *(19)*.

Many *E. coli* O157:H7 strains are tolerant of low pH environments. The pathogen has survived in apple cider (pH 3.6 to 4.2) for 7 to 21 days or 10 to 31 days at 20° C and 8° C, respectively *(28)*. The acid-induced oxidative system, acid-induced arginine-dependent system, and glutamate-dependent system are believed to be responsible for *E. coli* O157:H7 acid tolerance *(28)*. The arginine-dependent and glutamate-dependent systems are better at protecting the organism from acid than the oxidative system *(28)*. The acid-tolerant state can continue for an extended time period (≥ 28 days) at refrigeration temperatures *(28)*. Acid tolerance is also known to increase

the organism's tolerance to other environmental stresses (heat, radiation, and antimicrobials) *(28)*.

<u>*trans*-Cinnamic Acid (3-Pheynl-2-propenoic acid)</u>

Cinnamic acid (pK$_a$ of 4.37 to 4.44) is a phenolic compound found naturally in many spices (cinnamon and cloves), cranberries, and prunes, and provides a natural protection against pathogenic organisms. At low concentrations (31 mg/L) *(3)* cinnamic acid has been reported to have a flowery or cinnamon type flavor *(3, 8, 17)*. Storage conditions such as temperature, light, and pH should be controlled and kept constant for cinnamic acid to ensure the least amount of variability *(15)*. Cinnamic acid and related compounds have demonstrated antimicrobial and antifungal activities at *(3, 10, 15, 16)*.

At a pH of 1.6, cinnamic acid (pK$_a$ of 4.37 to 4.44) is mostly protonated, and at a pH of 8.2 it is dissociated. It is more favorable for cinnamic acid to form complexes in its protonated form rather than when dissociated *(53)*. The pH also affects the solubility of flavonoids, such as cinnamic acid. In an alkaline environment (higher than its pK$_a$), cinnamic acid is more soluble because it is dissociated. It has also been shown that natural phenolic compounds have greater antimicrobial activity at a lower pH (below 4.5) when they are undissociated acid molecules *(3, 51)*. Juices with low pH will favor both antimicrobial activity of cinnamic acid and complexation.

A Japanese patent states that cinnamic acid, in combination with other organic acids such as citric acid found in orange juice and malic acid in apple juice, have a greater bactericidal effect *(3)*. Cinnamic acid and other organic acids are used to preserve diakon (Japanese pickles), kamaboko (fish paste product), Vienna sausages, mixed bean

paste, and flour paste. According to Anslow and Stratford, 100 mg/L of cinnamic acid in a solution of pH 3 is effective at killing spoilage yeasts and molds. But the same amount of cinnamic acid has no antimicrobial activity in a solution of neutral pH; this illustrates the requirement of a low pH for cinnamic acid to be effective. The organic acids contribute to cinnamic acid antimicrobial effects by entering cells and increasing the H^+ concentration before the weak acid transport is able to finish. This transport is the movement of weak acid into the cells from the surrounding medium. This causes the internal and external pH of the cell to equalize which stops flow of weak acid into cell. The organic acid enables the internal pH to be reduced; this causes cellular enzymes to no longer function. As expected, cinnamic acid has a stronger effect when the cell is already weakened *(3)*.

Typically, weak acids in the undissociated form dissolve through microorganism membranes and dissociate in the cell which upsets the pH balance by causing pH to decrease. This lower pH negatively affects the enzymes, causing the cell to die *(3)*. It has been shown that cinnamic acid has the ability to uncouple the energy transducing membrane and stimulate non-specific membrane permeability, which allows influx of protons across the plasma membrane *(8)*. Noting that cinnamic acid is a phenol helps to characterize its mechanism for killing microorganisms. Phenols have been shown to change membrane permeability and to interfere with enzyme functions and metabolic pathways associated with energy production *(50)*.

In 1965, cinnamic acid was given Generally Recognized As Safe (GRAS) status for the Flavoring Extract Manufacturers Association (FEMA) as a flavoring agent in food by the Food and Drug Administration (FDA). This means the FDA deems cinnamic acid

12

safe under conditions of its intended use in beverages and as a food additive for flavor enhancement. Currently, there is no defined legal limit (acceptable daily intake (ADI) *(11)* is 'Acceptable') for cinnamic acid use but common flavor usage does not currently exceed 31 mg/L. Cinnamic acid has a cinnamon flavor, preservation capabilities, and is a natural compound. These characteristics make it an ideal candidate for a natural antimicrobial in juices *(3)*.

One dilemma is that *trans*-cinnamic acid is a lipophilic weak acid and is only slightly soluble in aqueous solutions and is has been shown to be largely insoluble in low pH tea solutions *(3, 12)*. The low pH of juice may influence the solubility of cinnamic acid. However, the chemical and physical properties of this acid, such as size, are ideal for complex formation *(12)*. The inventors of patent 6,042,861 state that spray drying cinnamic acid into a carrier powder (e.g. cyclodextrins) may help to increase solubility *(3)*.

Thus, this acid, which has been shown to have significant antimicrobial effects, could significantly reduce the amount of heat treatment required for juices. Cinnamic acid has already shown to have inhibitory growth effects on both pathogenic and spoilage microorganisms. Use of an antimicrobial would eliminate the need of high or extended heat treatment. The addition of cinnamic acid as a preservative would naturally, without heat treatment, improve the overall quality of the juice products while also ensuring microbial safety. Cinnamic acid is an ideal source of natural protection. All that needs to be done is to enhance the acid's solubility; this would make it very applicable for industry use.

<u>Cyclodextrins</u>

Cyclodextrins are non-reducing, cyclic oligosaccharides derived by enzymatic (cyclodextrinase) degradation of starch by *Bacillus macerans (6, 41)*. These molecules are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose *(25)*. Cyclodextrins come in various sizes, two of which are α-cyclodextrin and β-cyclodextrin, which have 6 and 7 units of cyclohexaamylose, respectively. Cyclodextrins with less than 6 carbons do not exist, as this causes excessive strain on the ring *(4)*. The melting points of these two fall within the range of 240 – 265 °C *(25)*.

Alpha-cyclodextrin, the smallest, has a molecular weight of 972 g, intrinsic solubility of 14.5 g/100 mL, and cavity diameter of 4.7 – 5.2 Å. Beta-cyclodextrin has a molecular weight of 1135 g, water solubility of 1.85 g/100 mL, and cavity diameter of 6.0 – 6.4 Å. Cyclodextrins have been shown to exhibit physiochemical advantages to a guest molecule. Such improvements include increased solubility, stability, and masking of unwanted tastes and smells. The hydrophobic microenvironment of cyclodextrins is what enables the molecule to house a guest, this leads to improved characteristics for the guest.

Their hydrophilic exterior and hydrophobic interior enable the cyclodextrins to host a variety of smaller hydrophobic molecules through non-covalent bonds. The cyclodextrins are soluble in water because hydroxyls are located on the rim of the cone. The interior of the cavity is lined with C(3)-H, C(5)-H hydrogens and ether-like oxygens O(4), which gives it its hydrophobic nature. No real specifications, such as subsistent position, size, charge, etc., have been made to the identity of the guest. The only

requirement seems to be that the guest either fit completely or partially within the cavity of the host *(41)*.

Alpha-cyclodextrin was listed as GRAS in June 2004 for use as a fiber supplement, flavor adjuvant, to improve mouth feel, and as a carrier or stabilizer for vitamins, fatty acids, and colors. It is intended for use in solid, semi-solid, and liquid foods and has the nutritional value of fermentable dietary fiber. It is ideal for liquids because it is stable under food processing conditions and has a low viscosity in aqueous solutions *(34)*.

Alpha-cyclodextrin is fermented by intestinal microflora, mainly in the caecum, and is not digested in the gastrointestinal tract. Alpha-cyclodextrin studies on mice, rats, and rabbits show that with a diet (concentration 20%) there were not any teratogenic effects and the assays for genotoxicity were negative. Long-term studies and human studies for alpha-cyclodextrin were not conducted. However, its toxicity compared to beta-cyclodextrin is lower (tolerance studies have been done) and the fermentation manner is similar to beta-cyclodextrin. This supports the hypothesis that it is fermented (demonstrated by laboratory animals) to metabolites before absorption by humans. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) assessed alpha-cyclodextrin in June 2001 and determined that there was ample information to allocate an acceptable daily intake (ADI) of "not specified" *(39)*.

Beta-cyclodextrin is the more limited in solubility than alpha-cyclodextrin and typically tends to yield type B solubility curves (see Complex and Complex Formation) while alpha-cyclodextrinn typically has shown to yield a type A curve. β-cyclodextrin has a low water solubility because it forms hydrogen bonds with secondary hydroxyl groups,

distracting it from bonding with water molecules. Substitution of the hydroxyl groups will increase water solubility *(25)*.

Beta-cyclodextrin was listed as GRAS in March 2001 for use as a flavor carrier *(43)*. They are inexpensive, so they will not inflate the price of the product to which they are being added *(48)*. Beta-cyclodextrin, similar to alpha-cyclodextrin, is utilized by intestinal microflora and is not hydrolyzed or absorbed by the upper intestinal tract *(54)*. JECFA studied the No Observed Adverse Effect Level (NOAEL) in a 1-year toxicity study with dogs; the diet consisted of 1.25% beta-cyclodextrin (equal to 470 mg/kg body weight/day). This data permitted JECFA to allocate an acceptable daily intake (ADI) of 0 to 5 mg/kg body weight/day for beta-cyclodextrin, which is equal to 300 mg/person/day for a 60 kg person *(43)*. This ADI was determined by reviewing a number of acute and short-term toxicity studies. These studies indicated a low toxicity of beta-cyclodextrin by the oral route; this is why an ADI was declared *(54)*.

Complex Properties, Complex Formation, and Complex Detection

Inclusion complexes between a cyclodextrin and a guest molecule occur when a hydrophobic guest interacts with the non-polar cavity of the cyclodextrin. Solubility data shows that complex formation is thermodynamically favored, due to the release of water from the cyclodextrin cavity, in an aqueous solution. Therefore, majority of complex formation occurs in water, as the water is the driving power behind complexation. The release of the enthalpy rich water from the cyclodextrin cavity causes formation of a complex consisting of a non-polar guest molecule and a cyclodextrin. It is also thought that α-cyclodextrin complexation releases ring strain. The cyclic structure of a hydrated

16

α-cyclodextrin is disturbed due to the internal hydrogen bonding of the water molecules *(25)*.

The guest molecules are weakly bound; no covalent bonds are formed or broken within the cyclodextrin. Thus, the guest molecule is not significantly altered in structure or function when encapsulated *(6)*. However, complexation may cause a small shift of the UV absorption maximum which affects the molar absorption coefficient (ε). A shift in the molar absorption coefficient will provide an incorrect absorbance; consequently using this measured absorbance to calculate other unknowns (e.g. concentration) yields inaccurate values. The complex should be dissolved in ethanol to separate the guest from the cyclodextrin; this is known as dissociation *(47)*. When the complex is dissociated, calculated guest content is more accurate.

There are a few factors that seem to affect solubility and complexation. Since the rate of molecular encapsulation is typically not high, addition of excess guest has shown to improve complexation. Also, un-ionized molecules are better guests since they typically form a more stable inclusion complex *(51)*. Hydroxy acids, such as citric and malic, not only help to keep cinnamic acid un-ionized (which may stabilize the inclusion complex) but also contribute to the antimicrobial effects of cinnamic acid as stated above *(25)*. Concentrations of complex and host, temperature, and pH have been shown to affect complexation.

Nuclear magnetic resonance (NMR) studies have shown that slight changes with guest and host occur when complex formation occurs. Protons (H-3 and H-5) within the cyclodextrin cavity shift to a higher field when complexed with cinnamic acid. These chemical shifts suggest that the phenol is most commonly within the cavity of the

cyclodextrin (See Figure 1) *(53)*. The pK_a's of compounds change in the presence of cyclodextrins. The pK_a's of acids are larger (or equal) when complexed compared to those in the absence of a cyclodextrin. Phenols have a decreased (or equal) pK_a in the presence of cyclodextrins. The pK_a of cinnamic acid increases ($\Delta pK_a = + 1.37$), which is characteristic of acids, in the presence if α-cyclodextrin *(6)*.

As briefly mentioned in the previous section, complexation has been shown to exhibit physiochemical enhancements to the guest molecule. The most noted modification for this study is the enhancement of solubility. This characteristic is gained because the non-polar guest is embedded within the hydrophobic interior cavity, the exterior of the complex is hydrophilic which allows the entire complex to become readily soluble *(25)*.

Phase solubility diagrams are constructed to determine if addition of a soluble compound, ligand (L), increases the solubility of an insoluble or slightly soluble compound, substrate (S). The slightly soluble (S) compound is added in excess to the various sample vials containing a constant volume of solvent. Then, increasing concentrations of the complexing agent (cyclodextrin in this case) is added to each vial. Analysis of the solutions at different phases for total concentration of S typically yields one of the two types of diagrams, A or B *(21)*.

The diagrams plot total concentration of S found in solution on the vertical axis and the concentration of L on the horizontal axis. Type A diagrams represent solutions where soluble complexes were formed. In type A diagrams, the total amount of the insoluble complex (S) rises above its intrinsic solubility. There are three type A phase solubility curves. A linear curve is represented by A_L, a positive curve by A_P, and a

negative curve by A_N (See Figure 2). Type B diagrams (Figure 2) correspond to insoluble complexes or complexes where substrate solubility increases and then hits a maximum, plateaus, and then drops *(21)*.

Cinnamic acid and alpha-cyclodextrin have been shown to have a 1:1 stoichiometry. On the contrary, cinnamic acid and beta-cyclodextrin have been shown to have a 2:1 molar ratio; this may be due to the larger cavity of beta cyclodextrin *(12)*.

Behavior of complexes can be studied using various techniques. Some common practices include spectroscopic methods such as circular dichroism, UV absorbance, and nuclear magnetic resonance (NMR) *(21)*. The UV spectrum for cinnamic acid differs when complexed with α-cyclodextrin (due to an increase in ε; see explanation above) *(12)*. Formation of inclusion complexes might also be detected by titration due to the pK_a modification *(6)*.

Apple Cider and Orange Juice

Orange juice is believed to be the most popular fruit drink in North America. In 1995, 60% of all juice consumed was orange juice. This is estimated to be 20.7 L of orange juice per person annually *(14, 24)*. It is important for popular products such as this to be safe for consumers.

Weak acids such as citric and malic acid have a slight antimicrobial effect when present in either high concentrations (>3000 mg/L) or when they are dissociated. They help to lower the pH in juices. The weak acids inhibit microbial organisms by acting as acidulants as well as acting in a synergistic relationship with cinnamic acid *(3)*. Until recently, the low pH (high acid) in apple and orange juice allowed them to be unlikely

vehicles for organisms that cause foodborne illness. Consequently, this lead processors to believe a high heat treatment or preservation step was unnecessary. These beliefs resulted in improperly treated juice and pathogen outbreaks.

Acid tolerance responses (ATR) are being recognized as a characteristic amongst many pathogens. This response is triggered under sublethal pH conditions and leads to the expression of acid shock proteins. The ATR system not only protects the bacterial cells from high acid content but may also protect against stresses (heat, osmotic stress, and nisin activity) and may help increase pathogen virulence and survival *(36)*. The major factor that has lead to numerous outbreaks associated with juice is the lack of proper treatment to kill or inactivate bacterial pathogens.

Thermal treatment increases shelf life, but also degrades the organoleptic and nutritional attributes and may produce disagreeable off-flavors. Pasteurization is generally done at 90 to 95° C for 15 to 60 seconds for orange juice and 77 to 88° C for 25 to 30 seconds for apple juice *(23, 24)*. Sensory studies have shown that panelists can identify heat treated juice, as it has a significantly different flavor than fresh and frozen juices *(37)*. The public's preference for fresh tasting, unpasteurized juice has lead processors to seek alternatives to heat treatments.

A few techniques have been studied for nonthermal pasteurization. These include isostatic high pressure, pulsed light, pulsed electric fields, irradiation, and filtration. Pulsed electric field (PEF) treatment has been shown to reduce the cell count in juices. It is dependent on temperature and number of pulses. Increasing the temperature and pulse number raises cell death. PEF treatment, however, does not require temperature as high as pasteurization. The combination of antimicrobials and PEF treatment yields increased

20

bactericidal effects *(24)*. Irradiation is accepted in many countries as a method to eliminate food pathogens. Pathogenic organisms are generally sensitive to irradiation and this technique preserves the raw characteristics of the food. This practice is not extensively used due to consumer reservations of irradiation *(28)*. Some of these methods have not been thoroughly studied and are not currently being commercially used or are not accepted (e.g. irradiation) by the majority of consumers *(36)*.

The Florida Department of Citrus has rules to control the oranges used in the production of fresh-squeezed, non-pasteurized juice. Oranges picked from the ground are banned, fruits must be rinsed with hypochlorite or equivalent bactericide, the juice processing area must be completely enclosed, and nonpasteurized juice must be routinely checked for microbial growth. Along with these practices, documented quality control, good manufacturing practices, or a Hazard Analysis Critical Control point (HACCP) program must be implemented *(14)*.

Typically apple juice has a pH less than 4, which means that it is a high acid product and bacteria growth is not normally a concern. But problems have still been encountered with pathogen survival. Along with a HACCP program, the FDA requires that a 5-log pathogen reduction must be achieved for the "pertinent microorganism" in the juice. According to the FDA a pertinent pathogen is the most resistant illness causing microorganism that has the ability to survive the specific treatment; some examples are *E. coli* O157:H7, *Salmonella* species, and *Listeria monocytogenes*. This reduction must occur in the processing facility and immediately prior to packaging, and must occur directly in the juice product (except for citrus juices) *(18)*.

Pathogenic bacteria can be introduced into juice products during growing (through organic fertilizers), picking, processing, and packaging. This causes contamination and dangerous bacteria are allowed to subsist, which can lead to both health and economic problems *(49)*. Results of previous studies show that oranges with contaminated peels yielded contaminated juices. This may seem unlikely because the peel never comes in contact with the juice (unlike apple juice), but the outside contaminates the surrounding equipment, utensils, area, and people *(26)*. It has been shown that pathogens have the capability to contaminate, internalize, survive, and grow in intact fruits *(18)*. The survival of organisms within fruit leads to production of contaminated juices.

Recent studies show that both *Salmonella* and *E. coli* O157:H7 counts increased within orange fruits when the culture was introduced to fruit wounds *(18)*. Fruits can be damaged in many ways. Harvesting, washing, and packing are easy ways for the fruits to be damaged during processing. Although these damages may be determined visually, small wounds caused by insects, thorns, hail, and falling to the ground might not be as noticeable. These small punctures and splits can allow for pathogens and spoilage bacteria to enter into the fruits *(2)*. Internalization of pathogens protects the bacteria from the damage of external sanitation methods *(45)*.

In the hope to eliminate pathogens from juice, it has been suggested to make juice pasteurization mandatory, have stringent HACCP plans, better labeling of products, and educate the public on the dangers of unpasteurized juices. Although these steps may solve most outbreak problems, the sensory quality of the juices will be compromised if pasteurization is mandatory. The processors who have been making juice for years

22

without any problems will be hesitant to comply with rules that may alter their juice characteristics which may lead to reduced sells. Also, just because products are pasteurized does not mean they are completely safe. Improper pasteurization or post-pasteurization contamination may lead to contaminated juices *(36)*. As mentioned above juices are a popular beverage; their nutrient value, safety, and taste must be preserved.

MATERIALS and METHODS

Cinnamic Acid Water Standard Curve (for determining intrinsic solubility)

A 1000 mg/L stock solution of cinnamic acid in water was prepared by dissolving 0.25 g *trans*-cinnamic acid 98+% (Acros Organics, New Jersey, USA) into 50%, 200 proof, absolute ethyl alcohol (Acros, New Jersey, USA). This mixture was stirred at a medium speed for 15 minutes on a stir plate to ensure all cinnamic acid was dissolved. An appropriate amount of stock solution was added to the desired amount of water (Table 1) to reach preferred concentrations. Concentrations were made in triplicate.

Analyses of all concentrations were performed in quartz cuvettes (Fisherbrand, Loughborough, Leicestershire) using a Shimadzu UV-2101PC UV-Vis Scanning spectrophotometer (Shimadzu, Columbia, MD) at 272 nm. The absorbencies of three samples at each concentration were averaged. The average absorbance was used to construct the standard curve. Concentration vs. absorbance were plotted and both the equation of the line ($y = mx + b$) and the R^2 were calculated.

Ethanol Standard Curve (for quantification of cinnamic acid in inclusion complexes)

The procedure for preparing the ethanol standard curve was similar to the water standard curve. A 100 mg/L stock solution of *trans*-cinnamic acid in ethanol was prepared by dissolving 0.025 g cinnamic acid into 99.5% ethyl alcohol. The cinnamic acid and ethyl alcohol were stirred at a medium speed for 15 minutes on a stir plate until all cinnamic acid was dissolved. Using Table 2 the desired concentrations were made in triplicate.

Analyses were performed in quartz cuvettes using a spectrophotometer at 269.5 nm. The absorbencies of three samples at each concentration were averaged to construct the standard curve. Concentration vs. absorbance were plotted and both the equation of the line (y = mx + b) and the R^2 were calculated.

Cinnamic Acid Intrinsic Solubility (IS)

To determine intrinsic solubility (IS), cinnamic acid (0.05 g) was added in excess of its literature IS (~0.4 g/L) *(12)* to three vials containing 10 mL distilled water. The mixtures were shaken in an Innova 4230 refrigerated electronic shaker (New Brunswick Scientific, Edison, NJ) for 72 hours at 250 rpm at 25° C with the shaker window covered with aluminum foil for protection against light. The absorbance, using a wavelength of 269.5 nm, was read at 24, 48, and 72 hours.

At each time (24, 48 and 72 hours) the mixtures were removed from the refrigerated electronic shaker and filtered using sterile, latex free, 5 mL disposable syringes (Becton Dickinson & Co., Franklin Lakes, NJ) and two sterile polypropylene, sizes 0.45 and 0.22 μm, disposable filters tips (Whatman, Schieicher & Schuell, Florham

Park, NJ) to obtain a clear filtrate. The solution was then diluted once in 50% ethanol

(1:10) and then again in 99.5% ethanol to obtain a 1:100 dilution. The absorbance was

read using a spectrophotometer at a wavelength of 269.5 nm and using quartz cuvettes.

The IS was determined using the cinnamic acid ethanol standard curve.

α-cyclodextrin Phase Solubility Diagram

A phase solubility diagram was constructed by dissolving increasing amounts of

α-cyclodextrin (Wacker Fine Chemicals, Adrian, Michigan) (0, 0.1, 0.2, 0.25, 0.3, 0.4,

0.5, 0.6, 0.7, 0.75, 1.0, 1.25, and 1.45 g) in10 mL distilled. Vials were placed in an

electronic shaker (25° C at 250 rpm, light protected with foil) to dissolve the

cyclodextrin. After the carbohydrate was dissolved (24 hrs), cinnamic acid in excess of

its intrinsic solubility (0.05 g/10 ml) was added to the vials containing the dissolved α-

cyclodextrin, vortexed, and placed in the electronic shaker (25° C, 250 rpm, protected

from light, for 48 hours) *(21)*. After 48 hours shaking, the samples (protected from light)

were left undisturbed until all solids settled to the bottom. The samples were filtered (5

mL syringe and piggybacked 0.45 and 0.22 μm filter tips), diluted first using 50%

ethanol, and then placed into the electronic shaker (25° C, 250 rpm, protected from light,

for 30 minutes). After shaking, samples were again diluted, in 99.5% ethanol to obtain a

dilution that yielded an absorbance between 0.1 and 2.0 at a wavelength of 269.5 nm

(47). A 1:100 dilution was used for 0 g/10mL and 0.7 g/10mL through 1.45 g/10mL and

a 1:1000 dilution was used and for 0.1 g/10mL through 0.6 g/10mL. The dilutions were

analyzed on the spectrophotometer at 269.5 nm using quartz cuvettes. Cinnamic acid

concentrations were determined using the ethanol standard curve equation. A phase

solubility diagram was made with cinnamic acid concentrations on the y axis and increasing α-cyclodextrin cyclodextrin concentrations on the x axis.

The stability constant (also known as equilibrium or binding constant and denoted as $K_{1:1}$) was calculated using the linear portion of the phase solubility diagram. The complex was assumed to be a 1:1 inclusion complex. The equation in Figure 3 was used to determine an estimated stability constant for α-cyclodextrin/cinnamic acid inclusion complex.

β-cyclodextrin Phase Solubility Diagram

The same procedure used to generate the α-cyclodextrin phase solubility diagram was used for generating β-cyclodextrin phase solubility diagram. Increasing concentrations of β-cyclodextrin were dissolved (Wacker Fine Chemicals, Adrian, Michigan) (0, 0.03, 0.06, 0.09, 0.12, 0.15, and 0.185 g) in 10 mL of distilled water. The electronic shaker (25° C at 250 rpm, light protected) was used to shake and dissolve the cyclodextrin. After 24 hours cinnamic acid, in excess of its intrinsic solubility (0.05 g/10 ml), was added to the vials containing the dissolved beta-cyclodextrin, the mixture was vortexed, and placed back into the electronic shaker (25° C, 250 rpm, protected from light, for 48 hours) *(21)*. After 48 hours, the light protected vials were left undisturbed until the solids settled to the bottom. The complex was filtered using a 5 mL syringe and piggybacked 0.45 and 0.22 μm filter tips. All concentration were diluted (1:10) using 50% ethanol, and then placed into the electronic shaker (25°C, 250 rpm, protected from light, for 30 minutes). After 30 minute shaking, the samples were diluted to yield a 1:100 dilution, this time using 99.5% ethanol. A 1:100 dilution for this specific inclusion

complex yielded an absorbance between 0.1 and 2.0 *(47)*. The spectrophotometer at 269.5 nm (quartz cuvettes) was used to determine the absorbance at each β-cyclodextrin concentration. The cinnamic acid concentrations were determined using the equation generated from the ethanol standard curve. A phase solubility diagram was made with cinnamic acid concentrations on the y axis and increasing β-cyclodextrin cyclodextrin concentrations on the x axis.

Stability constant for β-cyclodextrin/cinnamic acid conclusion complex was also calculated using the equation seen in Figure 3. It was assumed that the inclusion complex was a 1:1complex.

Inclusion Complex Formation

An inclusion complex containing α-cyclodextrin and cinnamic acid was prepared by adding 0.4 g/L α-cyclodextrin (predetermined with phase solubility curve) and allowing the cyclodextrin to dissolve in the electronic shaker (25° C, 250 rpm, protected from light, for 24 hours). Cinnamic acid in excess of its intrinsic solubility (5 g/L) was then added to the dissolved cyclodextrin solution and shook (25° C, 250 rpm, protected from light, for 48 hours).

The mixture was filtered using sterile vacuum 500 mL filter devices (Nalgene, Rochester, New York). 0.45 μm and 0.22 μm disk filters (Whatman International Ltd., Maidstone, England) were both used within the Nalgene containers. The filtered solution was poured into sterile Pyrex glass pans (1 L per pan) and covered tightly with plastic wrap. The pans were then placed in a freezer (-20° C) and left undisturbed until frozen (overnight). When frozen, the mixtures were removed from the freezer. The top of

plastic wrap covering was punctured many times to allow moisture transfer. The mixtures were placed in a freeze-drier for approximately 72 hours (more time required for larger quantities of complex). Once removed from the freeze drier the dry samples were removed from the Pyrex glass pan and placed into a sterile stomacher bag. The powder was compressed while in the bag by stomaching the complex. Dried samples were stored in labeled, sterile, amber, glass bottles. The lids were either wrapped with parafilm or placed in a desiccator to prevent moisture uptake.

Quantification of Cinnamic Acid in Complex

0.1 g of the dried complex (powder form) was dissolved in 9.9 mL 50% ethanol. 0.1 mL of dissolved complex solution was then diluted in 9.9 mL 50% ethyl alcohol and placed into the shaker (25° C, 250 rpm, protected from light, for 30 minutes). The complex was then diluted again, 1 mL into 9 mL 99.5% ethanol to obtain an absorbance between 0.1 and 2.0. The dilution was analyzed on the spectrophotometer at 269.5 nm using quartz cuvettes. Cinnamic acid concentration was determined using the ethanol standard curve.

The percent weight of cinnamic acid present in the complex was calculated by dividing the calculated concentration of cinnamic acid (g/10mL) found in the complex by the starting weight of the complex added (g/10mL) and multiplying by 100 to obtain a percent value. The molar ratio was determined by dividing moles of α-cyclodextrin present in the complex by moles of cinnamic acid present. Moles of α-cyclodextrin were calculated by subtracting the calculated concentration of cinnamic acid from the initial amount of complex added; this number was then converted to moles.

Stock Cultures and Initial Identification

Escherichia coli O157:H7 isolated from apple cider, was used in this study. *S. enterica (*serovars: Baildon, Agona, Michigan, and Gaminara) were also utilized in this study. *S.* Baildon was isolated from lettuce and tomatoes, *S.* Agona from alfalfa sprouts, *S.* Michigan from cantaloupe, and *S.* Gaminara from orange juice. A single strain of *E. coli* O157:H7 was used alone in apple cider and a cocktail of the *Salmonella* strains were used in the orange juice. All frozen cultures were obtained from storage (-80° C in 50% glycerol) at Virginia Polytechnic Institute and State University.

Cultures of each strain were grown separately in 9 mL Tryptic Soy Broth (TSB) (Difco^{TM}, Sparks, MD) and transferred at 24 hour intervals three successive times. Cultures were then streaked onto differential media for isolation, Levine Eosin Methylene Blue Agar (EMB) (Becton Dickinson and Co., Sparks, MD) for *E. coli* and Hektoen Enteric Agar (HE) (Difco^{TM}, Sparks, MD) for *Salmonella*. One colony from each plate was tested using API 20E (bioMérieux, Marcy l'Etoile, France) biochemical tests. Once pure cultures were confirmed, the organisms were streaked onto Tryptic Soy Agar (TSA) (Difco^{TM}, Sparks, MD) slants and stored for two weeks at 4° C. The organisms were transferred onto new slants every two weeks to ensure healthy cultures.

Visual Testing of α-cyclodextrin/cinnamic acid Complex in Juices

Twelve, 10 mL samples of both apple cider and orange juice were aseptically put into clear, glass, screw cap vials. Six vials of each sample were held overnight at 25.6 ± 0.42° C and 4° C to be tempered. After the samples reached appropriate temperature, varying concentrations of complexed cinnamic acid (0 mg/L, 250 mg/L, 500 mg/L, 1000

mg/L, 2000 mg/L, and 3000 mg/L) were added to the vials. Immediately following the addition of the inclusion complex the samples were vortexed for 2 minutes; visual descriptions and pH were recorded. The samples were stored at appropriate temperature (at $25.6 \pm 0.42°$ C and $4°$ C) and visually observed every 24 hours for 7 days.

Agar Diffusion Method

The minimum inhibitory concentration (MIC) of the cinnamic acid/cyclodextrin complex was determined by using the agar diffusion method. Molten Brain Heart Infusion Agar (BHIA) (Becton, Dickinson, and Co., Sparks, MD) (19.8 mL) was dispensed into 20 tubes and allowed to cool overnight in an incubator tempered at $45°$ C. The BHIA was stored at $45°$ C until inoculated with target organism. The cooled agar (19.8 mL) was inoculated with 0.2 mL of the culture (approximately 10^5 to 10^6 CFU/mL) and mixed to equally distribute the organism. Inoculated BHIA was poured into labeled plates and allowed to solidify (approximately 10 minutes). The plates were utilized immediately. A series of complexed cinnamic acid concentrations (0 mg/L, 60.4 mg/L, 120.9 mg/L, 241.8 mg/L, 483.4 mg/L, 966.8 mg/L, 1933.6 mg/L, and 3867.3 mg/L) were made from the cinnamic acid/cyclodextrin complex stock solution. 50 μL of the varying complex concentrations were added to wells cut out of the agar using a flame sterilized #3 cork borer (6.8 mm) *(40)*. There were three wells per BHIA plate; two plates per concentration.   Control plates (2) without antimicrobial addition to wells, were made to ensure ample growth of the microorganism. Two plates containing only BHIA were incubated to ensure sterile media. Plate surfaces were allowed to dry and the plates were incubated for 18 hours at $35°$ C.

After incubation, the zone of growth inhibition was measured from the edge of the well to the edge of growth a plastic millimeter ruler. The experiment was done in duplicate and six zones were measured for each complex concentration *(40)*.

Organism Preparation for Juice Assay

A loopful of *E. coli* O157:H7 from the stored TSA slant was inoculated into 9 mL TSB and incubated at 35° C for 24 hours. The *E. coli* O157:H7 culture underwent two successive 24 hour transfer before it was used in this experiment; the first transfer was 1 mL culture into 9 mL TSB, incubated 35° C for 24 hours. A loopful of *E. coli* O157:H7 from the first transfer was inoculated into 12 mL Brain Heart Infusion Broth (BHIB) (Difco^{TM}, Sparks, MD) and incubated at 35° C for 20 hours. The culture was then centrifuged (5000 x g for 5 minutes at 25.6 ± 0.42° C), the supernatant was decanted. The pellet was washed once with 10 mL of sterile 0.85 % saline solution, centrifuged again, and the supernatant decanted. The pellet was re-suspended in 12 mL BHIB; the cell concentration was approximately 10^9 CFU/mL. This culture was then used to inoculate apple cider; 2.5 mL culture into 247.5 mL cider.

From the *E. coli* O157:H7 inoculum produced; serial dilutions (using 9.9 mL 1% peptone blanks) were made. The dilutions were then spread plated onto TSA plates (in duplicate), and incubated at 35° C for 24 hours to determine initial cell count.

The transfers and preparation methods for *Salmonella enterica* serovars (Baildon, Agona, Michigan, and Gaminara) are the same as the *E. coli* O157:H7 culture, refer to explanation above. Each serovar was transferred separately. Once the cultures were re-suspended in the BHIB, three milliliters from each culture was aseptically removed and

combined to create the *Salmonella* cocktail totaling 12 mL. The cocktail was then used to inoculate the orange juice; 2.5 mL culture into 247.5 mL orange juice.

From the *Salmonella* cocktail produced; serial dilutions (using 9.9 mL 1% peptone blanks) were made. The dilutions were then spread plated onto TSA plates (in duplicate), and incubated at 35° C for 24 hours to determine initial cell count.

Complex in Juice

Apple cider (Murray Cider Company Inc., 103 Murray Farm Road, Roanoke, Virginia 24019) and orange juice (Tropicana, Tropicana Manufacturing Company, Bradenton, Florida) were obtained from Kroger, a local grocer in Blacksburg, VA. The cider and juice were aseptically portioned and held at test temperatures (4° C and 25.6 ± 0.42° C). Initial microbial populations were determined by surface plating juices onto TSA in duplicate. The plates were incubated at 35° C for 24 hours. The juices were aseptically divided into six 247.5 mL portions and tempered to 4° C and 25.6 ± 0.42° C. After tempering four of the 247.5 mL jars of cider were inoculated with 2.5 mL of the *E. coli* O157:H7 inoculum and four of the 247.5 mL jars of orange juice were inoculated with 2.5 mL of the *Salmonella* cocktail while being mixed on a stir plate for two minutes. Desired amounts of the complexed cinnamic acid, 1000 mg/L for treatment 1 and 400 mg/L for treatment 2, were aseptically added to two of the four inoculated juices for each temperature and mixed for two minutes. The weight of complex added varied slightly depending on which batch of complex was used; the weight needed was calculated for each treatment. Inoculated juices were stored at 4° C and at 25.6 ± 0.42° C (plated daily). Samples of the juices were extracted at day 0 and then every 24 hours for 7 days, serially

diluted in 0.1% peptone, and surface plated, in duplicate on TSA plates. To obtain a 10^0

plate dilution 0.2 mL of sample was spread on five TSA plates, in duplicate. The

colonies on the five plates were totaled for the reported count. The plates were incubated

at 35° C for 24 hours.

Organism Identification

Typical and atypical *E. coli* O157:H7 colonies on the TSA plates from the apple

cider were confirmed by T-streaking onto Tellurite Cefixime ((selective supplement)

OXIOD Ltd., Basingstoke, Hampshire England) MacConkey (Becton, Dickinson and

Co., Sparks, MD) (TC MAC) agar and incubated at 35° C for 24 hours. A typical colony

from the TC MAC plates were further confirmed using the Dry Spot O157 agglutination

test (Oxoid Ltd., Basingstoke, Hampshire England) and an API 20E strip.

Typical and atypical *S. enterica* colonies on the TSA plates from the orange juice

were tested by T-streaking onto supplemented XLT4 agar (Becton, Dickinson and Co.,

Sparks, MD) and incubated at 35° C for 24 hours. A typical colony from the

supplemented XLT4 agar was further confirmed using an API 20E strip.

Plates containing growth on either TC MAC or XLT4 agar that was not typical of

E. coli or *Salmonella* respectively were discarded. Juice sample reported counts for

plates containing colonies that were positively identified as not either *E. coli* O157:H7 or

Salmonella were not reported in the write-up of this experiment.

<u>Testing Complex Effect on Juice pH</u>

The pH of both apple cider and orange juice were tested alone, with 1000 mg/L complexed cinnamic acid, and with 400 mg/L of complexed cinnamic acid. For each run, four screw cap glass vials were filled with 10 mL of both apple cider and orange juice. Two of the four vials of each juice were stored at $25.6 \pm 0.42°$ C and two were stored at $4°$ C overnight to temper. Once tempered the calculated amount (the weight of cinnamic acid required varied between complex batches) of complex was added to one vial for each temperature for both juice types and vortexed. The pH of both the juice alone and the juice with complex at $25.6 \pm 0.42°$ C and $4°$ C were tested daily for each juice for days 0 through 7. This experiment was done in triplicate for both 400 mg/L and 1000 mg/L complexed cinnamic acid; the pH values were averaged to create a line graph.

<u>Statistical Analysis</u>

The test of difference between the reported counts (CFU/mL) of the positive control orange juice and apple cider combined and orange juice and apple cider with complex combined were performed for both treatments (1000 mg/L and 400 mg/L) at the statistical significance level $\alpha = 0.05$. The test of difference between reported counts (CFU/mL) between temperatures ($25.6 \pm 0.42°$ C and $4°$ C) was also conducted for both treatments (1000 mg/L and 400 mg/L) at the statistical significance level $\alpha = 0.05$. The reported counts for both treatments are averages from three replicated, independent experiments. Analysis was done using repeated measure, three-way ANOVA; calculations were performed by Proc Mixed in SAS statistical software (SAS Institute

Inc., Cary, N.C.). To meet the model requirements (i.e. normality assumption of measurements), the counts were converted to logarithmic units.

RESULTS and DISCUSSION

Cinnamic Acid Water Standard Curve

A cinnamic acid water standard curve was constructed to determine the intrinsic solubility of cinnamic acid in water and the concentration of cinnamic acid present in the prepared inclusion complex. The water standard curve was not needed after primary experimentation due to necessitate for complex dissociation for accurate cinnamic acid quantification.

Reading at a wavelength of 272 nm yielded a cinnamic acid water standard curve with $y = 0.1405x - 0.0382$ ($R^2 = 0.9983$) as the equation of the line.

Ethanol Standard Curve

An ethanol standard curve was constructed for the same purpose as the original water standard curve; to determine the concentration of cinnamic acid that was present within the inclusion complex. Ethanol was used as both the diluting material and to dissociate the complex. Formation of an inclusion complex altered the molar extinction coefficient of cinnamic acid thus shifting the UV absorption maximum. Therefore, invalid results were obtained if the complex was not dissociated. In Szente's Analytical Methods for Cyclodextrins, Cyclodextrin Derivatives, and Cyclodextrin Complexes it was suggested to dilute complexes in 50% ethanol and than again in 99.5% to dissolve

and dissociate the complex *(47)*. This method resulted in more accurate UV spectrophotometric determination.

The calculated equation, reading at a wavelength of 269.5 nm, for the ethanol standard curve was y = 0.1361x – 0.0095 (R^2 = 0.9994) (Figure 4).

Cinnamic Acid Intrinsic Solubility (IS)

The average intrinsic solubility in distilled water was calculated to be 496.18 mg/L, 499.07 mg/L, and 532.29 mg/L at 24, 48, and 72 hours respectively.

Solubility slightly increased with time. The slight solubility increase is not greatly beneficial because the time required to achieve the increase is notable; especially when time is limited. The experimentally determined intrinsic solubility required addition of excess cinnamic acid which would be need to be removed; this is both time consuming and costly. Previous experimentation states that the intrinsic solubility of cinnamic acid is $3.01*10^{-3}$ M *(12)*, this is equivalent to 446 mg/L which is less than was calculated in this experiment. The solubility differences between studies could be due to a number of factors (time allowed to dissolve, temperature, physical form of cinnamic acid, etc.).

The solution was prepared in the electronic shaker versus the on stir plate, as seen with standard curve procedures, to decrease variables. It was noticed that the stir plate generated heat (was warn to the touch) which may have yielded a falsely high intrinsic solubility value. The cinnamic acid water solution was diluted in 50% ethanol and then 99.5% ethanol when run on the spectrophotometer to mimic procedures used for phase solubility techniques; this was to make the data easily comparable.

Cyclodextrin/Cinnamic Acid Phase Solubility Diagrams

Inclusion complex formation between α-cyclodextrin and cinnamic acid yielded a type B phase solubility diagram (Figure 5). Solubility of cinnamic acid peaked when complexed with 0.4 g/10mL α-cyclodextrin; complexation with greater amounts of α-cyclodextrin caused the solubility to decrease. With the addition 0.4 g/10mL α-cyclodextrin the concentration of cinnamic acid reached 3,786.43 mg/L; this is approximately 7.5 times its intrinsic solubility. Complexation with excess cinnamic acid and α-cyclodextrin at its maximum solubility (1.45g/10mL) did not notably increase the solubility of cinnamic acid; the cinnamic acid concentration was very close to its intrinsic solubility.

In vials containing complex that correspond to the linear portion (cinnamic acid solubility increase) of the α-cyclodextrin/cinnamic acid phase solubility diagram (0 through 0.4 g/10mL α-cyclodextrin), the solution appeared clear, gritty, and particles were gathered at the top of the solution. The remaining vials, where the solubility of cinnamic acid decreased appeared cloudy, thick, and shiny.

Phase solubility results of the α-cyclodextrin/cinnamic acid inclusion complex are typical of a type B curve; solubility increased with increasing cyclodextrin addition, peaked, and then decreased with addition of increasing cyclodextrin. Other work studying complexation between both α- and β-cyclodextrin with cinnamic acid derivatives have also yielded type B curves *(52)*. Connors and Rosanske calculated the solubility of cinnamic acid and α-cyclodextrin complex to be $1.7*10^{-2}$ M (2,518.89 mg/L) which is less than the solubility determined in this experiment. As mentioned earlier the

solubility variations between studies may be due to a number of reasons (temperature, amounts of cyclodextrin and cinnamic acid added, etc.).

Complexation between β-cyclodextrin and cinnamic acid only slightly increased the solubility of cinnamic acid (Figure 6). Again, a type B curve was observed. However, only a 133.5 mg/L difference was seen between the intrinsic solubility of cinnamic acid and the highest increase achieved by complexation with β-cyclodextrin. At the addition of 0.15 g/10mL of β-cyclodextrin the solubility of cinnamic acid increased most; this point has a large standard deviation (demonstrated with the error bars). When observing the β-cyclodextrin/cinnamic acid phase solubility diagram it seems that the plateau would better emulate previous studies if it were a straighter horizontal line. Typical type B diagrams have a horizontal plateau. The two points (at 0.6 g/10mL and 0.15 g/10mL β-cyclodextrin) that skew the plateau have larger standard deviations then the other points and may be falsely high or low. If this observation is correct then the addition of 0.3 g/10mL β-cyclodextrin (start of the plateau) may be all that is needed to obtain the small increase in cinnamic acid solubility that complexation with β-cyclodextrin can achieve.

As seen with the α-cyclodextrin/cinnamic acid phase solubility diagram there was also a difference in solution appearance at higher concentrations of β-cyclodextrin. Vials containing 0 through 0.6 g/10mL β-cyclodextrin and cinnamic acid appeared gritty at the end of the complexation period; the remaining vials with higher β-cyclodextrin concentrations were gritty, slightly cloudy, thick, and shiny.

The type B curve constructed using β-cyclodextrin and cinnamic acid did not exhibit great increase in solubility. Complexation between β-cyclodextrin and cinnamic

38

acid may be poor because the cavity size of β-cyclodextrin may be too large interact with the phenyl moiety of cinnamic acid. This theory is supported by a ^{13}C-nulear magnetic resonance (NMR) study *(52)*. β-cyclodextrin/cinnamic acid phase solubility diagram demonstrates why the α-cyclodextrin/cinnamic acid complex would be most ideal. Cinnamic acid complexed with β-cyclodextrin does not show significant solubility increase and β-cyclodextrin has a slight toxicity at high levels of consumption.

Wettability of the complexing agent may play a role in the solubility of the inclusion complex. Solubility of beta-cyclodextrin is about eight times that of alpha-cyclodextrin *(52)*. This may help explain why α-cyclodextrin/cinnamic acid complexation increases the solubility of cinnamic acid better than β-cyclodextrin/cinnamic acid inclusion complexes.

Visual differences noted in solutions containing cinnamic acid and cyclodextrins may be due to precipitation. Differences were observed in both α-cyclodextrin/cinnamic acid and β-cyclodextrin/cinnamic acid phase solubility diagrams at higher cyclodextrin levels. A study by Uekama reported precipitation of solid complex when higher concentrations of the cyclodextrin were added during phase solubility diagram formation between β-cyclodextrin and cinnamic acid derivatives *(52)*. Precipitation of the solid complex may have caused the cloudy, thick, milky appearance noted at higher cyclodextrin concentrations.

After formation of the cinnamic acid/α-cyclodextrin and cinnamic acid/ β -cyclodextrin complexes they were diluted in 50% ethanol and placed into the electronic shaker for 30 minutes. This step was performed to ensure that the complex dissociated before being tested for cinnamic acid concentration. As mentioned earlier, inclusion

complex formation alters the molar extinction coefficient which will yield incorrect absorbencies.

Using the slope and y-intercept from the equation of the line (y = 0.7141x + 0.0035; R^2 = 0.9976) (Figure 7) from the linear portion of the α-cyclodextrin/cinnamic acid phase solubility diagram, the calculated stability constant ($K_{1:1}$) was 722.03 M^{-1}. Stability constant calculated from β-cyclodextrin/cinnamic acid inclusion complex, using slope and intercept from y = 0.4784x + 0.0026, was 273.78 M^{-1}. Reported stability constants have ranged from 70 to 3700 M^{-1} for inclusion complexes containing α-cyclodextrin and cinnamic acid *(12, 13, 52, 53)* and 60 to 371 M^{-1} for complexes composed of β-cyclodextrin and cinnamic acid *(52, 53)*. These values vary because formation environments (temperature, pH, etc.) were not consistent between studies. The binding constant measures the strength of the cyclodextrin complex. Thus, larger values for cinnamic acid with α-cyclodextrin show that it is a stronger complex than β-cyclodextrin/cinnamic acid complexes.

Uekama, Otagiri et al reported the stability constant for an α-cyclodextrin/cinnamic acid complex formed at pH 1.6 and pH 8.2 to be 3460 and 109 M^{-1}, respectively. Likewise, with the same pH conditions for formation of a β-cyclodextrin/cinnamic acid inclusion complex stability constant ranged from 371 to 313 M^{-1}. This illustrates that lower pH environments facilitates inclusion complex formation *(53)*. The calculated stability constants of 722.03 M^{-1} for α-cyclodextrin/cinnamic acid complex and 273.78 M^{-1} for β-cyclodextrin/cinnamic acid inclusion complex is expected because the inclusion complexes were synthesized in an unbuffered neutral pH

environment (distilled water) so is it predictable that stability constants would lay between maximum and minimum literature values.

Connors *(13)* reported a normal distribution curve of $K_{1:1}$ values for 663 different α-cyclodextrin complexes (Figure 8) previously formed. The log value of the calculated $K_{1:1}$ for α-cyclodextrin/cinnamic complex from the current study (2.86) falls past the mean (2.11), μ, of the distribution, meaning the stability constant is larger than average. This implies that the α-cyclodextrin/cinnamic complex formed in this study is stronger than average α-cyclodextrin inclusion complexes previously reported. Connors *(13)* also constructed a normal distribution curve for β-cyclodextrin complexes (Figure 9). The log of the stability constant of β-cyclodextrin/cinnamic acid complex (2.44) formed in this study is lower than the average (2.69) seen in the distribution.

Inclusion Complex Formation and Quantification of Cinnamic Acid in Complex

After shaking, the complex appeared cloudy and thick but was clear once filtered. The complex had a strong aroma of concentrated cinnamic acid. Once removed from the freeze drier the complex was a very light weight, fluffy, white, powder. It was difficult to transfer it into the stomacher bags because it was so light and easily carried away by air. After being compressed in the stomacher, the powder was denser and was easier to handle.

In dried α-cyclodextrin/cinnamic acid complex, the average concentration of cinnamic acid was calculated to be 1022.93 mg/L. The average percent weight of cinnamic acid in the dry complex was 10.29% and the average molar ratio was 1.35. A

molar ratio of 1.35 implies that there are 1.35 moles of cyclodextrin for every 1.0 mole of cinnamic acid present in the solid complex.

Stock Cultures and Initial Identification

   E. coli O157:H7 and *Salmonella enterica* serovars (Agona, Michigan, Baildon, and Gaminara) all illustrated turbidity when inoculated into 9 mL TSB and then again when transferred. Control, uninoculated, TSB showed no turbidity. When streaked onto EMB agar, the *E. coli* O157:H7 demonstrated typical green metallic colonies due to precipitation of dye caused by lactose fermentation. Both *S.* Agona and *S.* Baildon showed typical growth on HE agar, hydrogen sulfide production, and the colonies were blue-green because lactose, sucrose, and salicin were not fermented. *S.* Gaminara and *S.* Michigan yielded blue-green colonies with hydrogen sulfide production, slight fermentation of the carbohydrates present was observed; this caused yellowing of the agar.

   The *E. coli* strain was positively identified with 99.2% confidence with an API 20 E code of 7144072. Both *S.* Agona and *S.* Baildon had the same an API 20 E code (6704752) that identified them as *Salmonella* species with 99.9% confidence. *Salmonella* Gaminara was identified as *Salmonella* species with 99.9% confidence with an API 20 E code of 6706752. Last, *S.* Michigan with an API 20 E code of 6704552 was confirmed to be *Salmonella* species with 89.4% confidence.

<u>Visual Testing of α-cyclodextrin/cinnamic acid Complex in Juices</u>

Originally the apple cider had a small amount of sediment and was transparent; the orange juice was pulp free and not translucent. At day 0, after complex addition, the complexed cinnamic acid was only greatly visually noticed in the cider at concentrations greater than 2000 mg/L. At these concentrations chunked white powder was visible in the cider which caused the juice color to become lighter. At both 500 and 1000 mg/L a few small white particles remained after vortexing but dissolved into solution within 24 hours. The orange juice was visually unchanged with the addition of complex. The observations remained constant in both orange juice and apple cider until 48 hours; the control apple cider (without complex) showed sediment that would mix into solution when stirred making the juice slightly cloudy and then settle back out over time. Likewise, concentrations of 250 through 1000 mg/L also showed sediment that behaved the same as seen in the control cider. However, the sediment amount seen in concentrations of 250 through 1000 mg/L was not as great as was seen in the control cider. Vials containing 2000 and 3000 mg/L complexed cinnamic acid showed high amounts of particle sediment that would also mix into solution but would settle out quickly. At 48 hours no change was noticed in the orange juice. With increased time, separation in the orange juice was noticed; this separation was equally observed in all vials for both temperatures. The cider with no complex continued to show sediment and the 2000 and 3000 mg/L vials showed precipitation at the end of 7 days. The 250 through 1000 mg/L vials showed very little sediment at the end the seven day experiment (Tables 3 and 4).

The visible complex seen in the apple cider vials containing higher concentrations may be either complex that did not initially go into solution or precipitated out of solution over time. α-cyclodextrin may have dissociated from the cinnamic acid and bound to other particles in the juice. Complex solubility studies were done in distilled water alone. The pH and consistency differences between the distilled water and juices may have altered the solubility or binding properties of the complex. This experiment was only quantified visually so the true identity of the sediment, amount of complex in solution, or what molecules were bound in inclusion complexes is unknown.

From observing the interaction of the complex at differing concentrations with both orange juice and apple cider it was determined that 1000 mg/L of complexed cinnamic acid would be the maximum concentration used against the target organisms, *E. coli* O157:H7 and *Salmonella enterica*. This concentration was chosen because it was the highest tested that did not show any visual alterations of the both the orange juice and apple cider.

Agar Diffusion Assay

None of the wells, with or without α-cyclodextrin/cinnamic acid complex, displayed inhibition of the target organism (*E. coli)* that was seeded in the cooled agar. The target organism demonstrated equal growth on all plates regardless of complex concentration. The near neutral pH of the BHIA (7.4 ± 0.2) may have effected the antimicrobial ability of the cinnamic acid. Cinnamic acid (pk_a of $4.37 - 4.44$), like many weak acids, is a more potent bactericide when used in low pH environments and has also shown synergy with weak acids. When in the undissociated form, cinnamic acid is a

powerful antimicrobial because it can dissolve into the membranes of microorganisms. Once inside the cell it dissociates and disrupts the pH balance. At neutral pH weak acids form equilibrium between the charged (ionized) form and the uncharged (undissociated) form. However, in acidic solutions the undissociated form is dominate causing the acid to be effective *(3)*. This is why cinnamic acid shows synergy with other weak acids; the low pH causes cinnamic acid to become undissociated which allows it to easily enter through cell membranes.

Organism Preparation for Juice Assay

The average reported count for the *S. enterica* inoculum for treatment 1 (1000 mg/L complexed cinnamic acid) was 9.08-log CFU/mL and the initial count for the *E. coli* O157:H7 inoculum was 9.18-log CFU/mL.

Treatment 2, 400 mg/L complexed cinnamic acid, the average reported count for the *S. enterica* inoculum was 8.62-log CFU/mL and the average count for the *E. coli* O157:H7 inoculum was 8.60-log CFU/mL.

Complexed Cinnamic Acid Complex in Juice

Stirring for two minutes allowed the majority of the 1000 mg/L complexed acid to go onto solution; any remaining complex particles dissolved within 24 hours. However, at high agitation (vortex) the complexed cinnamic acid at the same concentration went into solution within two minutes. Juices containing inoculum were not vortexed; this would have caused unwanted physical stress on the cells. The addition of complexed cinnamic acid at 1000 mg/L did not affect the visual characteristics of the apple cider or

orange juice. Over the seven experimentation days equal cell accumulation (assumed to be *E. coli*) was visual in the positive control apple cider and the cider with a combination of complex and inoculum. The negative control cider showed little sediment (possible bacterial contamination or natural juice particles) and slight darkening due to visible mold growth. No mold growth was observed in the cider containing inoculum only or inoculum and complex. The orange juice showed sediment of the juice particles which caused a separation in the juice; this was equal in all jars, regardless of temperature or inoculum and complex addition. No growth or precipitation was observed in any of the orange juice samples.

Orange juice alone caused a 4-log decrease of *Salmonella* cells from day 0 to the end of 7 days at $25.6 \pm 0.42°$ C and a 3.7-log decrease at $4°$ C (Figures 10 and 11). Apple cider alone decreased *E. coli* cells approximately 1-log at $25.6 \pm 0.42°$ C and 1.5-log at $4°$ C over the 7 day period (Figures 12 and 13). At day 7 there was nearly a 1-log difference seen between the positive control and the orange juice combined with 1000 mg/L complexed cinnamic acid at $25.6 \pm 0.42°$ C (Figure 10). At $4°$ C a 3.2-log difference was noted (Figure 11). The combination of apple cider and 1000 mg/L complexed cinnamic acid yielded a 6-log decrease from the positive control at $25.6 \pm 0.42°$ C (Figure 12), and a 3.3-log difference was observed at $4°$ C (Figure 13).

Similar visual observations seen for 1000 mg/L were made over the seven experimental days for 400 mg/L complexed cinnamic acid in orange juice and apple cider. Cell accumulation was again seen in the positive control and treatment apple cider jars. Complex precipitation was not observed in any of the jars. Orange juice separation due to juice particle sedimentation was again observed. A difference between treatments

was that mold growth was not observed in negative control apple cider jars; however slight darkening was noticed in $25.6 \pm 0.42°$ C negative control cider jars.

Again survival of microorganisms was affected by orange juice and apple cider. Orange juice at $25.6 \pm 0.42°$ C reduced *Salmonella* 5.5-log CFU/mL and 3.7-log CFU/mL at 4° C (Figures 14 and 15). A 1.6-log CFU/mL and 1.2-log CFU/mL reduction was observed for *E. coli* at $25.6 \pm 0.42°$ C and 4° C respectively (Figures 16 and 17). At $25.6 \pm 0.42°$ C there was only a 0.5-log CFU/mL difference at day 7 between the orange juice positive control jar and 400 mg/L treatment jar (Figure 14). There was however a 2.7-log CFU/mL difference noted in jars for the 4° C orange juice (Figure 15). A 5-log CFU/mL difference between positive control and treatment jars was observed for the 25.6 $\pm 0.42°$ C apple cider (Figure 16). As expected, as smaller difference of 3-log CFU/mL was seen in the apple cider held at 4° C (Figure 17).

At 1000 mg/L complexed cinnamic acid there was 0.5-log CFU/mL greater reduction seen in both $25.6 \pm 0.42°$ C and 4° C for the orange juice than with 400 mg/L complexed cinnamic acid (Figures 10 – 11 and 14 – 15). 1000 mg/L complexed cinnamic acid, at $25.6 \pm 0.42°$ C, caused a 1.0-log CFU/mL more decrease in *E. coli* than 400 mg/L complexed cinnamic acid (Figures 12 and 15). There was only a 0.3-log CFU/mL increase, at 4° C, seen with using 1000 mg/L complexed cinnamic acid compared to 400 mg/L complexed cinnamic acid (Figures 13 and 16). This data indicates that the lowest concentration used in this study, 400 mg/L complexed cinnamic acid may, may be near the minimum inhibitory concentration. This assumption was made because when the cinnamic acid concentration was increased by a factor of 2.5 to attain 1000 mg/L complexed cinnamic acid the highest increase in cell death was only 1-log

CFU/mL. Increasing the concentration of complexed cinnamic acid to a value between 400 mg/L and 1000 mg/L may be all that is required to obtain the same antimicrobial results that 1000 mg/L complexed cinnamic acid yielded. At 400 mg/L a 5-log CFU/mL decrease was achieved for the apple cider held at $25.6 \pm 0.42°$ C; this meets the FDA requirement of a 5-log reduction. Complex addition, along with other safety measures could possibly be a "natural" solution for juice preservation.

Day 0 reported counts (log CFU/mL) between positive control jars and treatment jars vary. This may be because after addition of the inoculum in the positive jars the juice was only stirred for a total of two minutes. However, in the treatment jars the liquid was stirred initially for two minutes after addition of the inoculum and then again for two more minutes after addition of complex, totaling four minutes. Addition stirring in the treatment jars may have caused physical stress decreasing cell counts. It was also observed that jars held at $25.6 \pm 0.42°$ C demonstrated a larger difference in reported counts (log CFU/mL) between positive control and treatment jars for day 0.

As expected, juice and cider alone affected the survival of microorganisms. Orange juice was effective alone at killing some of the inoculated *Salmonella*; however the cinnamic acid complex reduced organism counts to 0-log CFU/mL for both 400 mg/L and 1000 mg/L complexed cinnamic acid. On the other hand, the apple cider alone was not as efficient at reducing the *E. coli* cells. Orange juice used in this experiment naturally had a pH lower than apple cider; this may have contributed to greater *Salmonella* reduction compared to the *E. coli* O157:H7. *E. coli* O157:H7 has experimentally demonstrated acidity endurance by surviving in apple cider at 4° C for 14 to 21 days *(27)*. *E. coli* O157:H7 survival in cider was also noted in the present

experiment. Addition of both 400 mg/L and 1000 mg/L complexed cinnamic acid to apple cider greatly reduced pathogen survival compared to the positive control. This shows that another hurdle, besides the natural low pH of the apple cider, is required to ensure microbial safety.

As anticipated, the complex had greater bactericidal effect at $25.6 \pm 0.42°$ C versus $4°$ C for the apple cider for both treatments. Antimicrobial activity of weak acids is low at refrigeration temperature and increases as temperature increases to room temperature *(29, 38)*. As temperature decreases there is a loss in cell membrane function because the membrane becomes increasingly viscous and less fluid *(31)*. The loss of membrane movement may be preventing or slowing passive transportation of cinnamic acid.

Temperature differences did not seem to have the same effect on the *S. enterica* in the orange juice. The combination of the complex and orange juice was bactericidal at both temperatures. For both treatments, at $25.6 \pm 0.42°$ C the orange juice with out complex had a greater antimicrobial effect than at $4°$ C. This made log differences between positive control juices and treatment juices less at $25.6 \pm 0.42°$ C than at $4°$ C which gives the impression that the both 400 mg/L and 1000 mg/L complexed cinnamic acid were more bactericidal at $4°$ C; this may not be true. It is difficult to compare log differences when the positive control varied immensely between temperatures. This could be tested by inoculating the juices with a higher cell concentration and then determining log differences at the end of the experimental trial. The problem perceived here was that, at both $25.6 \pm 0.42°$ C and $4°$ C, *Salmonella* treated with complex reached 0-log CFU/ml so a true comparison could not be made.

Cinnamic acid at 741 mg/L and 1000 mg/L has shown to have antimicrobial properties against both *E. coli* and *Salmonella* respectively *(35)*. The minimum reported concentration at which cinnamic acid has shown inhibitory effects against *E. coli* is 388 mg/L *(30)*. Using this literature value it was decided that treatment 2 would be performed using 400 mg/L complexed cinnamic acid. This concentration, an amount that literature has proven to work, was chosen to help prove that the complexed cinnamic acid is also bactericidal.

Antimicrobial mechanism of cinnamic acid is the same as other weak acids. The cinnamic acid is passively transported, non-dissociated form, into the microorganism's cell where it begins to dissociate. Dissociation of cinnamic acid upsets the pH balance, causing enzyme function to cease, which kills the cell. Some "preservative resistant" organisms have the ability to readily pump out acids. However, the unsaturated side chain of cinnamic acid is highly reactive which makes cinnamic less sensitive to the pump *(9)*.

Testing Complex Effect on Juice pH

Addition of 1000 mg/L and 400 mg/L complexed cinnamic acid caused minor fluctuations within a 0.5 pH unit range from the original pH of either orange juice or apple cider. Also, a significant pH change was not noted over the seven testing days with the addition of complexed cinnamic acid at 1000 mg/L and 400 mg/L. The line graphs created were scattered and did not show a decipherable pattern.

The pH of cinnamic acid was expected to increase when complexed. It was assumed that a large pH change did not occur because an alteration in the juice pH was

50

not observed. Literature states that uncomplexed cinnamic acid has a pk_a of 4.35, when complexed the pk_a rises to 5.66. It has been shown that acid strength of cinnamic acid deceases when complexed with α-cyclodextrin *(12)*. The acid dissociation constant (Ka) for complexed cinnamic acid is less than uncomplexed cinnamic acid; this may be due to the fact that the α-cyclodextrin has a hydrophobic cavity. When the cinnamic acid group is housed within the hydrophobic cavity this apolar environment causes the extent of ionization to be lowered *(12)*.

The theories that antimicrobial properties of cinnamic acid are decreased, which maybe attributed to the pH increase, when complexed were not tested in this experiment. However, when not bound within an inclusion complex the natural solubility properties of cinnamic acid would make it difficult to work with. Excess amounts of both the acid and time would be required to get the acid into solution at a low concentration; then remaining cinnamic acid solids would have to be manually removed. These conditions would not be ideal with an industry work schedule. Even if complexation somewhat alters the antimicrobial activity of cinnamic acid the benefits of increased solubility ought to outweigh the loss.

Statistical Analysis

For both orange juice and apple cider combined there was significant statistical difference between reported counts for the positive control juices (inoculated only) and the juices containing either 400 mg/L or 1000 mg/L complexed cinnamic acid and inoculum. The p-value of the test was extremely small (< 0.0001) for both treatments at the significance level $\alpha = 0.05$. Significant statistical difference was also seen between

25.6 ± 0.42° C and 4° C for the combined juice reported counts for both 400 mg/L and 1000 mg/L complexed cinnamic acid. Increasing complexed cinnamic acid concentration 2.5 times more, 400 mg/L to 1000mg/L, did not show an increase in statistical significance difference.

CONCLUSION

Inclusion complexation of cinnamic acid with α-cyclodextrin greatly enhanced the solubility of the acid, demonstrated with a phase solubility diagram. As expected, complexation with β-cyclodextrin did not greatly enhance solubility of cinnamic acid. The complexed cinnamic acid significantly contributed to the death of both *S. enterica* and *E. coli* O157:H7 at concentrations of 1000 mg/L and 400 mg/L. In apple cider at 25.6 ± 0.42° C, the sample with the largest microorganism decline, there was only a 1.0-log CFU/mL greater pathogen reduction seen using 1000 mg/L versus 400 mg/L complexed cinnamic acid. Overall, the cell counts were significantly lower when the α-cyclodextrin/cinnamic acid inclusion complex was present in the juice. At both 1000 mg/L and 400 mg/L the complexed cinnamic acid did not visually alter either the orange juice or apple cider characteristics. Cinnamic acid complexes are an ideal "natural" hurdle for juice processors.

The next step in experimentation will be to determine the minimum inhibitory concentration (MIC) needed for the target organism in question. Once the MIC is determined sensory work should be performed to ensure that the product is organoleptically acceptable. If desired, testing of the complex in liquid form may be

done to determine the reactions in differing food systems. Last, knowledge of the

antimicrobial efficacy of the complexed cinnamic acid versus the uncomplexed cinnamic

acid would be greatly beneficial.

TABLES and FIGURES

Table 1: Volumes needed to make standard curve in water.

Stock Solution (C_1) (mg/L)	Volume Stock Added (V_1) (L)	Desired Conc. (C_2) (mg/L)	Desired Volume (V_2) (L)	Water Needed (L)
1000	0	0	0.1	0.1
1000	0.0001	1	0.1	0.0999
1000	0.0002	2	0.1	0.0998
1000	0.0003	3	0.1	0.0997
1000	0.0004	4	0.1	0.0996
1000	0.0005	5	0.1	0.0995
1000	0.0006	6	0.1	0.0994

Table 2: Volumes needed to make standard curve in ethyl alcohol.

Stock Solution (C_1) (mg/L)	Volume Stock Added (V_1) (L)	Desired Conc. (C_2) (mg/L)	Desired Volume (V_2) (L)	Ethanol Needed (L)
100	0	0	0.01	0.0100
100	0.0001	1	0.01	0.0099
100	0.0002	2	0.01	0.0098
100	0.0003	3	0.01	0.0097
100	0.0004	4	0.01	0.0096
100	0.0005	5	0.01	0.0095
100	0.0006	6	0.01	0.0094
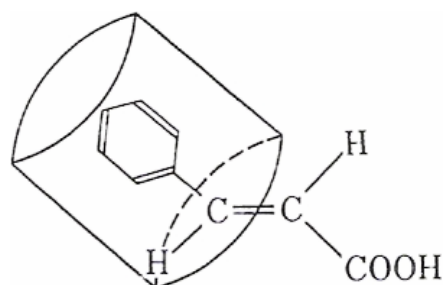
Figure 1: Proposed structure of cinnamic acid/cyclodextrin inclusion complex *(53)*.
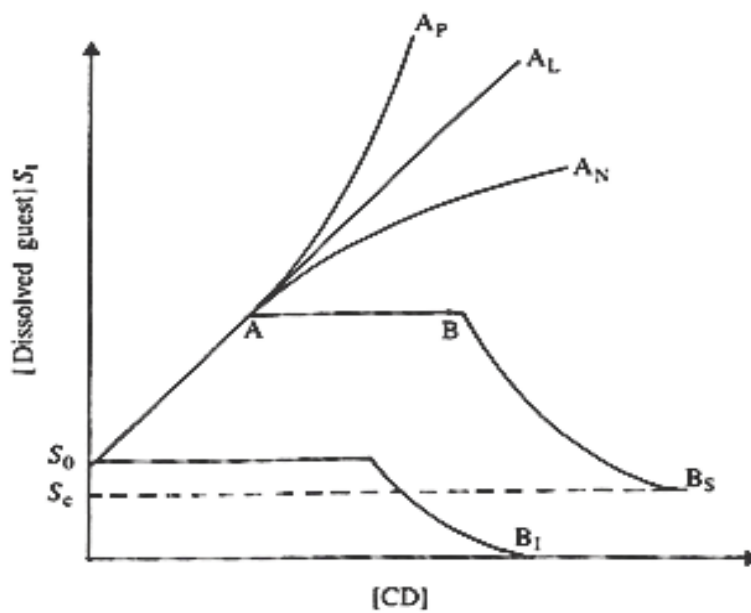
Figure 2: Phase-solubility diagram of type A and B systems showing increase in solubility of S caused by L *(21)*.

$$K_{1:1} = \frac{slope}{intercept \cdot (1-slope)}$$

Figure 3: Equation used to calculate stability constant of 1:1 inclusion complexes.

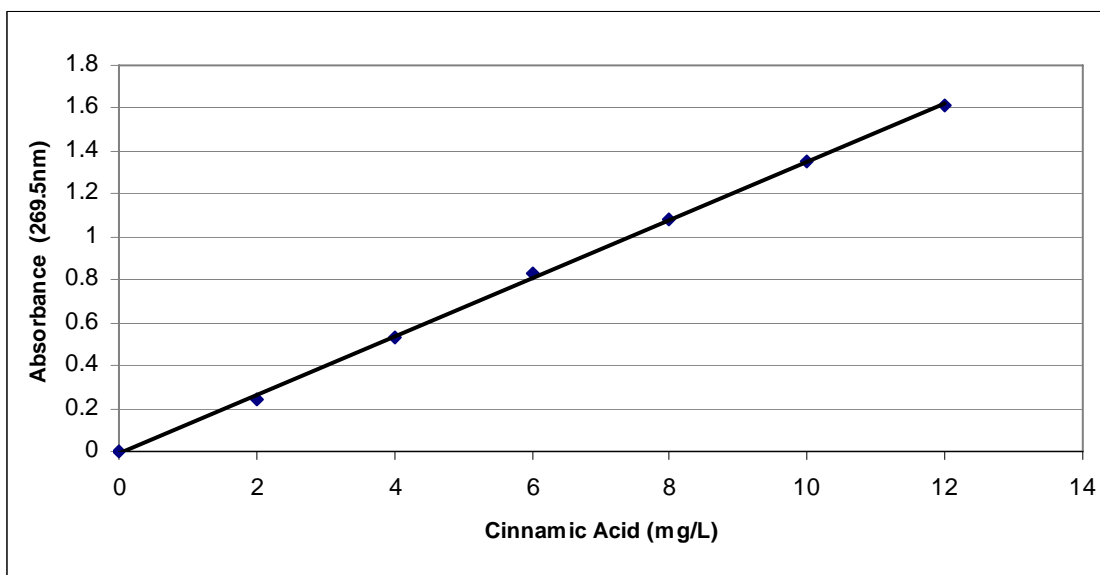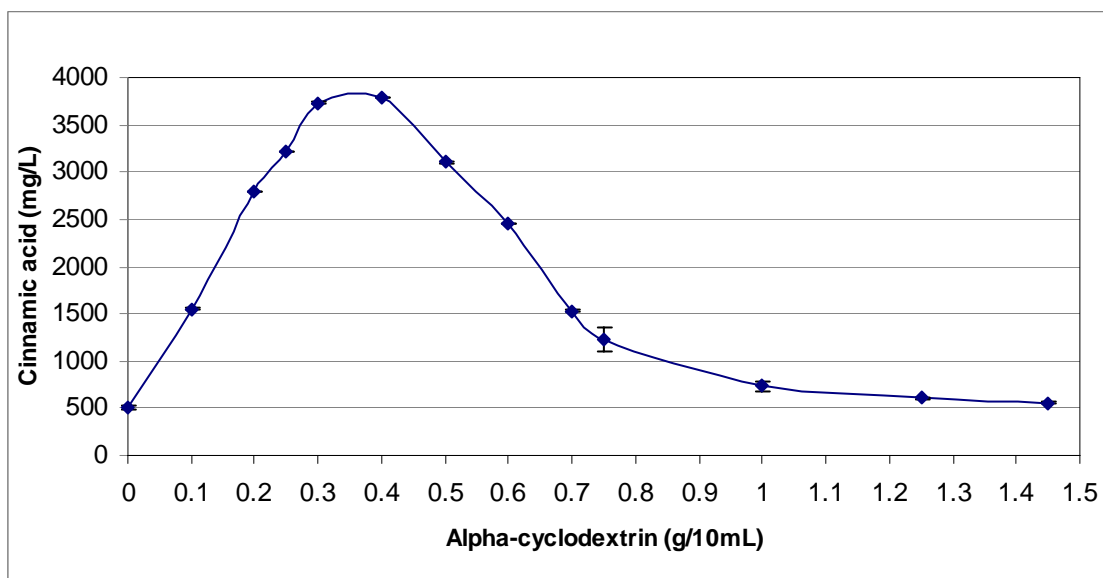**Figure 4: Cinnamic acid standard curve prepared in ethanol. The equation of the line is y = 0.1361x - 0.0095 and the R^2 = 0.9994.**

Figure 5: Alpha-cyclodextrin + cinnamic acid phase solubility curve prepared in distilled water.
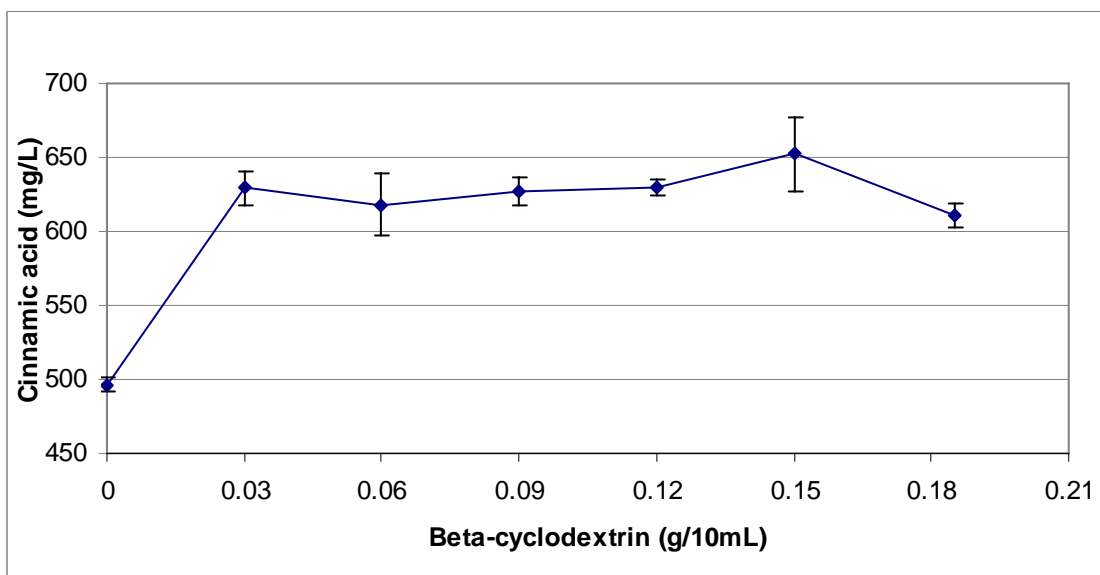
Figure 6: Beta-cyclodextrin + cinnamic acid phase solubility curve prepared in distilled water.
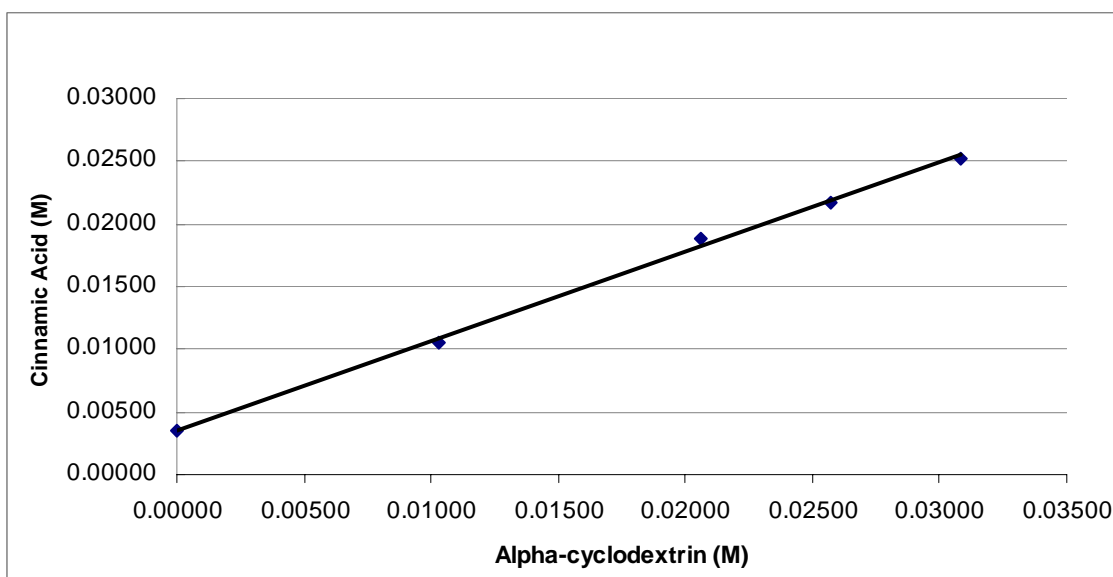
Figure 7: Linear portion of the alpha-cyclodextrin/cinnamic acid phase solubility curve. The equation of the line is $y = 0.7141x + 0.0035$ and the $R^2 = 0.9976$. The linear portion of the curve was used to calculate the complex stability constant ($K_{1:1}$).

Table 3: Visual descriptions of varying concentrations of complexed cinnamic acid in apple cider.

Apple Cider	Observations Time (Hours)							
Complexed CA (mg/L)	0	24	48	72	96	120	144	168
0	Slight natural sediment, transparent	No change	Natural sediment increase	No change	No change	No change	No change	No change
250	No change	No change	Slight natural sediment	No change	No change	No change	No change	No change
500	Slight complex sediment	No complex noticed	Slight natural sediment	No change	No change	No change	No change	No change
1000	Slight complex sediment	No complex noticed	Slight natural sediment	No change	No change	No change	No change	No change
2000	Complex sediment	Complex sediment	High complex sediment	No change	No change	Complex precipitation	No change	No change
3000	Complex sediment	Complex sediment	High complex sediment	No change	No change	Complex precipitation	No change	No change

Table 4: Visual descriptions of varying concentrations of complexed cinnamic acid in orange juice.

| Orange Juice	Observations Time (Hours)							
Complexed CA (mg/L)	0	24	48	72	96	120	144	168
0	Pulp free, not translucent	Pulp free, not translucent	Separation, no complex seen	No change	No change	No change	No change	No change
250	No change	No change	Separation, no complex seen	No change	No change	No change	No change	No change
500	No change	No change	Separation, no complex seen	No change	No change	No change	No change	No change
1000	No change	No change	Separation, no complex seen	No change	No change	No change	No change	No change
2000	No change	No change	Separation, no complex seen	No change	No change	No change	No change	No change
3000	No change	No change	Separation, no complex seen	No change	No change	No change	No change	No change
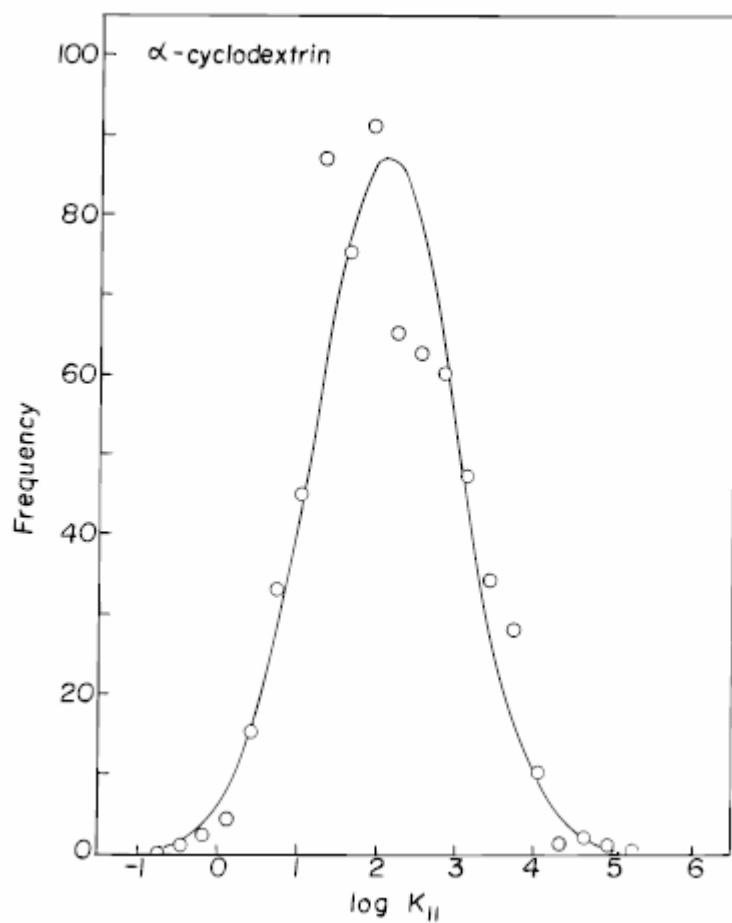
Figure 8: Normal distribution curve of log values of alpha-cyclodextrin inclusion complex stability constants.   Parameters n = 663 and μ = 2.11 *(13)*.
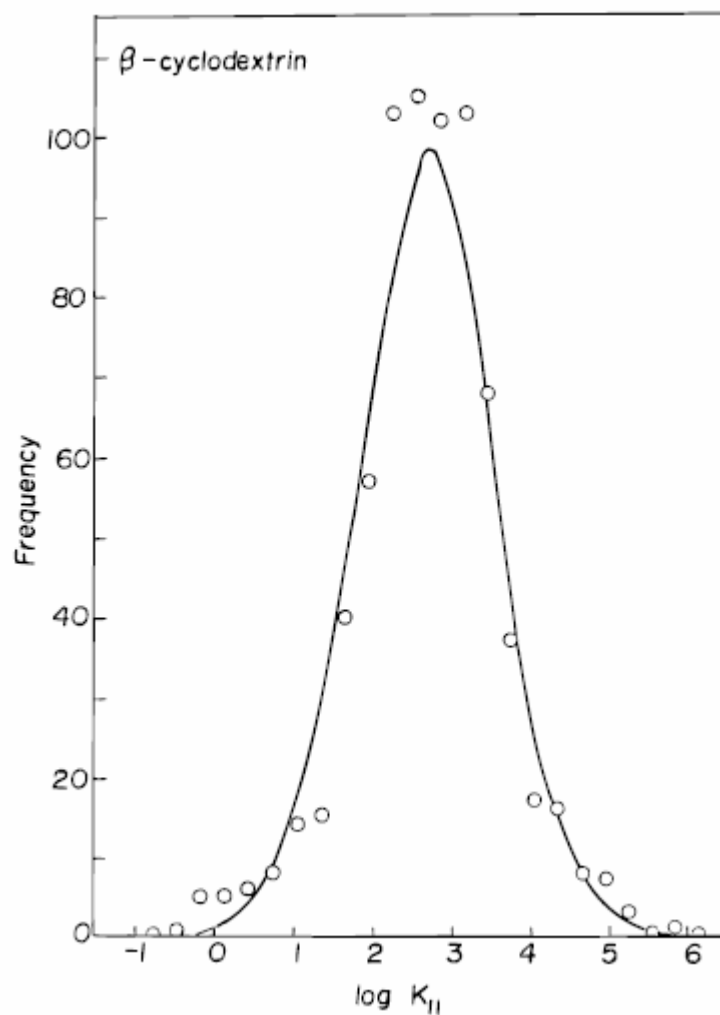
Figure 9: Normal distribution curve of log values of beta-cyclodextrin inclusion complex stability constants.   Parameters n = 721 and μ = 2.69 *(13)*.
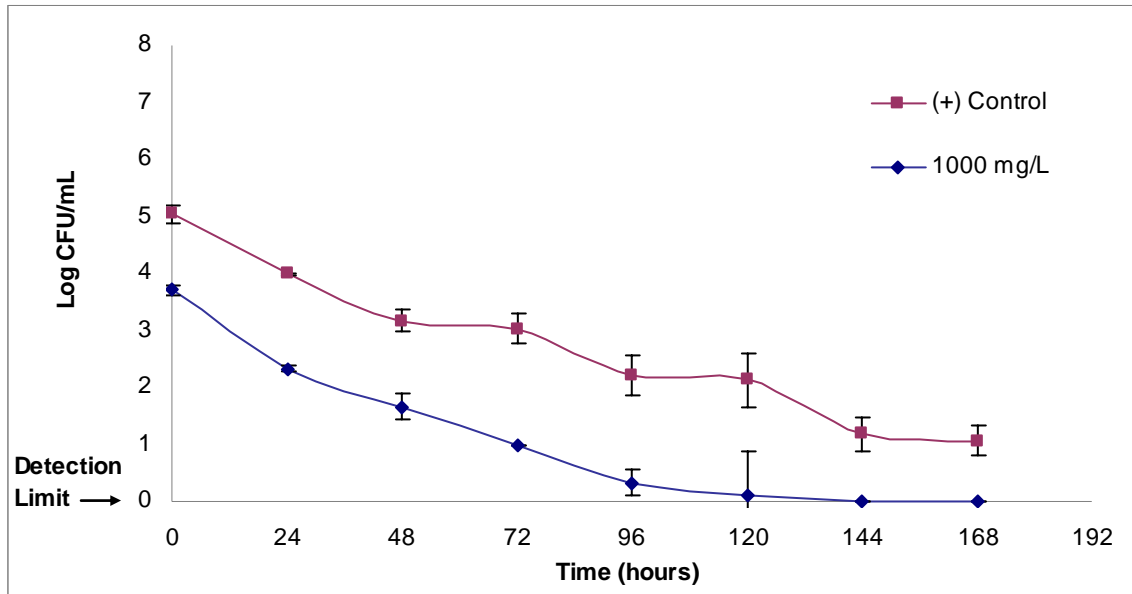
Figure 10: Results from orange juice held at 25.6 ± 0.42° C. ♦ = Combination of orange juice, 1000 mg/L complexed cinnamic acid, and *Salmonella* cocktail. ■ = Orange juice and *Salmonella* cocktail.

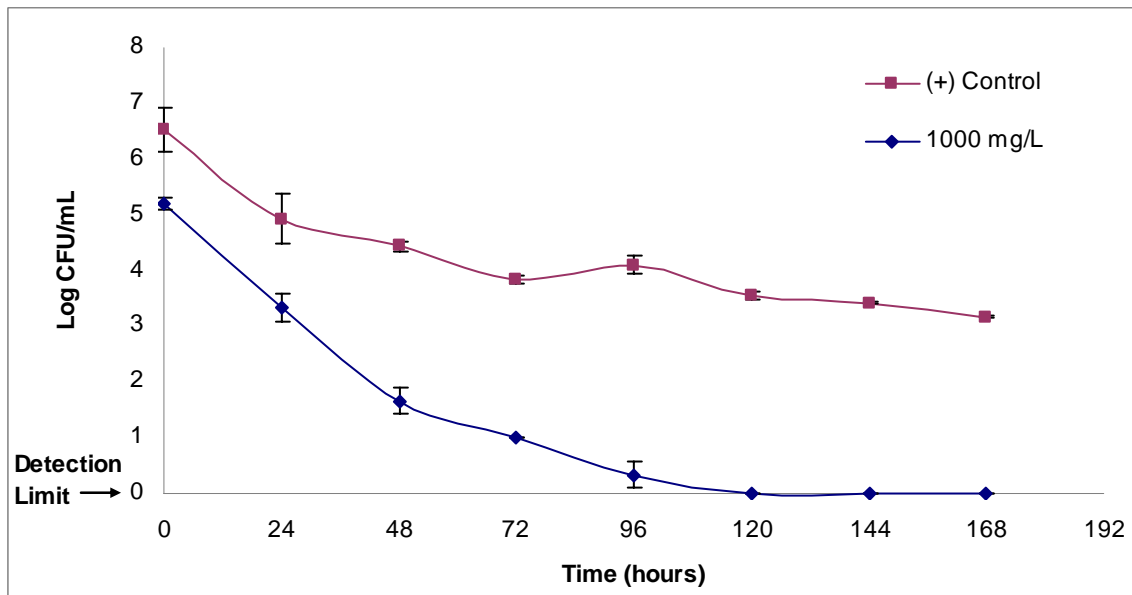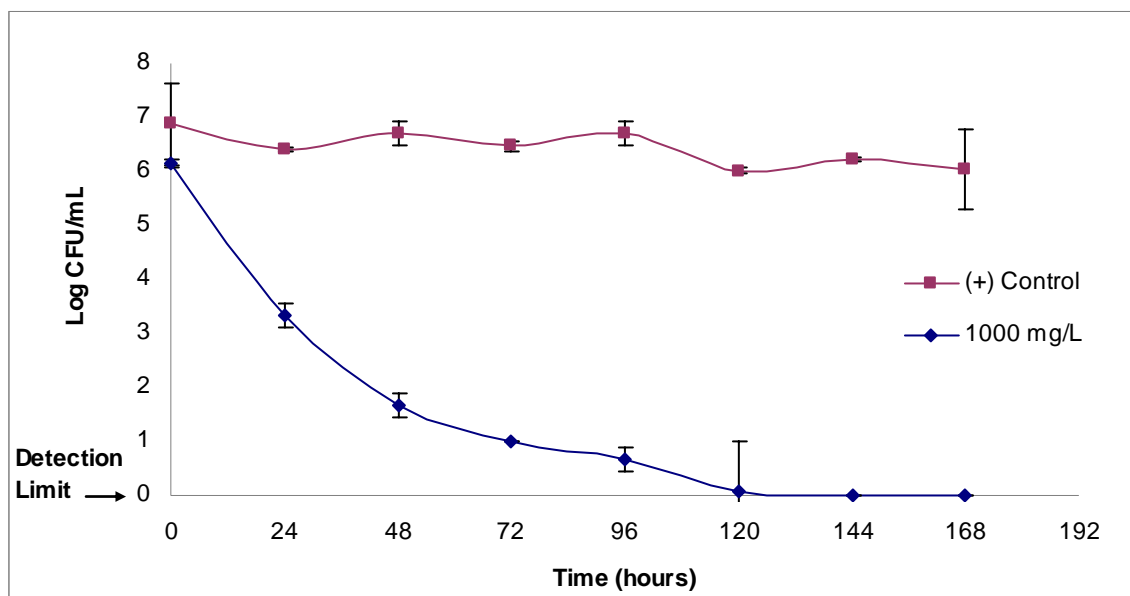**Figure 11: Results from orange juice held at 4° C. ♦ = Combination of orange juice, 1000 mg/L complexed cinnamic acid, and *Salmonella* cocktail. ■ = Orange juice and *Salmonella* cocktail.**

Figure 12: Results from apple cider held at 25.6 ± 0.42° C. ♦ = Combination of apple cider, 1000 mg/L complexed cinnamic acid, and *E. coli* O157:H7 inoculum. ■ = Apple cider and *E. coli* O157:H7 inoculum.
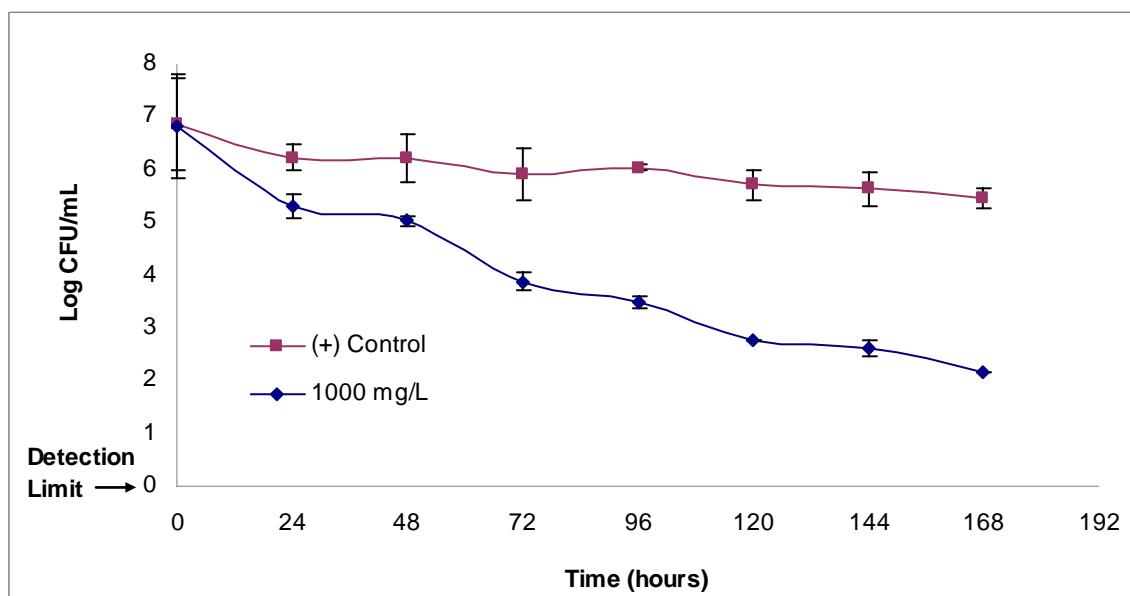

Figure 13: Results from apple cider held at 4° C. ♦ = Combination of apple cider, 1000 mg/L complexed cinnamic acid, and *E. coli* O157:H7 inoculum. ■ = Apple cider and *E. coli* O157:H7 inoculum.
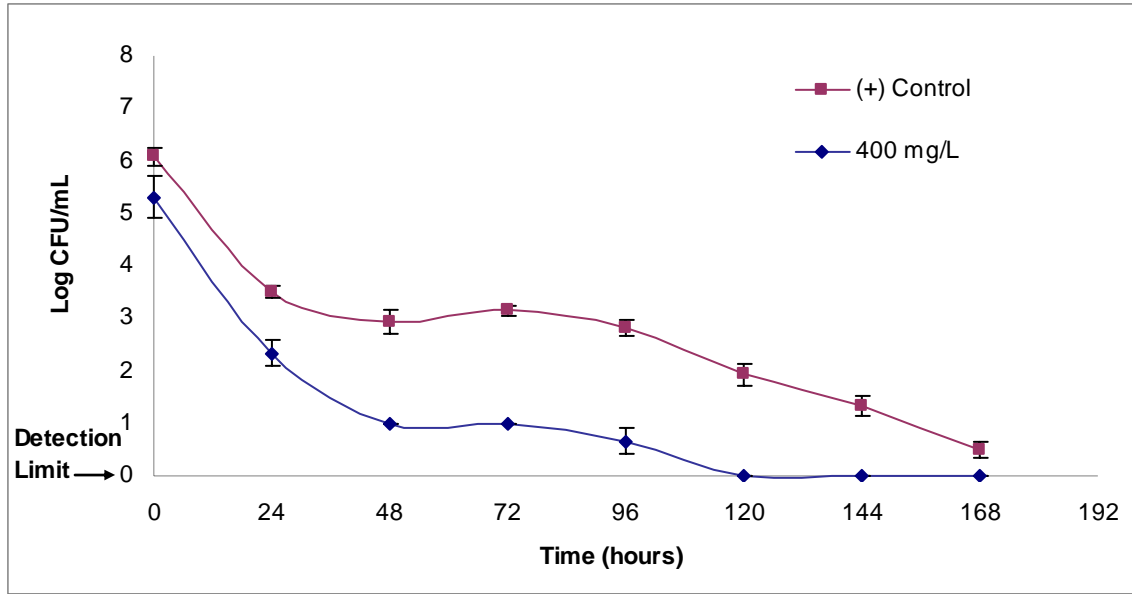
Figure 14: Results from orange juice held at 25.6 ± 0.42° C. ♦ = Combination of orange juice, 400 mg/L complexed cinnamic acid, and *Salmonella* cocktail. ■ = Orange juice and *Salmonella* cocktail.

Figure 15: Results from orange juice held at 4° C. ♦ = Combination of orange juice, 400 mg/L complexed cinnamic acid, and *Salmonella* cocktail. ■ = Orange juice and *Salmonella* cocktail.

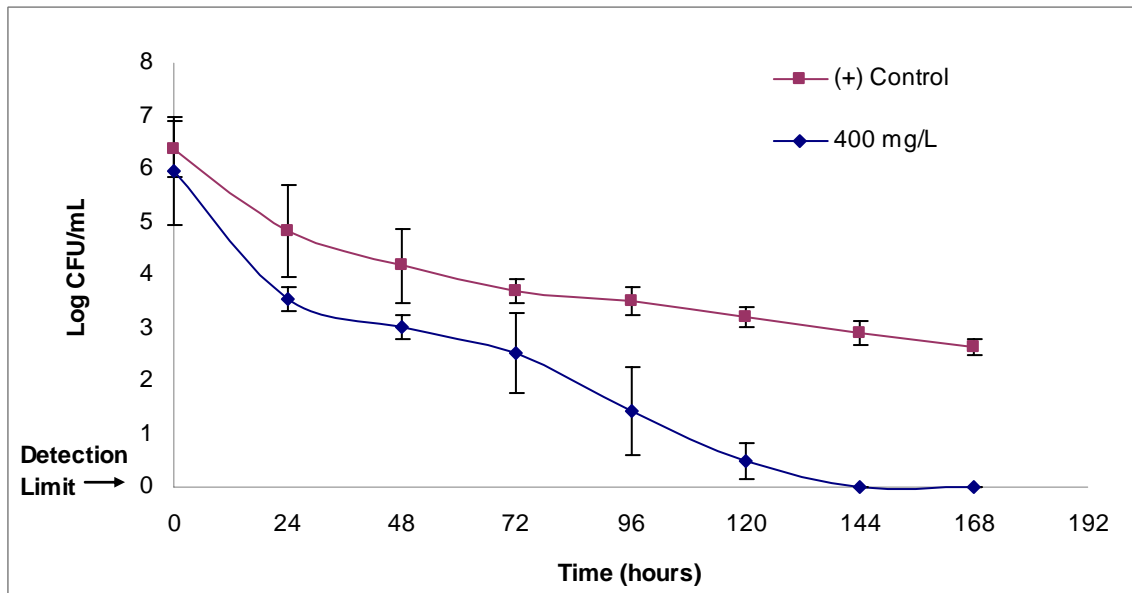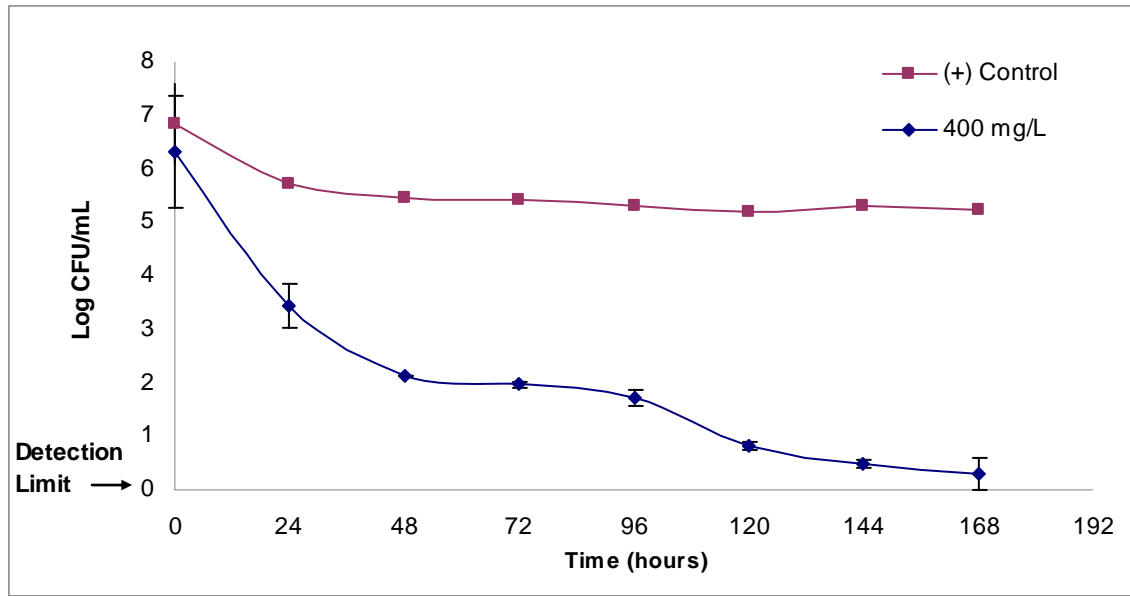**Figure 16: Results from apple cider held at 25.6 ± 0.42° C. ♦ = Combination of apple cider, 400 mg/L complexed cinnamic acid, and *E. coli* O157:H7 inoculum. ■ = Apple cider and *E. coli* O157:H7 inoculum.**

Figure 17: Results from apple cider held at 4° C. ♦ = Combination of apple cider, 400 mg/L complexed cinnamic acid, and *E. coli* O157:H7 inoculum. ■ = Apple cider and *E. coli* O157:H7 inoculum.
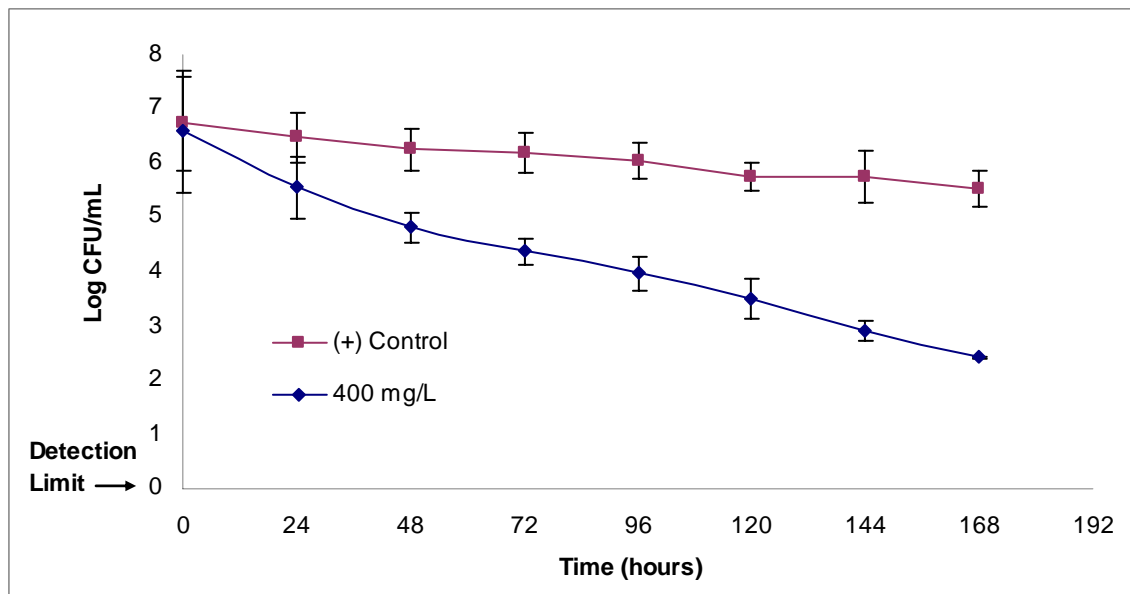
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additives towards selected pathogenic and beneficial gut bacteria. *Journal of Applied Microbiology*. 100:296–305.

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CURRICULUM VITAE

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EDUCATION

M.S. Food Science and Technology, Concentration: Food Microbiology
Expected Graduation: October 30 2007   GPA: 3.9/4.0
Advisor: Dr. Robert Williams
Thesis: Effect of cinnamic acid-cyclodextrin inclusion complexes on populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in fruit juices
Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA

B.S. Biological Sciences, Emphasis: Microbiology
Graduation: December 2005   GPA: 3.4/4.0, Cum Laude
Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA

SIGNIFICANT COURSEWORK

- Advances in Food Microbiology
- Food Packaging
- Food Chemistry
- Food Processing
- Microbial Genetics
- Pathogenic Bacteriology
- Microbial Physiology
- Food Microbiology

EMPLOYMENT

Tech Lab, August – December 2005
Corporate Research Center, Blacksburg, VA
- Prepared and packed diagnostic kits

RESEARCH

Thesis/Master's Research, January 2006 – present
- Synthesized inclusion complexes between alpha-cyclodextrin and cinnamic acid

- Produced standard curve and phase solubility isotherms
- Experimented to determine minimum inhibitory concentration
- Performed pathogen work

Undergraduate Research, August 2004 – May 2005
Department of Biology, Virginia Polytechnic Institute and State University
- Conducted general set-up and maintenance of experiments
- Researched antibiotic producing hunter bacterium isolates from Jordan and U.S. soils
- Measured and recorded zones of inhibition using target and predator microorganisms
- Organized data into spreadsheets and analyzed the results
- Followed lab procedures and protocols

WORKSHOPS

- HACCP Certified Training (Meat and Poultry), 2007
- GTA Training Workshop, 2007

TEACHING

Graduate Teaching Assistant, August – December 2007
Food Science and Technology Department, Virginia Polytechnic Institute and State University
- Demonstrated computer skills

Lead Laboratory Instructor, January – May 2007
Assistant Laboratory Instructor, January – May 2006
Food Science and Technology Department, Virginia Polytechnic Institute and State University
- Instructed students on laboratory procedures and theories
- Organized and presented class lectures
- Prepared and tested media

HONORS and AWARDS

- Outstanding Graduate Student, 2006 – 2007 Academic Year
- McNair Scholar, 2004 – 2005
- Phi Sigma Theta Honor Society, 2003 – 2005
- National Society of Collegiate Scholars, 2004 – 2005

PROFESSONAL MEMBERSHIPS

Institute of Food Technologists, 2006 – present

ADDITIONAL SKILLS

- Culturing bacterial, yeast, and fungal colonies
- Techniques for bacterial identification
- Media/dilution preparation
- Maintenance of laboratory notebooks
- Following procedures
- Testing for antibiotic resistant bacteria
- Aseptic techniques

ACTIVITES

- Food Science Club – Member, August – December 2007
- Food Science Club – Secretary, 2006 – 2007 Academic Year
- Product Development, Danisco Competition, January – May 2007
- Product Development, IFT Competition – Team Captain, August 2006 – February 2007
- Product Development, Danisco Competition – Second Place Award Winner, January – May 2006
- Product Development, IFT Competition, August – December 2005