2003

MINIMUM INHIBITORY CONCENTRATIONS OF TWO COMMON FOOD PHENOLIC COMPOUNDS AND THEIR EFFECT ON THE MICROBIAL ECOLOGY OF SWINE FECES IN VITRO

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ABSTRACT OF THESIS

MINIMUM INHIBITORY CONCENTRATIONS OF TWO COMMON FOOD PHENOLIC COMPOUNDS AND THEIR EFFECT ON THE MICROBIAL ECOLOGY OF SWINE FECES *IN VITRO*

Feeding sub-therapeutic levels of antibiotics to livestock has been associated with development and spread of antibiotic resistant bacteria. The present experiment was conducted to investigate the effect of antibiotic alternatives (caffeic acid, chlorogenic acid, and carbadox) on the microbial ecology of swine feces *in vitro*.

Minimum inhibitory concentrations of caffeic and chlorogenic acids were determined for several pathogens using macrobroth and agar dilution techniques. Gram-negative bacteria were not inhibited. Caffeic acid inhibited four *Staphylococcus aureus* strains at 200 ppm or less, and two *Clostridium perfringens* strains at 300 ppm. Chlorogenic acid inhibited all *S. aureus* strains at 500 ppm, and one *C. perfringens* strain at 400 ppm.

Effects of antibiotic alternatives on fecal microbial ecology were determined using an *in vitro* incubation. Caffeic acid lowered total anaerobes, *Bifidobacteria*, *Escherichia coli*, and percent *E. coli* ($p<0.01$). Chlorogenic acid lowered total anaerobes, *Bifidobacteria*, and lactobacilli ($p<0.01$), and increased acetate concentration ($p<0.0001$). Carbadox lowered total anaerobes, *Bifidobacteria*, *E. coli*, and coliforms ($p<0.01$), and lowered acetate, propionate, butyrate, valerate, and total volatile fatty acid concentrations ($p<0.01$). It can be concluded that addition of caffeic acid, chlorogenic acid, or carbadox effected bacterial and chemical components of the microbial ecology of swine feces.

KEYWORDS: Food phenolics, caffeic acid, chlorogenic acid, *in vitro*, microbial ecology.

Jennifer I. Zaffarano
13 June 2003

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THESIS

Jennifer I. Zaffarano

The Graduate School

University of Kentucky

2003

MINIMUM INHIBITORY CONCENTRATIONS OF TWO COMMON FOOD PHENOLIC COMPOUNDS AND THEIR EFFECT ON THE MICROBIAL ECOLOGY OF SWINE FECES IN VITRO

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture
at the University of Kentucky

By

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2003

ACKNOWLEDGMENTS

Many thanks –

To my advisor Dr. Melissa Newman for her continual guidance and encouragement. To my other committee members Dr. Joseph O'Leary and Dr. Clair Hicks. To Dr. Strobel for his advice and use of his laboratory. To Katherine Akers in the food microbiology lab for all her invaluable assistance. To Lori Heard on the 2nd floor. To fellow graduate students Susana Portocarrero, Nathan Clayton, Hesham Elgaali, Allen Pettey, and especially to Erica Pettey. To Ben von Dielingen for always believing in me. To Jerry and Carol von Dielingen for being life-savers. To my many family members for their unwavering support, including cousins, aunts and uncles, but foremost to my parents Gary and Sue DePew and my grandmother Irene Kaplon.

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CHAPTER 1

Introduction

Shortly after the introduction of antibiotics to the public, bacteria resistant to antibiotics emerged. Antibiotic resistance is a self-preservation technique inherent to the microorganism or acquired through microbial genetics. Complications associated with resistance include cross-resistance, multiple-drug resistance, increased virulence, and difficulty in treating disease.

The development of resistance occurs in two main reservoirs, humans and animals. Humans often misuse antibiotic therapy. Antibiotics are also frequently used in the livestock industry. They have been used as feed supplements for over 50 years at sub-therapeutic levels to promote growth. Scientists have linked this practice to the development and spread of resistance in humans.

Resistant bacteria can be transported from human to human, animal to animal, and animal to human through direct or indirect contact, including the consumption of food. Another problem emerges if the resistant bacteria are pathogens that cause foodborne illness. Each year, an estimated 5.2 million people suffer from foodborne bacterial gastroenteritis (Mead et al., 1999). *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Clostridium perfringens* are leading causes of bacterial foodborne illnesses. Antibiotic resistance in these pathogens may induce increased survivability and toxin production, resulting in prolonged illness or heightened severity of disease.

There are several ways to address the growing problem of antibiotic resistance. Discovery and creation of new antibiotics by scientists occur, but may not be practical. The role of antibiotic use in humans and animals can be altered. The prudent use of antibiotics and improved hygiene can decrease the development and spread of resistance. In addition, when antibiotics are removed from livestock diets, level of resistance decreases. For these reasons, The European Union (EU) has already banned several growth promoting antibiotics used in animal feeds.

Antibiotics are confirmed to improve growth performance in livestock compared to non-antibiotic supplementation, translating into a bigger profit for the farmer. However, growing public concern about antibiotic resistance is forcing farmers to seek out alternatives. Alternatives to antibiotics can be implemented without a lengthy approval process through the Food and Drug Administration (FDA). Well-studied alternatives include probiotics and

prebiotics, which have demonstrated improved growth characteristics and disease prevention. Yet, few studies exist on the effect of functional foods or herbs included in livestock diets.

To be included in a diet, a feed or feed supplement must be proven safe as well as improving growth performance or preventing disease. This can be accomplished through scientific studies. To develop a true understanding how diet effects an animal and the food it produces, studies must be done on many levels. One level includes specific chemicals found in food and how they may effect the animal in a variety of ways.

The chemical composition of a food plays a major role in its functionality. Phenolic compounds are a major group of biologically active chemicals found in plants (phytochemicals), and have been found to have antimicrobial, antioxidant, and anticarcinogenic activities. Some of the most common phenolic compounds found in foods of plant origin are the hydroxycinnamic acids (HCA), including caffeic acid (CFA) and chlorogenic acid (CGA).

Phytochemicals may have an effect of the microbial ecology of the gastrointestinal tract. The microbial ecology of animals plays a major role in the overall health of the animal. Healthy animals have decreased illnesses and improved growth characteristics. Understanding how diet affects microbial ecology is important to livestock producers as well as consumers. Therefore, the objectives of this study were:

1) To determine the minimum inhibitory concentrations of caffeic acid and chlorogenic acid against selected Gram-negative and Gram-positive pathogens.
2) To determine how caffeic acid and chlorogenic acid effect the fecal microbial ecology of swine *in vitro*.

CHAPTER 2
Literature Review

Antibiotic Resistance

Antibiotics were discovered as early as 1888 in Germany but were toxic to mammals and unstable. Although Alexander Fleming discovered penicillin in Britain in 1929, it was not introduced to the public until 1942. Shortly after, in 1945 Fleming warned of selecting for mutant bacteria resistant to penicillin (Levy, 1992). Since then, antibiotics have been widely used for the prevention and treatment of disease in humans. Pharmaceutical companies have provided large quantities for human and animal use. However, bacterial resistance to antibiotics threatens the ability of doctors to keep the human population healthy.

Definitions. Antibiotics are not novel biological agents, but very common microbial products. Using a strict definition, an antibiotic is a chemical produced by a microorganism that has the ability to inhibit the growth of bacteria, or kill bacteria and other microorganisms (Scholar and Pratt, 2000). This is a self-preservation technique used by many microbes. This definition has broadened to include synthetic and semi-synthetic antibiotics created by scientists.

Yet, the definition of an antibiotic fails to consider other materials such as plants and the compounds derived from them. These materials are considered chemotherapeutic agents (Jurgens, 1997), and can also be called antimicrobials. In the end, the results can be the same, bacteriostatic or bactericidal effects. *Bacteriostatic* compounds inhibit bacterial replication and growth, while *bactericidal* compounds cause bacterial cell death and lysis (Scholar and Pratt, 2000). Bacteriostatic drugs do not kill invading pathogens, but they allow the host's immune system to catch up in the infection battle. Growth of invading pathogens is put on hold giving the host's immune system more time to respond and rid the body of infection.

Mechanisms of resistance. Antibiotics work by targeting specific areas of the bacterial cell: preventing proper cell wall synthesis, interfering with nucleic acid or protein production, inhibiting chromosome replication and function, interference of folate metabolism, and compromising membrane permeability (Scholar and Pratt, 2000). Ideally, an antibiotic will only affect the infecting organism and will not harm host cells (selective toxicity).

Antibiotic resistance occurs when a bacterium is not sensitive to an antibiotic and continues to grow. Some bacteria have *intrinsic* resistance. The microorganism may lack a

specific drug receptor, or have protective characteristics such as the Gram-negative outer membrane which prevents the antibiotic from entering the cell (Scholar and Pratt, 2000). Gram-positive bacteria do not have this protective outer membrane (Hancock, 1997).

Resistance may also be *acquired.* The bacterium identifies an antibiotic as an environmental toxin. The microbe may initially be sensitive to an antibiotic, but undergoes a change in order to become less sensitive or completely insensitive. The change occurs through an assortment of biochemical mechanisms including decreased uptake of the drug, increased efflux of the drug, enzymatic inactivation of the drug, and altered target receptor (Scholar and Pratt, 2000).

Acquired resistance may occur by a mutation in the bacterial genome or through obtaining genetic information for resistance from another bacterium. Hundreds of different genes encode for biochemical mechanisms of resistance. Resistance may occur to only one antibiotic; still, one antibiotic can also trigger multi-drug resistance (George and Levy, 1983; Levy et al., 1976).

Development of resistance. The main reservoirs of antibiotic resistant bacteria include humans and animals. Causes for development of resistance include over-prescription by doctors, prophylactic treatment (prevention of disease) of hospitalized patients, misuse of antibiotics by patients at home, and use of antibiotics in agriculture. Today, doctors are better educated on consequences of over-prescription. Doctors are prescribing antibiotics less and educating their patients, making them more aware that following directions for the prescription is important (Williams and Heymann, 1998). However, the use of antibiotics in the livestock industry is commonplace around the globe and consequently contributes to the development and spread of antibiotic resistance.

Therapeutic and subtherapeutic antibiotic use in animals accounts for half of the world's antibiotic output (Perreten et al., 1997). Feeding antibiotics to animals was commercially introduced in 1959. In animal production, high doses of antibiotics are applied to feed, drinking water, or by injection for short periods of time to cure disease. The more common use is in low doses (below therapeutic levels) administered in feed for a long period of time to prevent disease and improve performance. The result is a decrease in the amount of feed required to produce a unit of gain in body weight, and lower morbidity and mortality rates. This translates into greater profit for the farmer (Strauch, 1987c).

Exposure to antibiotics in high or low doses creates resistance in bacteria. Some bacteria return to previous sensitivity, while others remain unchanged after hundreds of generations in antibiotic-free media (Strauch, 1987c). However, bacterial ecology of mammals is very complex. Skin/hide, respiratory, and gastrointestinal microbes include bacteria, fungi, protozoa, and viruses. These microbes are biologically linked, and when one group is affected, the others are as well. Resistance creates a selective pressure on the bacterial populations. The organisms with resistance will continue to reproduce, and the ones lacking resistance will perish. In the presence of the antibiotic, this selective pressure causes a shift towards resistant microflora of the host.

Intensively managed animals, such as swine, receive more antibiotics than other animals, such as cattle or sheep on pasture. They carry large numbers of antibiotic resistant *Escherichia coli* in their intestinal flora (up to 90%), and they become established components of the gut and persist even in the absence of antibiotics (Linton et al., 1976).

Spread of resistance. The spread of resistance occurs through transfer of genetic material from a resistant bacterium to a sensitive one. Genetic material may include plasmids, transposons, integrons, phages, and other elements capable of mobility.

Plasmids are circular, extrachromasomal (not on the chromosome), double-stranded DNA. They have the ability to replicate independently of the host bacterial cell. Most resistance in bacteria is plasmid-mediated, and one plasmid usually encodes for resistance to more than one antibiotic (Salyers and Shoemaker, 1994). Specifically, R-plasmids encode for antibiotic resistance. They can be transferred to other bacteria through the process of conjugation. Conjugation is a "mating" process used by bacteria to transfer genetic information from the donor to a recipient (Strauch, 1987c).

Transposable elements (transposons) are mobile extrachromasomal linear DNA that can recombine into the genome at random or at particular "hotspots." Transposons can occur on the chromosome or on plasmids. They often encode for enzymes involved in antibiotic resistance. The ability of a transposon to jump from one site to another (plasmid to plasmid, plasmid to chromosome, chromosome to plasmid) does not require help from the bacterial cell. The transposon's genes encode for enzymes needed for its own mobilization. Once the transposon is on a conjugative plasmid, it can be transferred to another bacterium. There can be multiple

transposons on a single plasmid (Lewin, 2002). Other transposons have the ability to conjugate themselves (Salyers and Shoemaker, 1994).

More recently discovered, integrons are linear segments of extrachromasomal DNA. Unlike transposons, they integrate at a specific site with an 8 base pair consensus sequence. They require plasmid-encoded enzymes to integrate into DNA and rely on the plasmid's promoter (a region of DNA involved in the initiation of transcription) to activate resistance genes. Several integron resistance genes can be inserted end to end and be controlled by one promoter. (Salyers and Whitte, 1994).

Another important mechanism of transfer is the bacteriophage. A bacteriophage is a virus that infects bacteria (also known as a phage). The phage binds to the outer bacterial membrane and injects its DNA (or RNA) into the cell. Once inside the cell, the genetic material can incorporate itself into the chromosome. Inserted phage DNA could contain a transposon encoding for antibiotic resistance as well as other virulence factors (Lewin, 2000; Salyers and Whitte, 1994).

There are numerous examples of bacteria able to transfer resistance, even in the absence of antibiotics. Virginiamycin resistant *Enterococcus faecium* isolated from pigs and broilers was able to transfer its resistance to a sensitive *E. faecium* (Hammerum, et al., 1998). The transfer, or spread, is not limited to specific bacterial strains or even species. Resistance can be transferred from one genera to another. Resistant *Salmonella* was able to transfer its resistance to an *E. coli* (Tessi et al., 1997). Gram-negative *E. coli* was able to transfer resistance into the Gram-positive *Clostridium perfringens* (Kaufmann et al., 1996). The multi-drug resistant *Lactococcus lactis* spp. *lactis* K214 isolated from a raw milk soft cheese was able to transfer its resistance to *Enterococcus faecalis* (Perreten et al., 1997). The ability of a non-pathogen to transfer its resistance to pathogens that can cause disease is an obvious concern to humans. There is even an example of trans-kingdom transfer of antibiotic resistance from an *E. coli* to the yeast *Saccharomyces cerevisiae* (Heinemann and Sprague, 1989).

Resistance can spread directly from animal to animal or animal to human. Bacteria can be excreted from an animal in a number of ways: mucosal secretions, feces, urine, milk, blood, and sperm (Strauch, 1987b). Bacteria and/or their spores may also become airborne (Strauch, 1987a). A farmer that works directly with livestock faces several microbial hazards. First, if the bacterium is a pathogen, he risks illness. Second, if the bacterium is resistant to antibiotics, the

resistance can be transferred to his own microflora. Farmers may also be in direct contact with animal feed that contains antibiotics. People working with farm animals have a higher percentage of antibiotic resistant *E. coli* in their intestinal microflora compared to the non-farming community (Levy et al., 1976).

Finally, resistance can spread from animal to food to human. Food is the direct link between microflora of an animal and the non-farming community. As previously discussed, antibiotic resistant bacteria have been isolated from animals as well as their food products. For meat products, it is not possible to slaughter an animal without contaminating the carcass with intestinal and skin/hide microflora (Jackson et al., 1997). Linton et al. (1977) were able to show resistant bacteria from chickens were carried along the food chain and colonized in man.

Significance of antibiotic resistant bacteria. Virulence is the degree to which a pathogen can cause disease. Antibiotic resistance has been associated with virulence factors. In one particular strain of *E. coli* isolated from piglets with diarrhea, genes controlling the production of enterotoxins were located on the same plasmid that encoded for tetracycline, streptomycin, and sulfonamide resistance (Gyles et al., 1977). The H2 plasmid carries resistance to chloramphenicol, kanamycin, streptomycin, and tetracycline. This plasmid was linked to enhanced survivability in calf intestines when tetracycline was introduced in the diet (Timoney and Linton, 1982).

Antibiotic resistance can not only increase the severity of disease it also makes treatment of diseases more difficult. *Staphylococcus aureus* can infect any part of the human body and is the main cause of bovine mastitis (Tueber, 1999). The most common cause of urinary tract infection is *E. coli* (Strauch, 1987c). Resistant strains are more difficult to eliminate and treatment becomes more costly.

Although some antibiotics fed to animals are not the same ones specifically prescribed to humans, there are other complications such as cross-resistance. For example, vancomycin is used for human therapy, while avoparcin is used in animal husbandry. Both antibiotics have the same mode of action by inhibiting cell wall synthesis, and resistance to one can render resistance to the other. The resistant bacteria can be transferred to humans by physical contact with the animal or through food (Witte, 1998).

Different components of the food chain are intricately intertwined (Figure 1). An example of this occurred in the former East Germany. The antibiotic streptothricin was used as a

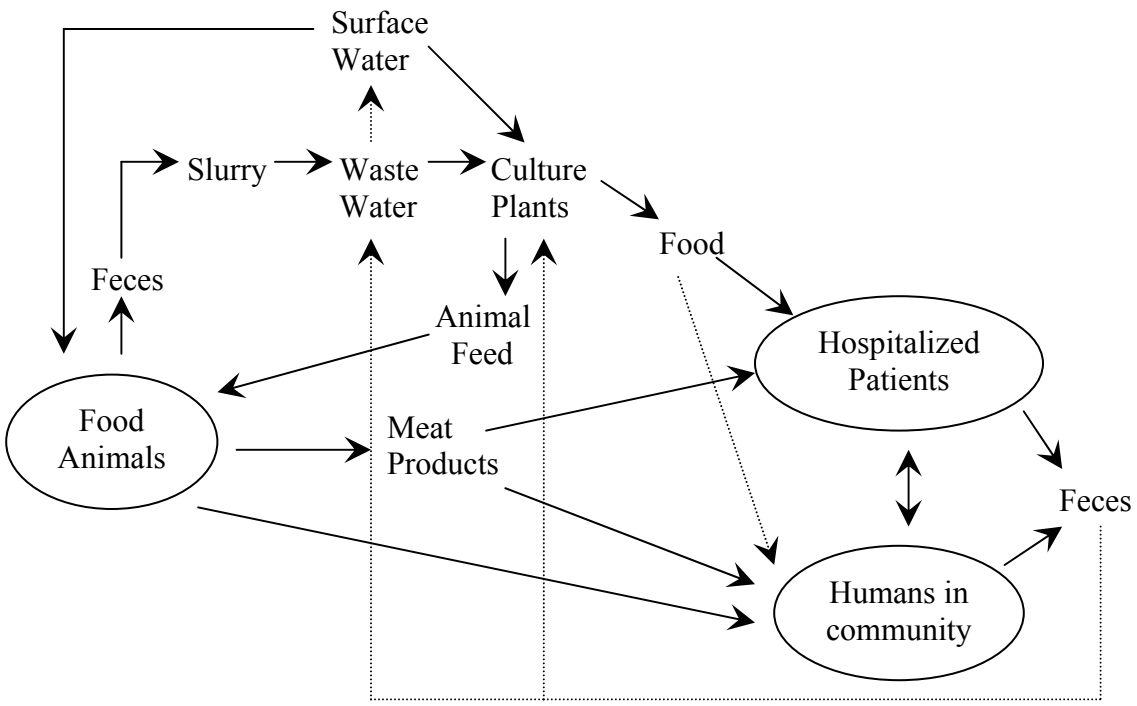


Figure 1. Network of Resistance. Adapted from Witte, W. 1998. Medical Consequences of Antibiotic Use in Agriculture. Science. 279: 996-997. Main reservoirs of antibiotic resistant bacteria include circled items. Antibiotics act as selective pressures on the main reservoirs. All arrows indicate routes of transmission**.**

growth promoter in pigs from 1982 to 1990. In 1984, resistance to the antibiotic was found in *E. coli* isolated from the gut flora of farm employees and their family members. By 1985, resistant *E. coli* were found in the urban population, milk and meat products, sick animals, and patients with urinary tract infections. Before the end of application in the early 1990's, resistance was found in bacteria causing the illnesses salmonellosis and shigellosis (Tschape, 1994). Interactions between human, animal, plant and environmental bacterial ecologies lead to an increase in the occurrence of antibiotic resistant pathogens. The threat of infection from a resistant, untreatable foodborne pathogen is increasing and becoming a significant risk in daily life (Mazel and Davies, 1999).

Foodborne Pathogens

Each year, an estimated 14 million people suffer from foodborne bacterial gastroenteritis (Mead et al., 1999). Food contains multiple nutrients that may support the growth of many pathogenic (disease-causing) bacteria. Gastroenteritis is a general term and refers to acute infectious diarrhea. The diarrhea usually lasts less that 2 weeks and may be accompanied by nausea, vomiting, fever, and abdominal pain (Schlossberg, 2001). From 1991 to 1993, bacterial foodborne illnesses cost Americans $2.9-$6.7 billion dollars annually, and the number of antibiotic resistant pathogens rises each year (CDC, 1998). A major challenge to human health is antibiotic resistance in foodborne pathogens. The resistance can develop by exposure to an antibiotic or occur through transfer of resistance genes from another bacterium as discussed in the previous section. Some of the major microorganisms causing foodborne illnesses are discussed here.

Salmonella. *Salmonella* species are Gram-negative rods that occur widely in nature. They are commonly found in the intestinal tracts of humans and animals. The organisms are excreted in feces and may be transported to numerous places, including water and food (Jay, 1996).

The genus *Salmonella* is divided into only 2 species, *S. enterica* and *S. bongori.* There are 6 subspecies within *S. enterica* (Table 1). Nearly all disease causing serotypes belong to this subspecies (Fierer and Guiney, 2001). Currently there are 1,478 serovars belonging to *S. enterica* subsp. *enterica* (Popoff et al., 2001). In this text, serovars are treated as species. For example, *S. enterica* subsp. *enterica* serovar Typhimurium will be referred to as *S. typhimurium*.

Table 1. Current number of *Salmonella* serovars.[a]

Species	Number
S. enterica	
subsp. *enterica*	1478
subsp. *salamae*	498
subsp. *arizonae*	94
subsp. *diarizonae*	327
subsp. *houtenae*	71
subsp. *indica*	12
S. bongori	21
Total	2501

[a]From Popoff et. al., 2001.

Salmonella species cause salmonellosis (gastroenteritis) and are responsible for an estimated 1.5 million bacterial foodborne cases each year. They cause an estimated 25% of hospitalizations and 31% of deaths caused by all foodborne illnesses, including bacterial, parasitic, and viral cases (Mead et al, 1999). *Salmonella typhimurium* and *S. enteritidis* are the most common isolated foodborne serovars (CDC, 2002). Foods associated with *Salmonella* involve raw meats, eggs, dairy products, raw fruits and vegetables, and water (CDC, 2001). Infective dose may be very low depending on age and health of host as well as differences among bacterial strains. In New York city, an outbreak of salmonellosis was due to non-dairy ice cream, and 150 viable salmonellae per gram of food were found (Armstrong et al., 1970). Symptoms usually appear in 12-72 hours and include nausea, vomiting, abdominal pain, chills, headache, and diarrhea and lasts 4-7 days (Schlossberg, 2001).

Most cases do not require treatment of antibiotics unless the infection does not clear up or the infection spreads. Complications following salmonellosis include septicemia (blood infection), reactive arthritis, and Reiter's syndrome (Petersen et al., 1996). Reiter's syndrome consists of urethritis (or cervicitis), conjunctivitis, and arthritis (Dworkin et al, 2001).

Resistance is important because of increased virulence and inability to treat the disease in humans and animals. Multi-drug resistance was found in 96% of *S. typhimurium* isolates from swine in North Carolina (Gerbreyes et al., 2000). A particularly virulent stain, *S. typhimurium* DT104, carries phage DNA with a typical multidrug-resistance pattern to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines. This phage type has also shown resistance to trimethoprim, spectinomycin, and ciprofloxacin (Teuber, 1999). Incidence of this multidrug-resistant organism is on the rise. In 1979-1980, occurrence of *S. typhimurium* isolates with a five-drug resistance pattern was 0.6%. In 1996, 34% of isolates displayed this pattern (Glynn et al., 1998). Fluoroquinolones are often prescribed to ill patients. However, an outbreak of DT104 in Denmark in 1998 proved difficult. The bacteria isolated had reduced sensitivity to fluoroquinolones. Out of 25 confirmed cases, 11 were hospitalized and 2 patients died (Mølbak et al., 1999).

Escherichia coli. *Escherichia coli* is a Gram-negative rod and is present as intestinal microflora of humans and animals. Fecal contamination of foods and water are responsible for *E. coli* gastroenteritis (Jay, 1996).

Five virulence types are recognized as a possible cause of gastroenteritis: enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and more recently enteroaggregative *E. coli* (EAggEC) (Levine, 1987). Foods associated with *E. coli* gastroenteritis include beef, unpasteurized milk and juice, raw fruits and vegetables, salad dressings, salami, and contaminated water (CDC, 2001).

EPEC and EIEC generally do not produce toxins. EPEC strains cause diarrhea in children under 1 year of age (Donnenberg and Kaper, 1992), and EIEC strains can cause voluminous bloody or non-bloody diarrhea (Levine, 1987). ETEC strains produce enterotoxins resulting in profuse watery diarrhea with a very sudden onset, and is a well known cause of traveler's diarrhea (Levine, 1987). Onset of symptoms for EPEC, EIEC, and ETEC is between 12 and 72 hours, and infective doses range between 10^5-10^7 viable cells (Eley, 1996). EAggEC strains produce enterotoxin (Savarino et al., 1991) and cause persistent diarrhea that lasts for over 2 weeks (Bhan et al., 1989).

EHEC strains produce Shiga-like toxins (SLT) and can cause hemorrhagic colitis (bloody diarrhea), severe abdominal cramps, nausea, and vomiting. Symptoms appear after an incubation period of 1-8 days and last for 5-10 days (CDC, 2001). The most well known strain, EHEC serotype O157:H7, was not recognized as a cause of human illness until 1982 (Bean and Griffin, 1990). Infective dose for EHEC O157:H7 is thought to be low, as few as 10 organisms causing illness (Keene et al., 1994; Willshaw et al., 1994).

Hemolytic uremic syndrome (HUS) is an illness that can develop from infection of SLT producing bacteria and may be associated with antibiotic therapy. The syndrome consists of hemolytic anemia, thrombocytopenia, and acute renal failure. Wong et al. (2000) conducted a study of children infected with *E. coli* O157:H7 gastroenteritis. HUS developed in 14%, half of which had previously received antibiotics. They concluded that antibiotic treatment of children with this type of infection increases the risk of developing HUS.

An estimated 270,000 illnesses per year are attributed to all *E. coli* infections, 74,000 from O157:H7, 37,000 from other Shiga-toxin producing *E. coli* (STEC) and 159,000 from non-STEC. However, STEC strains account for an estimated 5% of hospitalizations and 4% of deaths related to all foodborne illnesses, including bacterial, viral, and parasitic infections (Mead et al., 1999).

Antimicrobial therapy is generally not effective and could increase virulence. Antibiotic resistant *E. coli* have shown an increased ability to colonize the intestines (Timoney and Linton, 1982). Antibiotic treatment of EHEC infections is very controversial. SLT is encoded for by bacteriophage DNA (Schmidt, 2001). Antibiotics induce the phage lytic cycle via the SOS pathway (Fuchs et al., 1999). The SOS response is induced by DNA damage or inhibition of DNA replication. The lytic cycle involves mass-production of more phages, as well as SLTs. When cells lyse, phages and toxins are released, resulting in more damage to the patient (Neely and Friedman, 1998). Olaquindox and carbadox are used as growth promoters in the livestock industry and have been shown to increase toxin production in the same manner. This may influence transfer of virulence genes between microbes of the animal, increasing development and spread of new SLT producing bacteria (Köher et al., 2000).

There is evidence of increasing numbers of antibiotic resistant *E. coli* O157:H7 strains. Isolates collected between 1984-1987 were all sensitive to antibiotics. From 1989-1991, 7.4% of strains isolated were resistant to streptomycin, sulfisoxazole, and tetracycline (Kim et al., 1994). Not only is virulence increased, but the prevalence of this pathogen in food animals is increased.

Campylobacter jejuni. *Campylobacter jejuni* is a microaerophilic Gram-negative curved rod and is a common inhabitant of the intestinal tract of animals. This particular bacterium is quite fastidious and requires reduced oxygen (approximately 5%) and about 10% carbon dioxide for good growth in the laboratory. They are difficult to grow and stress such as exposure to air, drying, low pH, heating, and freezing hinder recovery (BAM, 1998). They do not grow at 25°C, and the optimum for growth is 42°C. Despite these hurdles, they can survive at 4°C for 2-4 weeks (Blaser et al., 1980).

Campylobacter infections cause campylobacteriosis (campylobacter enteritis or gastroenteritis). The majority of cases (over 90%) are attributed to *C. jejuni.* Other *Campylobacter* recognized as a cause of gastroenteritis include *C. coli, C. fetus,* and *C. upsaliensis* (McClellan et al., 2002). Foods associated with *Campylobacter* include poultry, unpasteurized milk, and contaminated water (CDC, 2001). However, *C. jejuni* has been isolated from many sources, including hamburger, cattle feces, sheep carcasses, and swine carcasses (Blaser, 1982). Symptoms of campylobacteriosis include watery diarrhea that may contain blood, fever, abdominal pain, nausea, headache, and muscle pain. Incubation period is 1-7 days

and the symptoms last about a week (Schlossberg, 2001). Infective dose is small, 800 cells being able to cause disease depending on bacterial strain (Black et al., 1988).

Surveillance has been limited due to a couple of factors. First, the organism requires special laboratory equipment to provide the proper environment for recovery and growth. In addition, this bacterium was not reported as a cause of foodborne illness until 1980 (Bean and Griffin, 1990). It is now thought that *C. jejuni* is the leading cause of bacterial diarrhea in the United States. Each year, estimates exceed those for salmonellosis at 2.5 million cases per year. They cause an estimated 17% of hospitalizations and 6% of deaths caused by all foodborne illnesses, including bacterial, parasitic, and viral cases (Mead et al, 1999). Complications of infection include septicemia, HUS, reactive arthritis, and Guillain-Barré syndrome, which can lead to temporary paralysis (Walder et al., 1982; Berden et al., 1979; Rhodes and Tattersfield, 1982).

Because campylobacteriosis is so prevalent, antibiotic resistance becomes a major concern. Sánchez et al. (1994) found a trend of increased quinolone resistance in *C. jejuni* isolates. On the average, 0% of strains tested were resistant in 1988 and 54% in 1992. Reina et al. (1994) had similar results, and reported an increase in tetracycline resistance. Tetracycline resistance is plasmid-mediated (Taylor et al., 1983) and reports of resistance may depend on specific strain and/or geographical area. The National Antimicrobial Resistance Monitoring System began surveillance on resistant *Campylobacter* in 1997. They discovered that fluoroquinolone resistance was on the rise, and patients infected with resistant bacteria had a longer duration of diarrhea compared to non-resistant infections (McClellan, 2002). Engberg et al. (2001) discuss emerging quinolone resistance in *C. jejuni* and link it to antibiotic use in the animal industry.

Clostridium perfringens. *Clostridium perfringens* is an anaerobic Gram-positive spore-forming rod that is widely distributed in the environment. It is frequently found in the intestines of humans and animals, and its spores persist in soils. This species is not a strict anaerobe and can tolerate some exposure to oxygen (Pearson, 1976).

The organisms are classified on their ability to produce certain types of toxins (types A, B, C, D, and E). Perfringens food poisoning is caused by type A strains. Gas gangrene strains are also of this type (Jay, 1996). Foods associated with this illness are meats, meat products, gravy, and dried or precooked foods (CDC, 2001). It has been found on 66% of fresh pork

products (NRC, 1999). Infective dose is high (approximately 10^8 viable cells) (Shandera et al., 1983). Symptoms appear 8-12 hours after consumption and include intense abdominal cramps and diarrhea. The illness runs its course usually within 24 hours (Schlossberg, 2001).

The illness is relatively mild and the true incidence of perfringens poisoning is unknown. It is estimated that 250,000 cases occur annually, and is one of the most commonly reported foodborne illnesses. Hospitalization and death rarely occur (Mead, 1999).

Ingested microbes produce enterotoxin in the intestine, and the toxin binds to intestinal epithelial cells. Binding is specific and leads to damaged host cell membranes which alters permeability properties. Glucose absorption is inhibited, while sodium and chloride ions are secreted (McDonel, 1980). For the farmer, decreased nutrient uptake means reduced feed efficiency. Eliminating *C. perfringens* from the intestines would improve growth performance (Strauch, 1987c). Another concern to poultry farmers is necrotic enteritis caused by type A or type C strains, which can lead to high mortality within a flock (Porter, 1998).

Antibiotic resistance increases the chance of survival and decreases elimination of the pathogen from the gastrointestinal tract. Tetracycline resistance is found on a conjugative plasmid and expression can be induced by exposure to subinhibitory levels of the antibiotic (Johanesen et al., 2001). Tetracycline and macrolide-lincosamide resistance in *C. perfringens* has been correlated to antimicrobials fed to pigs (Rood et al., 1985).

Treatment of bacterial foodborne gastroenteritis. Gastroenteritis is considered self-limited and usually does not require medical attention. However, it is recommended that a physician be consulted if there is fever, bloody stools, significant abdominal pain, dehydration, or risk factors. Treatment may include rehydration and administration of agents to control diarrhea. Treatment for specific microbes will depend on the clinical situation and/or identification of the pathogen. The latter requires time for growth of the organism. Often, empiric antibiotic therapy with a quinolone is prescribed before a complete diagnosis is made (Schlossberg, 2001). Without confirmation of a specific microbe and its resistance patterns, antibiotic treatment may create complications or increase severity of disease, such as prolonged diarrhea or HUS.

Risk factors for patients include age and disease state. Populations including the elderly, the very young, and patients recently treated with antibiotics are more susceptible to infection. Their intestinal flora is altered and the risk of gastroenteritis is increased. Populations with

compromised immune systems including the elderly, the very young, and people with certain diseases (i.e., AIDS and cancer) are more susceptible to complications (Schlossberg, 2002). A list of antibiotic treatments for foodborne illnesses can be found in Table 2.

Antibiotic use in food animals (meat and dairy) has implications for treatment of all bacterial infections, not just foodborne illnesses. Approved antibiotics used to treat infections in food animals include ampicillin, amoxicillin, erythromycin, oleandomycin, tetracyclines, gentamicin, streptomycin, penicillin, lincomycin, neomycin, spectinomycin, sulfonamides, and fluoroquinolones. Penicillin, ampicillin, some tetraclyclines, and oleandomycin are all approved for use to improve growth and feed efficiency. Some antibiotics (erythromycin and penicillin) are available over the counter and do not require a prescription from a veterinarian (NRC, 1999). All of the above mentioned antibiotics are used to treat a variety of human illnesses (Schlossberg, 2001).

Recall that resistance may develop to antibiotics in the same class. Although tetracycline may not be used for growth promotion, its derivatives (chlortetracycline and oxytetracycline) are used, and resistance to one can infer resistance to the other (Rood et al., 1985). The use of virginiamycin as a growth promoter selects for resistant *Enterococcus faecium* and staphylococci, and can confer cross-resistance to streptogramins used for human therapy (SCAN, 1998b). The use of tylosin and spiramycin as growth promoters can result in cross-resistance to erythromycin in Gram-positive and Gram-negative bacteria (SCAN, 1998a). Avilamycin is used as a growth promoter in swine and poultry, and has been linked to decreased sensitivity to evernimomicin, which has been under development for human use. Both compounds have the same mode of action (SCAN, 2000).

Reducing the Development and Spread of Antibiotic Resistance

There are several approaches to addressing the increasing problem of antibiotic resistant microorganisms. Each has advantages and disadvantages. Some proposed solutions only side step the true problem. Others are more difficult to achieve, but have positive and lasting results. Nevertheless, steps must be taken to prevent a post-antibiotic era. As previously discussed, pathogens not only become non-responsive to antibiotics, but their virulence may increase and severity of disease may intensify.

Table 2. Antibiotic therapy for food-borne gastroenteritis.[a]

Bacteria	Preferred	Alternatives	Comments
Salmonella typhimurium	Ciprofloxacin or Ceftriaxone[b]	Chloramphenicol, Amoxicillin or TMP-SMX[c,d]	
Campylobacter jejuni	Erythromycin	Ciprofloxacin, Aminoglycosides, Chloramphenicol	
EIEC, ETEC, EPEC[e]	Quinolone[f]	TMP-SMX, double strength	Antibiotic therapy is not recommended for EHEC strains
Clostridium perfringens			Antibiotic treatment not recommended

[a]Table adapted from Scholssberg, D., ed., 2001. Current Therapy of Infectious Disease.
[b]Ceftriaxone is a third-generation cephalosporin.
[c]TMP-SMX = trimethoprim-sulfamthoxazole.
[d]Amoxicillin or TMP-SMX treatment for neonate or immunocompromised child only after patient is stable and susceptibility data is known.
[e]EIEC = enterinvasive *E. coli*, ETEC = enterotoxogenic *E. coli*, EPEC = enteropathogenic *E. coli,* EHEC = enterohemorrhagic *E. coli.*
[f]Quinolone oral therapy options include ciprofloxacin, ofloxacin, and levofloxacin.

Discovery and creation of new antibiotics. A temporary solution is the discovery or creation of new antibiotics. Bacteria that were once difficult to grow in the laboratory can now be cultivated courtesy of newer, more successful culturing methods (Kaeberlein et al, 2002). When newly discovered bacteria are studied, chemicals they produce are also studied. In some cases, these chemicals are unique and may be antimicrobial in nature. Well-studied bacteria or fungi may also produce novel antimicrobials. A strain of the bacterium *Bacillus cereus* isolated from soil was found to produce a bacteriocin (a miniature protein) that inhibited the growth of pathogens and food spoilage bacteria (Bizani and Brandelli, 2002). Plus, scientists are constantly creating synthetic antibiotics and finding new drug targets (Silver and Bostian, 1993).

One advantage to this approach is good response to treatment. When new antibiotics are introduced effectiveness to treat or prevent disease in humans is very successful at first. When penicillin was first introduced it was considered a miracle drug and prevented many deaths due to common infections (Levy, 1992). However, microbes have the genetic ability to develop resistance quickly to any antimicrobial introduced. When penicillin resistance became prevalent in *Staphylococcus aureus*, methicillin became the drug of choice to treat infections. Only two years after being introduced, methicillin-resistant *S. aureus* (MRSA) isolates were discovered in the United Kingdom (Jevons, 1961). This led to the use of vancomycin to treat MRSA infections, and now vancomycin-resistant *S. aureus* strains have emerged (Sieradzki et al., 1999; Smith et al., 1999). Emerging resistance to antibiotics applies to other pathogens as well, such as *Streptococcus pneumoniae*, *Enterococcus* species, *E. coli*, and *Klebsiella pneumoniae* (Georgopapadakou, 2002).

Altering the use of antibiotics in human therapy. Antibiotics will always maintain a place in the treatment and prevention of diseases. Notwithstanding, there are circumstances where non-use of antibiotics is appropriate. Each year, millions of antibiotics are prescribed for colds, upper respiratory tract infections, and bronchitis (Gonzales et al., 1997). Antibiotics are only effective for treating bacterial or fungal infections and have little to no effect on these common ailments. Physicans' habits and pressure from patients cause over-prescription and lead to misuse resulting in antibiotic resistance (Williams and Heymann, 1998).

In Hungary in the 1980's, penicillin was cheap and overuse led to 50% of pneumococcus infections reported as penicillin resistant. By 1992, resistant infections were down by 16%. This occurred in part because physicians changed prescribing habits, relying on other classes of non-

β-lactam antibiotics (Nowak, 1994). This suggests that rotational use of antibiotics could alleviate some resistance problems.

Empiric antibiotic therapy is when antibiotics are administered early in an illness prior to culture identification and clinical response. These antibiotics are generally broad-spectrum, meaning they are active against a wide range of organisms. Conversely, narrow-spectrum drugs may target a specific genus or species of microbe (Conte, 2002). If the illness is caused by an antibiotic resistant strain, certain empiric antibiotics might not be appropriate. One author suggests that doctors use surveillance to make an educated decision on which antibiotic therapy to administer. Setting up surveillance systems could provide information to support decisions based on level of resistance in geographical regions as well as changes in the level of resistance. A web-based searchable database would provide doctors quick and easy access to this information (Felmingham, 2002).

Finally, a hospital in Finland was able to eliminate 10 strains of MRSA and prevent the spread of nosocomial (hospital acquired) MRSA infections. This was accomplished through strict and aggressive control measures. MRSA-positive patients were treated in contact isolation, and staff was intensively educated on hospital hygiene (Kotilainen et al, 2003).

Altering the use of antibiotics in livestock. There is without question a link between antibiotic use in livestock and antibiotic resistant bacteria affecting humans. Growing concerns were voiced by the Swann Committee in England in a 1969 report (Swann, 1969). The World Health Organization (WHO, 1997) and the United States Food and Drug Administration (FDA, 2000) have also reported possible medical risks resulting from the use of antimicrobials in animal feeds. The conclusions of these reports call for the limited use and elimination of antibiotics as growth promoters in animal feeds. More recently, Engberg et al. (2001) tracked macrolide and quinolone resistance emergence in *Campylobacter* species. They concluded that quinolone resistance in humans frequently coincides with or follows the approval of fluoroquinolone (a type of quinolone) use in livestock.

Several countries have already taken steps towards eliminating antibiotics as growth promoters. The European Union (EU) includes 15 member countries and 13 other eastern and southern European countries preparing for accession. The Council of the Union has passed several directives concerning additives in feedstuffs. The first Council Directive was passed in 1970 (EC, 1970). Since then there have been numerous amendments and regulations passed into

law (EC, 1994). Additives, including antibiotics, are authorized for a limited time and must be re-evaluated and re-authorized for continued use. Re-authorization requires the company to prove efficacy and safety. The additive must improve the feedstuffs or livestock without causing harm to the environment, animals, or humans. Harm includes development of antibiotic resistance to a particular antibiotic used in human medicine, or the development of cross-resistance to other antibiotics used in human medicine. The EU has already banned avoparcin (EC, 1997), bacitracin zinc, spriramycin, virginiamycin, and tylosin phosphate (EC, 1998a) due to antibiotic resistance. Carbadox and oliquindox were banned due to genotoxic, carcinogenic, and tumorigenic properties (EC, 1998b). The remaining growth promoting antibiotics flavophospholipol (flavomycin), salinomycin sodium, lasalocid sodium, and monensin sodium are being re-evaluated (EC, 2002).

While some antibiotic resistant genes will remain in the environment, there is evidence that resistance levels decrease after withdrawal of antibiotics in animal feeds. Some resistance is acquired through chromosomal mutation, as previously discussed. This mutation puts a strain on the cell and is conditional on continued growth under antibiotic pressure. When antibiotic pressure is removed, resistance may return to low levels. This has been shown for tetracycline and chloramphenicol resistance in *E. coli* (George and Levy, 1983). However, even with removal of antibiotics, low levels of resistance may remain. Low-level resistance is a precursor in adaptive evolution towards high-level, clinical resistance (Baquero, 2001). For example, a combination of low-level antibiotic resistance mechanisms may have a synergistic, additive effect resulting in high-level resistance.

The other method of resistance is through mobile genetic elements such as plasmids, transposons, integrons, and phages as previously discussed. One study has shown that exposure to antibiotics increases transfer rate of plasmids encoding antibiotic resistance in *Bacteriodes* species found in the human colon (Stevens et al., 1992). By removing antibiotic pressure there is less of a need for microbes to develop and/or transfer antibiotic resistance genes. Linton (1976) observed a decline in resistant *E. coli* isolated from swine feces after the removal of an antibiotic (aureomycin) from drinking water. Resistance declined from 90% resistant isolates at 93 days prior to slaughter to 35% on the day of slaughter. Resistance was attributed to transferable plasmids. In another study, six months after the removal of tetracycline from a chicken farm,

there was a reversal in selection for resistant organisms in human farm residents' fecal flora. Resistant bacteria contained transferable plasmids (Levy et al., 1976).

Alternatives to antibiotics. Animals carry bacteria in their feces and may contaminate carcasses, passing the organisms onto the meat products, and finally to the consumer. Bacteria that develop antibiotic resistance in the animal may also follow this route. If the resistant bacteria are pathogens, severe or untreatable illness may occur. Reducing pathogens in the animal decreases the chance of carcass contamination and illness in humans. However, antibiotic resistant bacteria may not be eliminated in animals by sub-therapeutic levels of antibiotics. Carcass contamination is unavoidable, but there are alternatives to antibiotics for the purpose of growth promotion in livestock. These alternatives can be used to prevent development and spread of antibiotic resistance as well as decrease the number of disease-causing organisms in livestock.

Animals that become stressed have a tendency to developed stress-related illnesses. Causes of stress include large numbers of animals of different origins in confined spaces, poor hygiene, climate conditions, and raising leaner but heavier animals. Sub-therapeutic levels of antibiotics are often fed to prevent stress-related illnesses. Reducing stress and/or replacing antibiotics with alternatives can eliminate the need for growth promoting antibiotics. A German study concluded that a homeopathic mixture of herbs and minerals was more effective at preventing diseases of the respiratory tract in pigs than sub-therapeutic levels of antibiotics (Albrecht and Schütte, 1999).

A probiotic is a "live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" (Fuller, 1989). Probiotics include many bacterial species and some fungi. Probiotics are associated with improved performance and reduction of disease. Piglets fed 10^6-10^7 viable spores of *Bacillus lichenformis* or *Bacillus cereus* var. *toyoi* exhibited reduced incidence and severity of diarrhea, significantly lowered mortality, and increased performance compared to negative controls (Kyriakis et al, 1999). In another study, piglets with rotavirus and *E. coli* associated diarrhea were fed 10^9 cfu/piglet/day (colony forming units per pig per day of *Bifidobacterium lactis* (Shu et al., 2001). The probiotic group exhibited reduced severity of diarrhea, increased performance, and lower concentrations of fecal rotavirus and *E. coli* compared to negative controls. Probiotics may also have a direct effect on the host's intestinal morphology and mucins (Baum et al., 2002).

A prebiotic is a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). Prebiotics include non-digestible but fermentable carbohydrates (oligo- and polysaccharides), some proteins, and certain lipids. The goal is to stimulate growth of advantageous bacteria (bifidobacteria and lactobacilli) while suppressing adverse bacteria (pathogens). In one study, rats fed resistant potato starch had stimulated growth of bifidobacteria (Kleessen et al., 1997). Another study indicated that swine treated with fructooligosaccharides in their drinking water had reduced fecal shedding of *S. typhimurium* (Letellier et al., 2000).

Food ingredients may be classified as a probiotic or prebiotic. Probiotic bacteria are used in cheeses, yogurts, sausages, and fermented milk. Dietary fiber is considered a prebiotic and is found in many foods of plant origin. Other ingredients may also have beneficial health effects. Phytochemicals are biologically active plant chemicals. For example, the anti-cancer compound lycopene is found in tomatoes (Love et al., 2000). Collectively, any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains can be called a "functional food" (Thomas and Earl, 1994). A variety of foods have anti-cancer properties, including soy, flaxseed, tomatoes, garlic, broccoli, tea, fish, dairy products, and beef (Hasler, 1998).

There are several terms used in the United States today to describe specific categories of foods (Table 3). The definitions often overlap and it can be difficult to categorize some particular foods. Currently, herbs are not considered foods or drugs, but "dietary supplements" by the Food and Drug Administration (DSHEA, 1994). However, in other countries such as Germany, herbs are considered to be drugs and are strictly controlled by the government (Blumenthal, 1998). In this text, functional foods and herbs will be considered separate categories, but collectively will be referred to as foods unless specifically noted.

Studies of foods in livestock diets are limited. There is some information available on clinical studies in humans, but studies on the effect of animal performance and gut microbiology are few. In some farming districts of Australia, it is a common practice to add raspberry juice or raspberry cordial (35% pure fruit juice) to the drinking water of livestock and humans (Ryan et al., 2001). This practice is thought to prevent or cure the symptoms of gastroenteritis. However, there is little to no scientific evidence to support this claim. Ryan et al. (2001) demonstrated the

Table 3. Definitions of food terms.

Term	Definition	Reference
Dietary Supplement	A product (other than tobacco) that is intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, and amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or concentrate, metabolite, constituent, extract, or combinations of these ingredients….is intended for ingestion in pill, capsule, tablet, or liquid form….is not represented for use as a conventional food.	DSHEA, 1994
Food	Any raw, cooked, or processed edible substance, ice, beverage, or ingredient used or intended for use or for sale in whole or in part for human consumption, or chewing gum.	FDA, 1999
Functional food	Any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains.	Thomas and Earl, 1994
Herb	A plant or plant part that is used to make medicine, food flavors (spices), or aromatic oils for soaps and fragrances.	Strohecker, 1994
Medical food	A food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirement on the basis of recognized scientific principles are established by medical evaluation.	U. S. Congress, 1988
Nutraceutical	Any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease.	Love et al., 2000
Prebiotic	Non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.	Gibson and Roberfroid, 1995
Probiotic	Live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance.	Fuller, 1989

antimicrobial nature of raspberry juice and cordial, inhibiting Gram-negative and Gram-positive pathogens.

Echinacea (purple coneflower) is an herb that can increase immune function (Rehman et al., 1999). One study demonstrated that inclusion of 3% echinacea in swine diets resulted in improved feed efficiency and daily gain compared with 0 or 1.5% echinacea. Daily gain of the pigs on the 3% diet was also equal to the control diet containing the growth promoter carbadox (Holden and McKean, 2000).

Advantages and Disadvantages of Functional Foods and Herbs

There are advantages and disadvantages to inclusion of food into livestock diets. Each must be studied before decisions are made, and the benefits must outweigh the risks. Scientific research must come first, followed by education of the community, then implementation on the farm.

Foods are not regulated as drugs are, and do not have to go through a lengthy approval process (NRC, 1999). Implementation of foods and herbs into the diet can begin without a waiting period. They can be readily available in mass quantities. They are also considered a "natural" alternative, which is becoming an increasing public demand.

Another problem is acceptance of new ideas by profit driven industries. Pharmaceutical companies would lose revenue from loss of antibiotic sales. Plus, farmers are leery of implementing new ideas. Antibiotics are confirmed to improve livestock performance. Studies have shown a 33 to 75% improvement in growth compared to non-antibiotic supplementation (Hays and Speer, 1960). The difference in percentages was due to environment. Cleaner conditions and healthier animals resulted in a smaller response to antibiotics. Other studies under experimental conditions have also shown growth improvement due to antibiotic supplementation (Cromwell, 2001).

A food must be proven to improve performance without side effects. Ephedra is an herb considered to be a dietary supplement and is not strictly regulated by the FDA. It is used to encourage weight loss or increase energy performance of athletes. This herb has been linked to stroke, heart disease, and sudden death (Samenuk et al., 2002). Garlic has been the subject of many studies and is thought to have multiple health benefits (Amagase et al., 2001). Yet, when included in a swine diet, animals had decrease performance and extremely objectionable off-

flavors in the meat (Holden et al., 1998). Animal studies must be done in order to get a true sense of the effects on treatment and prevention of diseases, growth performance, and quality of the final product.

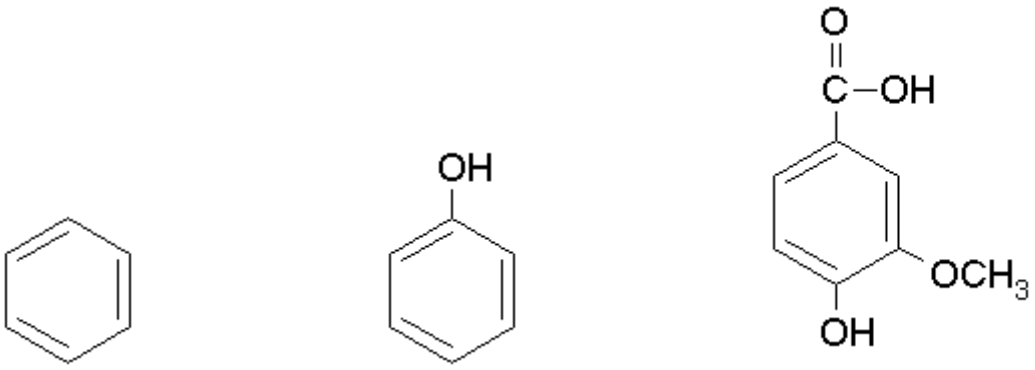
It is more costly to raise livestock without the use of growth promoting antibiotics (Larson and Kliebenstein, 2002). This does not take into account replacing antibiotics with alternatives. More importantly, it does not include the human cost of antibiotic resistance and the ability to effectively prevent and treat disease. Drugs of first choice are abundant and relatively cheap. If the first round of therapy is ineffective, a more costly drug might be the alternative choice (Conte, 2002). In extreme cases, the cost is loss of life due to an untreatable disease.

Another dilemma involves standardization. What are the active compounds in the food? How do they benefit the animal? What level of the compound is required in order to see a beneficial result? Are the levels consistent between cultivars? Many factors can affect the chemical composition of foods. Some herbal supplement companies impose standardization of active ingredients on their own products (Ternus, 1999).

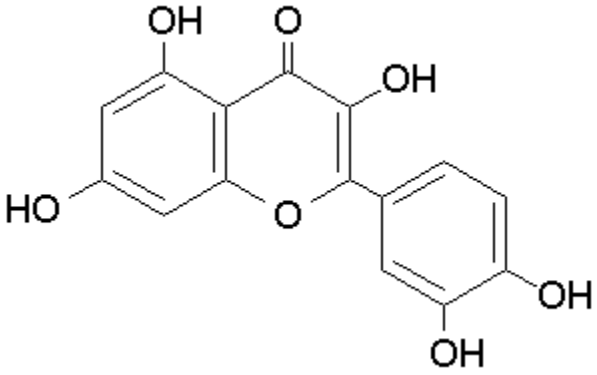
A major group of biologically active ingredients in plants is phenolic compounds (Shahidi and Naczk, 1995). These phytochemicals contain at least one aromatic (benzene) ring with one or more hydroxyl groups (OH) attached. Other compounds may also be attached (Figure 2). Level of phenolics may change depending on environmental conditions or plant cultivar.

Growing and harvesting conditions will have an affect on chemical composition. What type of soil is the plant grown in, and does is contain the proper nutrients? What are the weather conditions, has there been a drought, a late freeze, or ideal conditions? Geographic location can dictate soil type and climate to some degree. Crop load will affect chemical composition. High yielding apple trees have been shown to have smaller, less colorful fruits. Lower crop loads resulted in increased quality and higher phenolic concentrations in Jonagold Apples (Stopar et al., 2002). Maturity at harvest plays a role. Broccoli and cauliflower harvested from 3-day-old sprouts had higher concentrations of anticancer compounds compared to mature plants (Fahey et al., 1997).

Stress can cause chemical changes in plants. Physical stresses can occur from harvesting or insect infestation, and biological stresses can occur from fungal, bacterial, or viral infection.



Benzene Phenol Vanillic Acid



Quercetin

Figure 2. Benzene ring and phenolic compounds.

Phenolic compounds are a main defense chemical in many plants (Shahidi and Naczk, 1995). Stress response results in an increase in total phenolics content (Rhodes and Wooltorton, 1978).

Food processing can cause chemical changes. Thermally processed tomatoes into paste resulted in a decrease of the anti-cancer compound lycopene (Takeoka et al., 2001). Increased cooking time and presence of skin increased phenolic content of peach puree without affecting quality (Talcott et al., 2000).

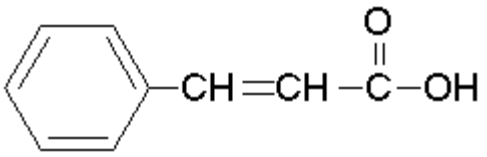
Regardless, active ingredients must be studied in the test tube (*in vitro*) and in living systems (*in vivo*) to get a complete picture of how a food is functional. Phenolic compounds are a very active group of phytochemicals and have been found to have antimicrobial, antioxidant, and anticarcinogenic activities. Phenolics extracted from the herb *Scrophularia* inhibited both Gram-positive and Gram-negative bacteria including *Bacillus subtilis, Staphylococcus aureus, E. coli, S. typhimurium* and *Moraxella lacunata* (Fernández et al., 1996). A direct relationship between antioxidant activity and total phenolic content of various vegetables, fruits, and herbs has been demonstrated (Velioglu et al., 1998). However, composition of the phenolics was different depending on plant type and was a factor in level of antioxidant activity. Antioxidant activity has been linked to anticarcinogenic properties due to the ability of phenolics to prevent DNA damage (Lodovici et al., 2001).

Caffeic Acid and Chlorogenic Acid

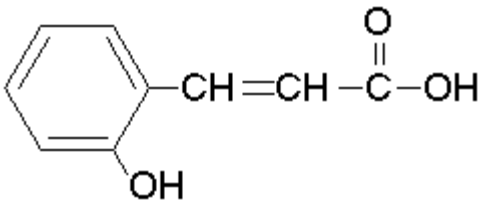
Some of the most common phenolic compounds found in foods of plant origin are the hydroxycinnamic acids (HCA), also called phenolic acids (Kroon and Williamson, 1999). HCAs contain at least one cinnamic acid and at least one –OH group (Figure 3). In particular, two prevalent HCAs are caffeic acid (CFA) and chlorogenic acid (CGA).

Caffeic acid is the simplest of the HCAs, bearing only a simple cinnamic acid group and two -OH groups (Figure 3). It has been found in numerous foods, including cereals, legumes, oilseeds, fruits, vegetables, herbs, and beverages (Table 4). CFA is the predominant HCA in most fruits (Shahidi and Naczk, 1995).

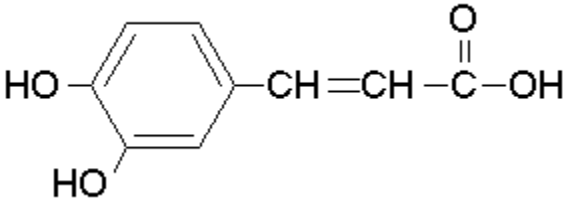
Caffeic acid has been shown to have antioxidant activities. Oxidative damage is caused by free radicals. Metal ions can initiate the formation of free radicals. CFA is able to chelate (bind) iron (Fe^{2+}) due to its hydroxyl groups (Kono et al., 1998) and has also been shown to chelate copper (Cu^{2+}) and scavenge free radicals (Nardini et al., 1995). In the latter study, CFA



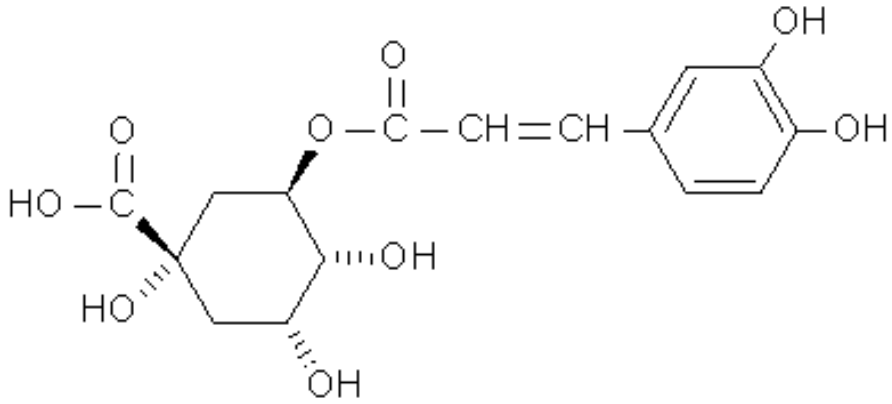
Cinnamic Acid



Coumaric Acid



Caffeic Acid



Chlorogenic acid

Figure 3. Cinnamic and hydroxycinnamic acids.

Table 4. Caffeic acid and chlorogenic acid content of selected foods and beverages.

Food/beverage	Caffeic Acid	Chlorogenic Acid	Reference
Apples	85-1270 mg/kg	173 mg/kg	Stich, 1991 van der Sluis et al., 2001
Artichoke	219 mg/kg		Radtke et al., 1998
Barley	7.0-18 mg/kg		Hernanz et al., 2001
Beer, German	1 mg/L		Shahidi and Nazck, 1995
Blueberry	977 mg/kg	1851-2075 mg/kg	Radtke et al., 1998 Schuster and Herrmann, 1985
Broccoli	19 mg/kg		Radtke et al., 1998
Brussel sprouts	34 mg/kg		Stich, 1991
Carrot, orange	85 mg/kg		Alasalvar et al., 2001
Carrot, purple	24 mg/kg	541 mg/kg	Alasalvar et al., 2001
Cherry, sour	121 mg/kg		Radtke et al., 1998
Coffee	631 mg/kg	1300 mg/L	Radtke et al., 1998 Stich, 1991
Corn	4.5 mg/kg		Shahidi and Naczk, 1995
Echinacea purpurea (herb)	125 mg/kg	320 mg/kg	Głowniak et al., 1996
Eggplant	330 mg/kg		Radtke et al., 1998
Endive	284 mg/kg		Radtke et al., 1998
Grapefruit	40 mg/kg		Radtke et al., 1998
Kiwi	384 mg/kg		Radtke et al., 1998
Lettuce	160-900 mg/kg		Stich, 1991
Oats	1-5 mg/kg		Shahidi and Naczk, 1995

Table 4 (continued)

Peach	159 mg/kg		Radtke et al., 1998
Pear	77 mg/kg	18-141 mg/kg	Raktke et al., 1998 Amiot et al., 1995
Plum	110 mg/kg		Raynal et al., 1989
Potato		96-187 mg/kg	Friedman, 1997
Prune	9-10 mg/kg	411-436 mg/kg	Donovan et al., 1998
Sorghum	6.0-8.7 mg/kg free 17-45 mg/kg bound		Shahidi and Naczk, 1995
Strawberries	15-39 mg/kg		Stich, 1991
Sunflower, meal	0.2%	2.7%	Cater et al., 1972
Tea	250 mg/kg		Stich, 1991
Tea, fennel		560-1000 mg/kg	Bilia et al., 2000
Tomato	24 mg/kg		Radtke et al., 1998
Wine, red	6-13 mg/L		Frankel et al., 1995
Wine, white	1-4 mg/L		Frankel et al., 1995

inhibited the oxidation of low-density lipoproteins (LDL), which is linked to atherosclerosis and heart disease.

CFA has been the focus of several antimicrobial studies. It has shown significant growth inhibition of *C. perfringens* without inhibiting *Bifidobacter* and *Lactobacillus* (Lee et al., 2001). Studies have shown inhibited growth of *E. coli* and *K. pneumoniae* at 300 ppm and the fungus *Aspergillus* at 200 ppm, and completely inhibited aflatoxin production at 200 ppm (Aziz et al., 1998). The inhibitory activity was attributed to presence of –OH groups. *E. coli* O157:H7 and *S. typhimurium* were also inhibited by 350 ppm CFA using an agar dilution method (Tun◊el and Nergiz, 1993). *Staphylococcus aureus*, *Bacillus subtilis*, and the yeast *Candida albicans* were inhibited at 200 ppm, while *Cornybacterium diptheria*, *Aspergillus niger*, *Aspergillus flavus*, and *Pullularia pullularis* were inhibited by 100 ppm of CFA (Binutu et al., 1995). Studies vary on whether CFA is an effective inhibitor of Gram-negative organisms. Regardless, phenolic content of some foods may affect survivability of Gram-negative pathogens. CFA in apple juice had a concentration dependent affect on the survival of *E. coli* O157:H7 (Reinders et al., 2001). Antiviral activity against Vesicular Stomatitis Virus (VSV) has also been reported at the level of 500 ppm CFA (Cheminat et al., 1988).

Chlorogenic acid is made up of two subunits, caffeic acid and quinnic acid (Figure 3). These subunits are released with hydrolysis. CGA is the most abundant soluble form of HCAs in the diet (Faulds and Williamson, 1999) and is thought to be the source of dietary CFA (Kroon and Williamson, 1999). CGA has been found in many of the same foods as caffeic acid (Table 4). CGA is also an antioxidant, with the same mechanisms as CFA (Kono et al., 1998). It has been shown to inhibit DNA damage due to oxidation (Kasai et al., 2000) and prevent colon cancer in rats (Mori et al., 2000). One study suggests its usefulness for controlling glucose levels in non-insulin-dependent diabetes (Hemmerle et al., 1997). CGA also has antimicrobial activity, though it is less studied than CFA. *Shigella sonnei* (Group D) was 78% inhibited by 400 μM of CGA (approximately 145 ppm; Tsou et al., 2000).

Influence of Diet on Health

The microbial ecology of an animal's gastrointestinal tract is a major factor in maintaining animal health. The natural flora in the intestine helps animals resist infections by competing with pathogens (Fuller, 1989). Volatile fatty acids (VFAs) are produced as a result of

microbial carbohydrate fermentation in the gut, and are absorbed and used as a source of energy in the large intestine of non-ruminants (Wolin, 1981). Diet composition greatly influences the microbial balance (Varel et al., 1984; Varel et al., 1987), and therefore influences disease resistance and available energy. Studying the effect of diet on microbial populations can be accomplished by studying live animals (*in vivo*), or environments in a test tube that mimic real life situations (*in vitro*). Studies on whole foods may provide an overall picture. However, in order to know specifically which phytochemicals have an effect, individual compounds must be studied separately.

Many species of animals are used to produce meat or dairy products. Pork consumption accounts for 40% of the annual total world consumption of meat (Pond and Lei, 2000). Improving growth characteristics are a main concern for farmers. However, studying swine may have human health implications. Despite some differences, pigs have an omnivorous digestive system similar to humans. Anaerobic fermentation of fiber and VFA production are comparable, and pig models are often used to predict what might happen in humans (Pond and Lei, 2000).

CHAPTER 3
Methods Development

Introduction

Certain aspects of this study required preliminary work prior to the main projects. Particular details of established and standard methods did not work upon first or second attempts. This required that the methods be adjusted slightly in order to achieve repeatable results before conducting the main study. The major factor was media composition.

Study 1. Aerobic Culture Growth in Ethanol

Introduction

The minimum inhibitory concentrations (MICs) of antimicrobial compounds on the growth of aerobic cultures were tested using the macrobroth dilution method as previously described (NCCLS, 2000). This method required that the compounds be solubilized in broth used to grow the bacteria. The compounds used in this study, caffeic acid (CFA) and chlorogenic acid (CGA) were not water soluble. CFA and CGA were previously reported to be freely soluble in alcohol (Budavari, 1989), and therefore the broth was reformulated to contain a specific amount of alcohol. Ethanol is lethal to aerobic bacteria in high concentrations. The objective of this preliminary study was to determine the ability of aerobic cultures to survive and grow in broth containing low concentrations of ethanol.

Materials and Methods

Media

Mueller-Hinton Broth (MHB; Difco Laboratories, Detroit, MI) was mixed with distilled water, 10.3 mL (10 mL after autoclaving) distributed into tubes, and autoclaved for 10 min at 121°C.

Ethanol (95%) was cold sterilized using a sterile Swinnex-25 syringe apparatus (Millipore Corporation, Bedford, MA) containing an Isopore™ membrane filter with 0.2µm pore size (Millipore). The volume of several MHB tubes were averaged and used as a base to determine the amount of filtered 95% ethanol to be aseptically added to achieve a final

concentration of 3% ethanol in the MHB tubes. An aliquot (330 μL) of 95% ethanol was added for every 10 mL MHB.

Bacterial cultures and conditions

Cultures tested were *Staphylococcus aureus* ATCC 12600, *S. aureus* ATCC 25923, *S. aureus* ATCC 27543, *S. aureus* ATCC 43300, *Escherichia coli* ATCC 11775, *E. coli* ATCC 25922, *E. coli* 25404, *E. coli* 43895, *Salmonella enteritidis* 108A (University of Kentucky Food Science stock culture), *Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Typhimurium ATCC 13311, *S. choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Typhimurium ATCC 700408, and *Salmonella sp.* TT42 (University of Kentucky Food Science stock culture isolated from swine feces).

Frozen stock cultures were transferred twice into MHB and incubated at 35°C for 24 hr. Cultures were checked for purity, transferred to MHB containing 3% ethanol, and incubated in the same conditions. The controls contained MHB with no ethanol. Presence or absence of growth was visually observed at 24 hr and compared to controls.

Results and Discussion

Two of the bacteria tested, *S. enteritidis* 108A and *E. coli* ATCC 25404, were inhibited by ethanol (growth was less than 0.125 McFarland). None of the other cultures were affected by the addition of 3% ethanol, and growth was comparable to the control with no ethanol (growth was >0.5 McFarland).

It is important when testing the MIC of a compound to eliminate possible interference. First, susceptibility cannot be reliably determined unless there is adequate growth. Ethanol inhibited growth of the two bacterial cultures, and it would not be possible to determine whether CFA or CGA would inhibit growth. Therefore, the two bacteria inhibited by 3% ethanol were removed from further MIC testing.

Study 2. Compound and Agar Component Interaction

Introduction

The MICs of antimicrobial compounds on the growth of anaerobic cultures were to be tested using the agar dilution method as previously described (NCCLS, 2001). This method calls for the use of a supplemented blood agar for the testing of the compounds. A previous study showed that *Clostridium perfringens* ATCC 13124 was inhibited by 0.1 mg (100 μg) of CFA using a disk diffusion method (Lee et. al., 2001). Another study indicated that *C. perfringens* ATCC 3624 was inhibited by 500 ppm (500 μg/mL) of CFA using a broth dilution method (Debrauwer, et. al., 1989). In this study, 1000 ppm of CFA failed to inhibit *C. perfringens* ATCC 13124 and *C. perfringens* 92D.

Other studies have shown that CFA and CGA can chelate iron (Kono et. al., 1998) and bind to hemoglobin (Suryaprakash et. al., 2000). The objective of this preliminary study was to determine which component of the supplemented blood agar was interacting with the compounds and interfering with the testing.

Materials and Methods

Antimicrobial compound

A 10 mg/mL stock solution of CFA (Sigma-Aldrich, St. Louis, MO) was solubilized in 70% ethanol and stirred with a magnetic stir bar for 5 min with gentle heating (40°C). Ethanol solution was prepared using distilled water. A final concentration of 1000 ppm was achieved by adding 2 mL of CFA stock solution into 18 mL of medium.

Media

Mueller-Hinton broth was mixed with distilled water, distributed into tubes, and autoclaved at 121°C for 10 min. Brucella broth (BB; Difco) was mixed with distilled water, distributed into tubes, and autoclaved at 121°C for 15 min. Brucella broth plus 0.16% agar was prepared by mixing with distilled water, supplemented with 1.6 g/L granulated agar (Difco), distributed into tubes, and autoclaved at 121°C for 15 min.

Cooked Meat Medium (CM; Difco) was distributed (1.25 g) into tubes and 10 mL of distilled water was added. Medium was allowed to sit for 10 min at room temperature before

autoclaving at 121°C for 15 min. If medium was not used within 24 hr, tubes were steamed for an additional 10 min and cooled just before use.

Mueller-Hinton Agar (MHA; Difco) and Brucella Agar (BA; Difco) were mixed with distilled water, steamed, and autoclaved at 121°C for 15 min.

Vitamin K_1 Brucella Agar (v-BA) was prepared by the addition of 5 mg/L vitamin K_1 (Sigma-Aldrich) to BA as previously described (NCCLS, 2001). Vitamin K_1 (3-phytylmenadione) stock solution was prepared by adding 0.2 mL to 20 mL of 95% ethanol and stored at 4°C for no longer than one year. Vitamin K_1 working solution was prepared by adding 1 mL of stock solution to 9 mL of sterile distilled water, and stored at 4°C for no longer than 1 month. Brucella Agar was mixed with distilled water, supplemented with 1 mL/L vitamin K_1 working solution, and autoclaved at 121°C for 15 min.

Hemin Brucella Agar (h-BA) was prepared by the addition of 1 mg/L hemin (Sigma-Aldrich) to BA as previously described (NCCLS, 2001). Hemin stock solution was prepared by dissolving 0.1 g into 2 mL of 1.0 N NaOH (Fisher Scientific, Fairlawn, NJ), the volume brought up to 20 mL with distilled water, and sterilized at 121°C for 15 min. Hemin was protected from light by wrapping the bottle in aluminum foil and stored at 4°C for no longer than 1 month. Brucella Agar was mixed with distilled water, supplemented with 1 mL/L hemin stock, and autoclaved at 121°C for 15 min.

Supplemented Brucella Agar (s-BA) was prepared by the addition of 5 mg/mL vitamin K_1 and 1 mg/mL hemin. Brucella Agar was mixed with distilled water, supplemented with 1 mL/L vitamin K_1 working solution and 1 mL/L hemin stock solution, and autoclaved at 121°C for 15 min.

Supplement Brucella Blood agar (s-BBA) was prepared by the addition of 5% defribrinated laked sheep blood (Gibson Laboratories, Lexington, KY) to sterile s-BA as previously described (NCCLS, 2001). Blood and autoclaved agar were tempered to 48°C before the addition of 5% blood.

All tested agar media was distributed in 18.2 mL aliquots (to achieve 18 mL after autoclaving) into 50-mL polypropylene BlueMax™ Falcon® conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ). The media was autoclaved for 15 min at 121°C. After cooling to 48°C, either 2 mL of CFA stock solution plus 1 mL sterile distilled water, 2 mL CFA stock

solution plus 1 mL sheep blood, or 2 mL 70% ethanol plus 1 mL sterile distilled water (controls) were added and mixed gently. Contents of the tube were poured into a sterile 15 x 100 mm round petri plate (Fisherbrand; Fisher) and allowed to solidify. Agar plates were placed in a 35°C incubator with lids ajar for 30 min to dry and were prepared on the day of inoculation.

Bacterial cultures and conditions

Isolates tested were *S. aureus* ATCC 12600, *S. aureus* ATCC 25923, *S. aureus* ATCC 27543, *S. aureus* ATCC 43300, *C. perfringens* 92D (University of Kentucky isolate from poultry), *C. perfringens* ATCC 13124, and *Campylobacter jejuni* ATCC 33291.

Staphylococcus aureus cultures were transferred twice into MHB and incubated at 35°C for 24 hr. Cultures were then streaked onto MHA and incubated at 35°C for 24 hr. Typical colonies were selected and suspended in MHB broth until 0.5 McFarland standard was achieved. Cultures were then spotted (2 μL) onto the various agars containing CFA or ethanol and incubated for 48 hr. Agars tested included MHA, RCM, BA, v-BA, h-BA, s-BA, and s-BBA.

All incubations for *C. perfringens* cultures were in Gas-Pak Anaerobic System jars with CO_2 System Envelopes (BBL, Sparks, MD) at 35°C for 24 hr. Cultures were transferred twice into CM. Cultures were then streaked onto s-BBA and incubated. Typical colonies were selected and suspended in Brucella broth until 0.5 McFarland standard was achieved. Cultures were then spotted (2 μL) onto the various agars containing CFA or ethanol and incubated for 48 hr. Agars tested included MHA, RCM, BA, v-BA, h-BA, s-BA, and s-BBA.

Incubations for *C. jejuni* were in a Gas Pak jar with Campylobacter System Envelopes (BBL) at 42°C for 48 hr. The culture was transferred twice in BB plus 0.16% agar. Cultures were then streaked onto s-BBA and incubated. Typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. Cultures were spotted (2 μL) onto agars containing CFA or ethanol. Agars tested included MHA, RCM, BA, v-BA, h-BA, s-BA, and s-BBA.

Results and Discussion

Binutu et al. (1995) had previously found that *S. aureus* was inhibited by CFA using an agar dilution method. In this study, *S. aureus* cultures were used as an aerobic control using an agar method. The cultures were inhibited by 1000 ppm CFA on MHA.

A summary of results can be found in Table 5. Caffeic acid was effective at inhibiting growth of both *C. perfringens* cultures at the level of 1000 ppm on MHA, BA, h-BA, v-BA, and s-BA. Supplementation with vitamin K_1 had no effect on the availability of CFA and did not interfere with antimicrobial action. The addition of hemin to the agar also did not interfere with the antimicrobial action. The structure of hemin contains an iron atom, which is covalently bound to four nitrogen atoms surrounded by four pyrrole rings linked by methene bridges (Figure 4). The iron in hemin is also bound to a chloride atom. Although CFA and CGA can chelate iron, the iron atom in hemin is not available to interact with the compounds. However, free iron and hemoglobin proteins are abundant in blood and were available to bind to CFA (Nilsson et al., 2002; Moran et al., 1994a). This binding interfered with the ability of CFA to prevent microbial growth. The use of blood in agar dilution medium formulations was discontinued in all other tests.

Growth of *C. perfringens* was not supported on RCM containing CFA or on the control containing 7% ethanol. None of the media tested supported the growth of *C. jejuni.* The lack of growth could have been due to the high level of ethanol combined with type of media used.

Study 3. Media Combinations

Introduction

Results from Study 2 indicated that *C. perfringens* and *C. jejuni* growth was not supported on certain agars. The agars contained 7% ethanol and might have influenced growth. Also, Stern et. al. (1988) concluded that combinations of source and growth medium influenced the effect of antimicrobial compounds. The objective of this study was to determine which combination of media best encouraged *C. perfringens* and *C. jejuni* growth.

Materials and Methods

Media

Brucella Agar (BA; Difco) was mixed with distilled water and autoclaved at 121°C for 15 min. Cooked Meat Medium (CM; Difco) was distributed (1.25 g) into tubes and 10 mL of distilled water was added. Medium was allowed to sit for 10 min at room temperature before

Table 5. Summary of results from Study 2[a].

Medium	Isolate	1000 ppm CFA	Ethanol Control
MHA	*S. aureus* ATCC 25923	-	+
	S. aureus ATCC 27543	-	+
	S. aureus ATCC 12600	-	+
	S. aureus ATCC 43300	-	+
	C. perfringens 92D	-	+
	C. perfringens ATCC 13124	-	+
	C. jejuni ATCC 33291	-	-
RCM	*C. perfringens* 92D	-	-
	C. perfringens ATCC 13124	-	-
	C. jejuni ATCC 33291	-	-
BA	*C. perfringens* 92D	-	+
	C. perfringens ATCC 13124	-	+
	C. jejuni ATCC 33291	-	-
h-BA	*C. perfringens* 92D	-	+
	C. perfringens ATCC 13124	-	+
	C. jejuni ATCC 33291	-	-
v-BA	*C. perfringens* 92D	-	+
	C. perfringens ATCC 13124	-	+
	C. jejuni ATCC 33291	-	-
s-BA	*C. perfringens* 92D	-	+
	C. perfringens ATCC 13124	-	+
	C. jejuni ATCC 33291	-	-
s-BBA	*C. perfringens* 92D	+	+
	C. perfringens ATCC 13124	+	+
	C. jejuni ATCC 33291	-	-

[a]MHA = Mueller-Hinton Agar, BA = Brucella Agar, h-BA = hemin supplemented Brucella Agar, v-BA = vitamin K_1 supplemented Brucella Agar, s-BA = hemin and vitamin K_1 supplemented Brucella Agar, s-BBA = hemin and vitamin K_1 supplemented Brucella Blood Agar. Agars contained either 1000 ppm caffeic acid (CFA) or 7% ethanol. (+) indicates growth, (-) indicates no growth.

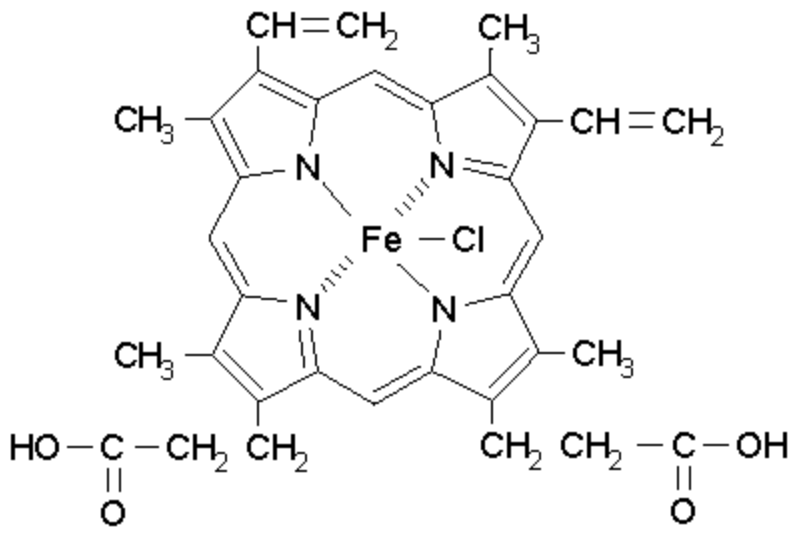


Figure 4. Structure of hemin.

autoclaving at 121°C for 15 min. If medium was not used within 24 hr, tubes were steamed for an additional 10 min and cooled just before use.

Reinforced Clostridial Medium (RCM; Difco) was mixed with distilled water, supplemented with 15 g/L granulated agar, and autoclaved at 121°C for 15 min. A final concentration of 1.5% agar was achieved in the medium.

Figures 5 and 6 illustrate the different combinations tested. Agar plates containing 3.5% ethanol were prepared by the addition of 1 mL of 70% ethanol to tubes containing 19 mL sterile, tempered (48°C) agar. Agar plates containing 7% ethanol were prepared by the addition of 2 mL of 70% ethanol to tubes containing 18 mL sterile, tempered agar. Plates were dried and as previously described.

Bacterial cultures and conditions

All incubations for *C. perfringens* were incubated in anaerobic Gas-Pak jars at 35°C for 24 hr unless otherwise stated. Initially, *C. perfringens* ATCC 13124 and *C. perfringens* 92G were streaked onto s-BBA, BA, and RCM containing no ethanol and growth was observed after 48 hr. Stock cultures were transferred twice into CM and incubated. Cultures were then streaked onto the appropriate agar. After incubation, typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. Cultures were then spotted (2 μL) onto the various agars containing ethanol and incubated for 48 hr.

All incubations for *C. jejuni* were in a microaeophilic incubator (5% O_2, 10% CO_2) for 48 hr at 42°C. Initially, *C. jejuni* ATCC 33291 was streaked onto s-BBA, BA, and MHA containing no ethanol and growth was observed after incubation. The stock culture was transferred twice in BB plus 0.16% agar and incubated. The culture was then streaked onto the appropriate agar. After incubation, typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. Cultures were then spotted (2 μL) to achieve a final inoculum of 2×10^5 bacteria onto the various agars containing ethanol and incubated.

Results and Discussion

A summary of results can be found in Table 6. *Clostridium perfringens* growth was supported on s-BBA, BA, and RCM containing no ethanol. Growth was inhibited on all agars containing 7% ethanol. Most of the agars containing 3.5% ethanol only supported the growth of

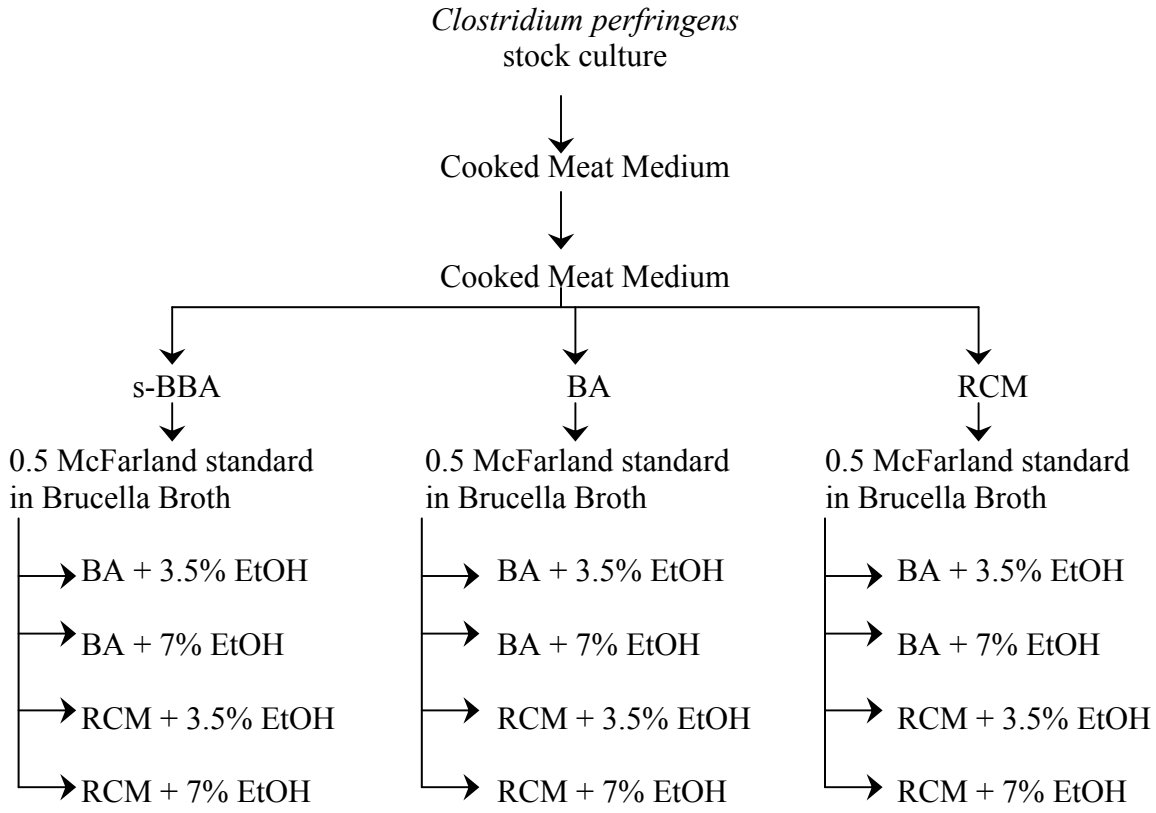


Figure 5. Flow chart of *Clostridium perfringens* for Study 3. s-BBA = supplemented Brucella Blood Agar, BA = Brucella Agar, RCM = Reinforced Clostridial Medium plus 1.5% agar, EtOH = ethanol, % EtOH represents total amount of ethanol in 20 mL agar plate. Incubations for transfers and initial agars were anaerobic at 35°C for 24 hours. Incubations for plates with ethanol were 48 hours.

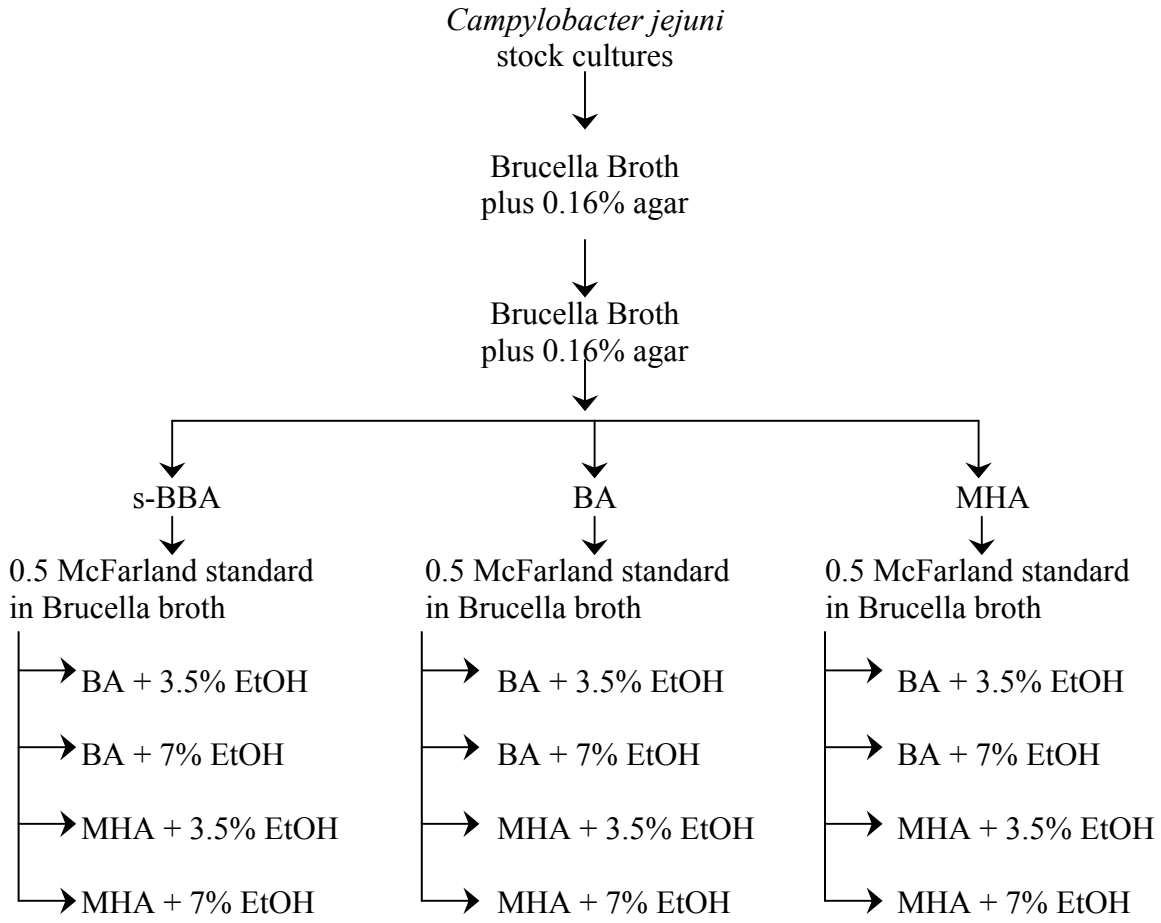


Figure 6. Flow chart of *Campylobacter jejuni* in Study 3. s-BBA = supplemented Brucella Blood Agar, BA = Brucella Agar, MHA = Mueller-Hinton Agar, EtOH = ethanol, % EtOH represents total amount of ethanol in 20 mL agar plate. All incubations were in a microaerophilic incubator (5% O_2, 10% CO_2) at 42°C for 48 hours.

Table 6. Summary of results for *Clostridium perfringens* cultures from Study 3[a].

Source Agar	Growth Agar	Isolate	Growth
s-BBA	BA + 3.5% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	+
	BA + 7% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	-
	RCM + 3.5% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	+
	RCM + 7% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	-
BA	BA + 3.5% EtOH	*C. perfringens* ATCC 13124	+
		C. perfringens 92D	+
	BA + 7% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	-
	RCM + 3.5% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	+
	RCM + 7% EtOH	C. *perfringens* ATCC 13124	-
		C. perfringens 92D	-
RCM	BA + 3.5% EtOH	*C. perfringens* ATCC 13124	+
		C. perfringens 92D	+
	BA + 7% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	-
	RCM + 3.5% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	+
	RCM + 7% EtOH	*C. perfringens* ATCC 13124	-
		C. perfringens 92D	-

[a]s-BBA = supplemented Brucella Blood Agar, BA = Brucella Agar, RCM = Reinforced Clostridial Medium plus 1.5% agar, EtOH = ethanol. (+) indicates growth, (-) indicates no growth.

C. perfringens ATCC 13124, but did not support the growth of *C. perfringens* 92D. Only two combinations, BA from BA and BA from RCM, were able to support the growth of both cultures.

The NCCLS (2001) standard for testing MICs with anaerobic cultures using the agar dilution method requires 2 mL of the selected compound be added to each agar plate. Recall that the compounds used in this project are solubilized with 70% ethanol. This results in 7% ethanol in the final agar. Because this will not support culture growth, a lower concentration for further MIC testing was used. A volume of 1 mL of the compound results in 3.5% ethanol in the final agar. This concentration supported the growth of *C. perfringens* cultures and therefore the standard method was adjusted. Brucella Agar from RCM containing 3.5% ethanol was used for further MIC testing for *C. perfringens* cultures.

Campylobacter jejuni growth was supported on s-BBA, BA, and MHA containing no ethanol. However, growth on MHA was minimal, and it was difficult achieving 0.5 McFarland needed for inoculation. There was no growth on any of the agars containing 3.5% or 7% ethanol. These percentages were too high to support growth.

Study 4. Ethanol and *Campylobacter jejuni* Growth

Introduction

Results from Study 3 revealed that *C. jejuni* cultures did not grow on any of the agar plates tested. This was attributed to amount of ethanol in the agar. The objective of this study was to determine what ethanol concentrations would allow the growth of *C. jejuni*.

Materials and Methods

Antimicrobial compounds

In order to achieve no more than 1.5% ethanol in the final media, a limit of 30% ethanol was set for solubility of the compounds. Caffeic acid stock solutions of 64 and 48 mg were mixed in 3 mL of distilled water. Seventy percent ethanol was added by 0.5 mL aliquots until the set limit was reached. Stock solutions were stirred with a magnetic stir bar for 5 min with gentle heating (40°C). Final concentrations of 640 and 480 ppm were achieved by adding 1 mL of CFA stock solution into 19 mL of medium.

Chlorogenic acid stock solutions of 64 and 48 mg were mixed with 3 mL distilled water. Ninety-five percent ethanol was added in 0.5 mL aliquots until CGA was solubilized. In addition, a CGA stock solution of 80 mg/mL was mixed with 4 mL of distilled water. Seventy percent ethanol was added by 0.5 mL aliquots until CGA was solubilized. Stock solutions were stirred with a magnetic stir bar for 5 min with gentle heating. Final concentrations of 800 and 600 ppm were achieved by adding 1 mL of CGA stock solution into 19 mL of medium.

Media

Brucella Agar was mixed with distilled water and distributed in 19.2 mL aliquots (to achieve 19 mL after autoclaving) into 50-mL polypropylene BlueMax™ Falcon® conical tubes. The media was autoclaved for 15 min at 121°C. After cooling to 48°C, 1 mL of CFA stock solution, 1 mL of CGA stock solution, or 1 mL of 30% ethanol (controls) was added and mixed gently. Contents of the tube were poured into a sterile 15 x 100 mm round petri plate and allowed to solidify. Agar plates were placed in a 35°C incubator with lids ajar for 30 min to dry and were prepared on the day of inoculation.

Bacterial culture and conditions

Campylobacter jejuni ATCC 33291 was incubated in a microaerophilic incubator (5%O_2, 10% CO_2) at 42°C for 48 hr. Stock culture was transferred twice in BB plus 0.16% agar and incubated. The culture was then streaked onto s-BBA. After incubation, typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. The culture was then spotted (2 μL) onto BA containing 1 mL of the stock solution, 1 mL of 30% ethanol, or 1 mL sterile distilled water as previously described and incubated.

Results and Discussion

Campylobacter jejuni growth was supported agar plates containing 1.5% ethanol and was comparable to growth on agar plates with no ethanol. Agar dilution plates containing 1.5% ethanol will be used for future MIC testing.

Chlorogenic acid went into solution at a concentration of 16 mg/mL in 14% ethanol. A volume of 1 mL was added to an agar plate for a final concentration of 800 ppm CGA and 0.7% ethanol. Growth was not inhibited.

Chlorogenic acid also went into solution at 16 and 12 mg/mL in 23% ethanol. A volume of 1 mL was added to agar plates for final concentrations of 800 and 600 ppm with 1.15% total ethanol. Growth was not inhibited.

Caffeic acid was not soluble at 12.8 and 9.6 mg/mL in 28% ethanol. A volume of 1 mL was added to agar plates for a final concentration of 640 and 480 ppm with 1.4% total ethanol. Despite the visible insolubility, CFA inhibited growth at 480 ppm.

The difference in the solubility of CFA and CGA lies in their structures (Figures 7 and 8). Chlorogenic acid includes a quinnic acid moiety and is more hydrophilic than CFA. This accounts for the ability of CGA to go into solution at a much lower ethanol percentage. Therefore, when conducting MICs, the solubility of CFA combined with the inability of *C. jejuni* to survive on agar containing ethanol are limiting factors.

Study 5. Compound Solubility

Introduction

There were two limiting factors for MIC testing of CFA on *C. jejuni* using the agar dilution method. First, *C. jejuni* did not grow on agar dilution medium containing more than 1.5% ethanol. Second, CFA was not soluble in some in ethanol/water mixtures. The highest concentration used for MIC testing was 1000 ppm. In order to achieve a maximum of 1.5% ethanol and 1000 ppm CFA in the agar dilution medium, the following combinations for CFA stock solutions would be required:

1) For 2 mL of stock solution into 18 mL agar, 10 mg/mL CFA in 15% ethanol
2) For 1 mL of stock solution into 19 mL agar, 20 mg/mL CFA in 30% ethanol
3) For 0.5 mL of stock solution into 19.5 mL agar, 40 mg/mL CFA in 60% ethanol

However, Study 4 demonstrated that 9.6 and 12.8 mg/mL CFA were not soluble in 28% ethanol. This excluded options 1 and 2 for the stock solution. Therefore, the objective of this study was to determine if 40 mg/mL CFA would solubilize in 60% ethanol.

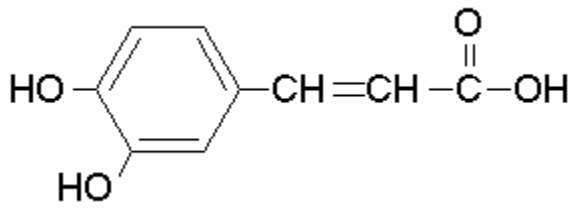


Figure 7. Structure of caffeic acid (3,4-dihydroxycinnamic acid).

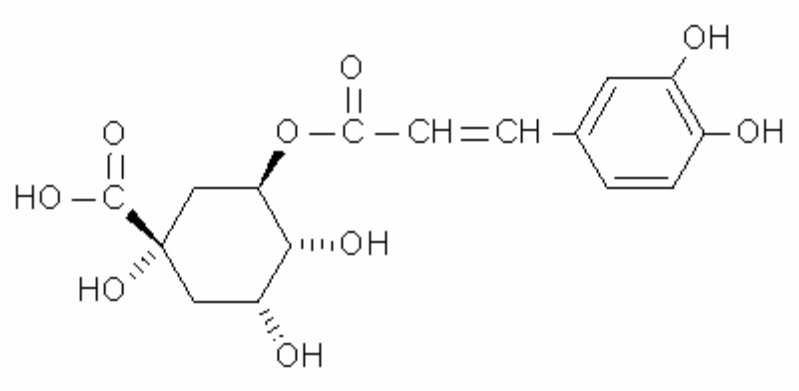


Figure 8. Structure of chlorogenic acid (5-caffeoylquinic acid).

Materials and Methods

Ethanol/water mixture was prepared with distilled water to achieve a 60% ethanol solution. Caffeic acid stock solution was prepared by the addition of 40 mg/mL CFA to 60% ethanol. The stock solution was stirred using a magnetic stir bar for 10 min with gentle heating.

Results and Discussion

Caffeic acid was soluble at the concentration of 40 mg/mL in 60% ethanol. Therefore, concentrations of less than 40 mg/mL CFA will also solubilize in 60% ethanol. This meets the requirements of 1.5% ethanol and 1000 ppm solubilized CFA or less in agar dilution medium. In this study, it was determined that distribution of 0.5 mL stock solution (60% ethanol) into 19.5 mL agar dilution medium was optimal for MIC testing of CFA on *C. jejuni*.

Study 6. *In Vitro* Survival of *Campylobacter jejuni*

Introduction

Initially, a main objective was set to determine the effect of CFA and CGA on survival of *C. jejuni* for an *in vitro* study using swine feces. However, the inoculated culture did not survive the incubation period. Because a minimal medium was used, it was thought that the culture could not survive in that particular medium. Thus, the objective of this study was to determine if *C. jejuni* cultures would survive the incubation period in a minimal medium.

Materials and Methods

Media

Phosphate and magnesium chloride dilution water was prepared as previously described (Marshall, 1992). Stock phosphate solution was prepared by dissolving 34 g KH_2PO_4 (Fischer) in 500 mL distilled water, pH adjusted to 7.2 with 1 N NaOH, the volume brought up to 1 L with distilled water, and sterilized at 121°C for 15 min. Stock $MgCl_2$ solution was prepared by dissolving 38 g $MgCl_2$ (Sigma-Aldrich) in 1 L distilled water, and autoclaved at 121°C for 15 min. Stock solutions were stored at 4°C until use. Class C dilution water was prepared by the addition of 1.25 mL stock phosphate solution and 5 mL $MgCl_2$ solution, and the volume brought up with distilled water to 1 L. The pH was adjusted to 7.4 (pH 7.2 after autoclaving). Media

was dispensed in aliquots of 10.2 mL into tubes or 105 mL into bottles (to achieve 9.9 and 99 mL blanks after autoclaving, respectively). Dilution blanks were then autoclaved for 15 or 30 min at 121°C for tubes or bottles, respectively. *Brucella stearothermophilus* spores, BT Sure biological indicator for steam sterilization (Barnstead/Thermolyne, Inc., Dubuque, IA), was used as an indicator of sterility. Spores were autoclaved with the media then incubated at 55°C for 24 hrs. Lack of growth indicated the autoclave was working properly.

Anaerobic dilution solution was prepared according to methods previously described (Holdeman and Moore, 1975). The salts solution contained 0.2 g/L anhydrous $CaCl_2$ (J. T. Baker Chemical Company, Phillipsburg, NJ), 0.2 g/L $MgSO_4 \bullet 7H_2O$ (Sigma-Aldrich), 1 g/L K_2HPO_4 (Fischer), 1 g/L KH_2PO_4, 10 g/L $NaHCO_3$ (Fisher), and 2 g/L NaCl (Fischer). Salts were dissolved in distilled water. For each liter of media, 500 mL of distilled water was combined with 2 g/L gelatin (Difco), 1 mL resazurin solution, and 500 mL of salt solution. Resazurin (Sigma-Aldrich) solution was prepared by dissolving 100 mg in 100 mL distilled water. The medium was boiled then cooled, and 5 g/L L-cysteine (Sigma-Aldrich) was added under a constant flow of CO_2 using gas jets. Medium continued under a flow of CO_2 until it turned from pink to colorless. A volume of 50 mL was anaerobically transferred into a 125-mL serum bottle under a flow of CO_2. The bottle was sealed by a butyl rubber stopper and crimp-sealed. The rest of the medium was transferred to the anaerobic chamber for fecal processing. The anaerobic chamber was under a CO_2 environment in the absence of O_2.

Campylobacter Agar Base (Karmali; Oxoid, Hampshire, England) was mixed with distilled water and sterilized at 121°C for 15 min. Sterile agar was tempered to 48°C and supplemented with freeze-dried Campylobacter Selective Supplement SR167E (Karmali; Oxoid). One vial of supplement contained 50 mg sodium pyruvate, 16 mg cefoperazone, 10 mg vancomycin, and 50 mg cyclohexamide. Vial components were dissolved by 2 mL of 50% ethanol and aseptically added to every 500 mL of sterile Karmali Agar. Karmali agar was used for the enumeration of *Campylobacter coli* and *C. jejuni* at 42°C.

Fecal samples

Swine feces were collected from a Duroc X Landrace/Yorkshire crossbred barrow. The animal was 4 months of age, weighed 60 kg, and was housed at the University of Kentucky Animal Laboratory. The diet consisted of 82% corn, 16% dehulled soybean meal, 1% dicalcium

phosphate, and less than 1% of lysine, limestone, salt, vitamin and mineral mix, and 0.05% Tylan-40. Pigs were kept in individual 5' x 10' pens with slatted floors, continuous lighting, and a constant temperature of approximately 25°C. Water was available *ad libitum.* A fresh stool sample was obtained, placed in a plastic bag, and delivered into the anaerobic chamber within 10 min of collection for processing. Anaerobic chamber was under a CO_2 environment in the absence of O_2.

Fresh feces (10 g) was blended with 100 mL anaerobic dilution solution in a Waring blender for 60 seconds to achieve a 10% (w/v) fecal slurry. The slurry (50 mL) was transferred into a 125-mL serum bottle under a constant flow of CO_2. The bottle was sealed by a butyl rubber stopper and crimp-sealed.

Bacterial culture and conditions

Campylobacter jejuni ATCC 33291 was transferred twice in BB plus 0.16% agar and incubated microaerophilically at 42°C for 48 hr. The culture was streaked onto s-BBA and incubated for another 48 hr in the same conditions. Colonies were suspended in sterile dilution water until 0.5 McFarland Standard was achieved. An inoculum amount of 1 mL was anaerobically transferred to serum bottles containing anaerobic dilution solution or fecal slurry. Serum bottles were incubated in a shaking water bath at 125 rpm for 24 hr at 39°C. Initial slurry and final samples were diluted using sterile dilution water, and 0.1 mL was plated using the spread plate method onto Karmali agar. Plates were incubated in a microaerophilic incubator (5% O_2 and 10% CO_2) at 42°C for 48 hr.

Results and Discussion

A summary of results can be found in Table 7. Bacterial counts for the initial slurry and slurry at 24 hr were comprised of both *C. jejuni* and *Campylobacter coli.* Karmali agar supports the growth of both organisms for these incubation conditions. Without further testing, it is not possible to distinguish between these two species.

Bacterial counts for the medium containing only the *C. jejuni* inoculum after 24 hr had good survival. The counts were reduced by only 10% (less than 10^1 CFU/mL). On the other hand, when feces were added to the combination, survival was drastically reduced. The counts were diminished by 64% (10^4 CFU/mL). The pH did not drop after 24 hr, and a pH of 6.5 is well

Table 7. *In vitro* survival of *Campylobacter jejuni* in medium[a] with and without feces.

	CFU/mL[c]	pH
Initial fecal slurry[b]	1.7×10^5	
Medium plus inoculum[d] at 24 hours	4.5×10^5	6.5
Fecal slurry plus inoculum[d] at 24 hours	2×10^2	6.5

[a]Medium consisted of anaerobic dilution solution.
[b]Fecal slurry was 10% (w/v) feces (wet weight) in the medium.
[c]CFU/mL = colony forming units per milliliter.
[d]Inoculum was *C. jejuni* ATCC 33291 at the level of 2×10^6 bacteria/mL.

within the range for supporting *Campylobacter* survival (optimum pH of 7.0). *Campylobacter* requires 5-10% oxygen; yet, they can survive, but not grow, under reduced-oxygen conditions (BAM, 2001). However, the microbial population of swine feces is very diverse and competitive. This particular environment may introduce too much competition for *C. coli* and *C. jejuni* to survive and grow.

CHAPTER 4

Minimum Inhibitory Concentrations of Caffeic Acid and Chlorogenic Acid and Their Effect on the Microbial Ecology of Swine Feces *In Vitro*

Introduction

Preliminary work in Chapter 3 described adjustments to established and standard methods needed to complete this study. These adjustments involved formulation changes to the different media types.

The first objective of this study was to determine the Minimum Inhibitory Concentrations (MICs) of caffeic acid and chlorogenic acid against potential pathogens. The MIC is the minimal concentration of an antimicrobial required to inhibit or kill a microorganism (Murray, 1995). This information is useful when studying specific compounds and specific microorganisms. It may also provide clues as to how an antimicrobial will affect overall microbial ecology.

The second objective was to determine the effect of caffeic acid and chlorogenic acid on the fecal microbial population of swine. *In vitro* studies can offer a general look at the microbial population without the use of live animals. *In vitro* studies sustain a more controlled and uniform environment compared to using a number of different animals. While this provides invaluable information, all aspects must be considered.

Materials and Methods

Minimum Inhibitory Concentration

Antimicrobial compounds. Caffeic acid (CFA; Sigma-Aldrich, St. Louis, MO) and chlorogenic acid (CGA; Sigma-Aldrich) were tested at the levels of 0, 50, 100, 200, 300, 400, 500, 600, 800, and 1000 ppm. CFA and CGA were solubilized in 70% ethanol and stirred with a magnetic stir bar for 5 min with gentle heating (40°C). A stock solution of 20 mg/mL was prepared and a portion was diluted with 70 % ethanol by a 1:2 ratio to achieve a solution of 10 mg/mL. A stock solution of 16 mg/mL was prepared and a portion was diluted with 70% ethanol in a series of 1:2 ratios to achieve concentrations of 8 mg/mL, 4 mg/mL and 2 mg/mL. Finally, a

stock solution of 12 mg/mL was prepared and a portion was diluted with 70% ethanol by a 1:2 ratio to achieve 6 mg/mL solution. For the testing of *Campylobacter jejuni,* CFA and CGA were solubilized and diluted in 60% ethanol. Ethanol solutions were prepared using distilled water.

For aerobic cultures, stock solutions were filter sterilized using a sterile Swinnex-25 syringe apparatus (Millipore Corporation, Bedford, MA) containing an Isopore™ membrane filter with 0.2μm pore size (Millipore).

Media. Brain Heart Infusion Broth (BHI; Difco Laboratories, Detroit, MI) was mixed with distilled water, 6 mL distributed into tubes, and autoclaved for 15 min at 121°C. Brucella broth (BB; Difco) was mixed with distilled water, 10 mL were distributed into tubes, and autoclaved at 121°C for 15 min. Brucella broth plus 0.16% agar was prepared by the addition of 1.6 g/L granulated agar (Difco), mixed with distilled water, 10 mL distributed into tubes, and autoclaved for 15 min at 121°C. Reinforced Clostridial Medium (RCM; Difco) plus 1.5% agar was prepared by the addition of 15 g/L granulated agar and mixed with distilled water before autoclaving at 121°C for 15 min.

Cooked Meat Medium (CM; Difco) was distributed (1.25 g) into tubes and 10 mL of distilled water added. Medium sat for 10 min at room temperature prior to autoclaving at 121°C for 15 min. If the medium was not used within 24 hr, tubes were steamed for an additional 10 min and cooled just before use.

Mueller-Hinton Broth (MHB, Difco) was mixed with distilled water. For culture growth tubes, 10 mL were distributed into tubes and autoclaved for 10 min at 121°C. For the macrobroth dilution tubes used for the testing of Minimum Inhibitory Concentrations (MICs) of aerobic cultures, 200 mL MHB were distributed into bottles and autoclaved for 10 min at 121°C. Then, 0.9 mL of sterile medium was aseptically transferred to sterile 13 x 100 glass tubes in order to avoid inconsistent volumes from autoclaving. The tubes were incubated at 25°C overnight to verify sterility. The CFA and CGA stock solutions were prepared, as described, and 0.1 mL was aseptically added to each sterile 13 x 100 broth tube on the day of inoculation.

Agar dilution plates for testing the Minimum Inhibitory Concentration (MIC) on *Clostridium perfringens* cultures contained Brucella Agar (BA; Difco). The medium was mixed with distilled water and distributed in 19.2 mL aliquots (to achieve 19 mL after autoclaving) into 50-mL polypropylene BlueMax™ Falcon® conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ). The medium was autoclaved for 15 min at 121°C, cooled to 48°C, and 1 mL of CFA

or CGA stock solution was added and mixed gently. Contents of the tube were poured into a sterile 15 x 100 mm sterile round petri plate (Fisherbrand, Fischer Scientific, Fairlawn, NJ) and allowed to solidify. Agar plates were placed in a 35°C incubator with lids ajar for 30 min to dry and were prepared on the day of inoculation.

Agar dilution plates for the MIC of *Campylobacter jejuni* contained Supplemented Brucella Agar (s-BA). The medium was prepared by the addition of 1 mg/L hemin and 5 mg/L vitamin K_1 to BA as previously described (NCCLS, 2001). Hemin (Sigma-Aldrich) stock solution was prepared by dissolving 0.1 g into 2 mL of 1.0 N NaOH (Fisher), the volume brought up to 20 mL with distilled water, and sterilized at 121°C for 15 min. Hemin was protected from light by wrapping aluminum foil around a dark glass bottle and stored at 4°C for no longer than 1 month. Vitamin K_1 (3-phytylmenadione; Sigma-Aldrich) stock solution was prepared by adding 0.2 mL to 20 mL of 95% ethanol and stored at 4°C for no longer than one year. Vitamin K_1 working solution was prepared by adding 1 mL of stock solution to 9 mL of sterile distilled water, and stored at 4°C for no longer than 1 month. Brucella Agar was supplemented with 1 mL/L hemin stock and 1 mL/L vitamin K_1 working solution. Supplemented Brucella Agar was distributed in 19.8 mL aliquots (to achieve 19.5 mL after autoclaving) into 50-mL polypropylene BlueMax™ Falcon® conical tubes and autoclaved. Agar was cooled to 48°C and 0.5 mL of CFA or CGA stock solution was added and mixed gently. Contents of the tube were poured into a 15 x 100 mm sterile round petri plate and allowed to solidify. Agar plates were placed in a 35°C incubator with lids ajar for 30 min to dry and were prepared on the day of inoculation.

Supplement Brucella Blood agar (s-BBA) was prepared by the addition of 5% defribrinated laked sheep blood (Gibson Laboratories, Lexington, KY) to sterile s-BA as previously described (NCCLS, 2001). After autoclaving, blood and agar were tempered to 48°C prior to addition of 5% blood. Plates were allowed to solidify and dried for 30 min at 35°C in an incubator with lids ajar. Unused plates were stored at 4°C for no longer than 48 hr. Blood was stored at 4°C for no longer than 1 month.

Bacterial Cultures and Conditions. A complete list of bacterial strains tested is presented in Table 8. Aerobic cultures, including *Salmonella, Escherichia coli,* and *Staphylococcus aureus* were tested for susceptibility using the macrobroth dilution method as previously described (NCCLS, 2000). Frozen stock cultures were transferred into Brain Heart

Table 8. List of bacterial strains used for Minimum Inhibitory Concentration testing.

Strain	Source	Incubation conditions
Campylobacter jejuni subsp. *jejuni* ATCC[a] 33291	Human feces	Microaerophilic[b], 42°C, 48 hrs
Clostridium perfringens ATCC 13124	Quality control strain, produces α-toxin	Anaerobic, 35°C, 24 hrs
Clostridium perfringens (92D)	University of Kentucky isolate from poultry	Anaerobic, 35°C, 24 hrs
Escherichia coli ATCC 11775	Urine	Aerobic, 35°C, 24 hrs
Escherichia coli ATCC 25922	Clinical isolate	Aerobic, 35°C, 24 hrs
Escherichia coli O157:H7 ATCC 43895	Hamburger	Aerobic, 35°C, 24 hrs
Salmonella choleraesuis subsp. *choleraesuis* (Smith) Weldin serotype Typhimurium ATCC 13311	Food Poisoning in Man	Aerobic, 35°C, 24 hrs
Salmonella choleraesuis subsp. choleraesuis (Smith) Weldin serotype Typhimurium, ATCC 700408	Phage type DT104, multi-drug resistant	Aerobic, 35°C, 24 hrs
Salmonella sp. (TT42)	University of Kentucky isolate from swine feces	Aerobic, 35°C, 24 hrs
Staphylococcus aureus ATCC 12600	Pleural fluid	Aerobic, 35°C, 24 hrs
Staphylococcus aureus ATCC 25923	Clinical isolate	Aerobic, 35°C, 24 hrs
Staphylococcus aureus ATCC 27543	Mastitic cow	Aerobic, 35°C, 24 hrs
Staphylococcus aureus ATCC 43300	Clinical isolate, methicillin resistant	Aerobic, 35°C, 24 hrs

[a] ATCC = American Type Culture Collection.
[b] Microaerophilic conditions were 5% O_2, 10% CO_2, 85% N_2.

Infusion broth (BHI, Difco) and incubated 24 hr at 35°C. Cultures were checked for purity, transferred to fresh BHI broth, and incubated 8 hr. Cultures were transferred into MHB containing 3% ethanol, and incubated for 24 hr at 35°C. After incubation, cultures were transferred to MHB and adjusted to 0.5 McFarland standard. One mL of culture was then transferred into macrobroth dilution tubes containing MHB and CFA or CGA, and incubated 24 hr at 35°C.

Clostridium perfringens cultures were tested using the agar dilution method for susceptibility of anaerobic bacteria according to NCCLS standards (NCCLS, 2001). All incubations were in anaerobic Gas-Pak jars (BBL, Sparks, MD) using CO_2 system envelopes (BBL) for 24 hr at 35°C. Frozen stock cultures were transferred twice in CM. The cultures were then streaked onto RCM containing 1.5% agar. After incubation, typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. Cultures were then spotted (2 µL) onto BA containing various concentrations of CFA and CGA stock solutions and incubated for 48 hr. Hemin, vitamin K_1, and blood were not added to the agar dilution plates used for susceptibility testing.

Campylobacter jejuni was tested using the agar dilution method for susceptibility of anaerobic bacteria according to NCCLS standards (NCCLS, 2001). All incubations were in a microaerophilic incubator (5% O_2, 10% CO_2) at 42°C for 48 hr. Frozen stock was transferred twice into in BB plus 0.16% agar, and then streaked onto s-BBA. Typical colonies were selected and suspended in BB until 0.5 McFarland standard was achieved. Cultures were spotted (2 µL) onto s-BA containing various concentrations of CFA and CGA stock solutions and incubated. Blood was not added to the agar dilution plates used for susceptibility testing.

In Vitro Incubation

Media. A complete list of media used is in Table 9. Anaerobic dilution solution was prepared according to methods previously described (Holdeman and Moore, 1975). The salts solution contained 0.2 g/L anhydrous $CaCl_2$ (J. T. Baker Chemical Company, Phillipsburg, NJ), 0.2 g/L $MgSO_4 \bullet 7H_2O$ (Sigma-Aldrich), 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 10 g/L $NaHCO_3$, and 2 g/L NaCl (Fisher Scientific). Salts were dissolved in distilled water. For each liter of media, 500 mL of distilled water was combined with 2 g/L gelatin (Difco), 1 mL resazurin solution, and 500

Table 9. Complete list of media and their use in this study.

Media	Purpose	Target Culture and Incubation Conditions
Anaerobic Dilution Solution[a]	*In vitro* incubation	Fecal microflora, shaking water bath, 125 rpm, 39°C for 24 hrs
BS-LV[b]	Enumeration	*Bifidobacteria*, anaerobic, 37°C for 72 hrs
Brain Heart Infusion broth (BHI)[c]	Enrichment	*S. aureus, E. coli, S. enteritidis, S. typhimurium, Salmonella* sp., 35°C for 8 or 24 hrs
Brucella Agar (BA)[c]	Agar dilution susceptibility testing	*C. perfringens*, anaerobic, 35°C for 48 hrs.
Brucella Broth (BB)[c]	0.5 McFarland standard for agar dilution testing	*C. perfringens, C. jejuni*, no incubation
Coliform/*E. coli* Petrifilm[d]	Enumeration/ Differentiation	*E. coli*, coliforms, 35°C for 24 hrs
Cooked Meat Medium[c]	Enrichment	*C. perfringens*, anaerobic, 35-37°C, 24 hrs
Granulated Agar[c]	Supplement to RCM and Brucella Broth	*C. perfringens*, Total Anaerobes, C. jejuni
Karmali Agar (KA)[e]	Enumeration	*C. jejuni and C. coli*, microaerophilic, 42°C for 72 hrs
Mueller-Hinton Broth (MHB)[c]	Enrichment, 0.5 McFarland standard for macrobroth dilution testing	*S. aureus, E. coli, S. enteritidis, S. typhimurium, Salmonella* sp., 35°C for 24 hrs
OPSP[e]	Enumeration	*C. perfringens*, anaerobic, 37°C for 72 hrs
Phosphate and Magnesium Chloride Dilution Water[f]	Dilution blanks	All, no incubation

Table 9 (continued)

Reinforced Clostridial Medium[c] with 1.5% agar (RCM)	Enrichment	*C. perfringens*, anaerobic, 35-37°C for 24 hrs
Reinforced Clostridial Medium with 1.5% agar	Enumeration	Total Anaerobic count, anaerobic, 37°C for 72 hrs
Rogosa SL Agar[c]	Enumeration	Lactobacilli, anaerobic, 37° for 72 hrs
Supplemented Brucella Agar (s-BA)[c, g]	Agar dilution susceptibility testing	*C. jejuni*, microaerophilic, 42°C for 48 hrs
Supplemented Brucella Blood Agar (s-BBA)[c, g]	Enrichment	*C. jejuni*, microaerophilic, 42°C for 48 hrs

[a]Holdeman and Moore, 1975.
[b]McCann, et al., 1996,.
[c]Difco Laboratories, Detroit, MI.
[d]3M Microbiology Products, St. Paul, MN.
[e]Oxoid limited, Hampshire, England.
[f] Marshall, 1992.
[g]NCCLS, 2001.

mL of salt solution. Resazurin (Sigma-Aldrich) solution was prepared by dissolving 100 mg in 100 mL distilled water. The medium was boiled then cooled, and 5 g/L L-cysteine (Sigma-Aldrich) was added under a constant flow of CO_2. Media was under a flow of CO_2 until media turned from pink to colorless. Media was then transferred to the anaerobic chamber and used for fecal processing.

Phosphate and magnesium chloride dilution water was prepared as previously described (Marshall, 1992). Stock phosphate solution was prepared by dissolving 34 g KH_2PO_4 in 500 mL distilled water, pH adjusted to 7.2 with 1 N NaOH, the volume brought up to 1 L with distilled water, and sterilized at 121°C for 15 min. Stock $MgCl_2$ solution was prepared by dissolving 38 g $MgCl_2$ (Sigma-Aldrich) in 1 L distilled water, and autoclaved at 121°C for 15 min. Stock solutions were stored at 4°C until use. Class C dilution water was prepared by the addition of 1.25 mL stock phosphate solution and 5 mL $MgCl_2$ solution, and the volume brought up to 1 L with distilled water. The pH was adjusted to 7.4 (pH 7.2 after autoclaving). Media was dispensed in aliquots of 10.2 mL into tubes or 105 mL into bottles (to achieve 9.9 and 99 mL blanks after autoclaving, respectively). Dilution blanks were then autoclaved for 15 min for tubes, or 30 min for bottles at 121°C. *Brucella stearothermophilus,* BT Sure biological indicator for steam sterilization (Barnstead/Thermolyne, Inc., Dubuque, IA), was used as an indicator of sterility. Spores were autoclaved with the media then incubated at 55°C for 24 hr. Lack of growth indicated the autoclave was working properly.

For the enumeration of *Bifidobacter* species, BS-LV agar was prepared as previously described (McCann et al., 1996). To each liter of Liver Veal agar (Difco), 5 g propionic acid sodium salt (Eastman Kodak Company, Rochester, NY), 10 g α-lactose (Sigma-Aldrich), 500 mg lithium chloride (Sigma-Aldrich), 400 mg L-cysteine, and 20 mg sodium lauryl sulfate (Sigma-Aldrich) were added before autoclaving at 121°C for 15 min. Media was tempered to 48°C and 0.1 to 1 mL of sample (in duplicate) was pour plated with approximately 10 mL of BS-LV. After solidifying, an additional 10 mL of agar was overlaid and allowed to solidify. Finally, an additional 10 mL of BS-LV plus neomycin sulfate solution was overlaid and allowed to solidify before incubation. Neomycin sulfate (Sigma-Aldrich) was prepared by dissolving 100 mg in 6.7 mL sterile distilled water and frozen in 0.5 mL amounts. Just prior to use, 9.5 mL sterile distilled water was added and mixed. For every 175 mL of BS-LV agar 1 mL of neomycin solution was added.

Campylobacter Agar Base (Karmali; Oxoid, Hampshire, England) was supplemented with freeze-dried Campylobacter Selective Supplement SR167E (Karmali; Oxoid). One vial of supplement contained 50 mg sodium pyruvate, 16 mg cefoperazone, 10 mg vancomycin, and 50 mg cyclohexamide. Vial components were dissolved by 2 mL of 50% ethanol and aseptically added to every 500 mL of sterile Campylobacter Agar Base. Karmali agar was used for the enumeration of *Campylobacter coli* and *C. jejuni* at 42°C. Samples (0.1 mL) were plated in duplicate using the spread plate method.

Oleandomycin Polymyxin Sulphadiazine Perfringens Agar (OPSP) was used for the enumeration of *Clostridium perfringens*. Perfringens Agar Base (OPSP; Oxoid) was mixed and autoclaved at 121°C for 15 min. Sterile agar was supplemented with freeze-dried Perfringens Selective Supplements A and B (OPSP; Oxoid). One vial of Supplement A (SR076E) contained 50 mg of sodium sulphadiazine and was dissolved in 2 mL of sterile distilled water. One vial of Supplement B (SR077E) contained 0.25 mg oleandomycin phosphate and 5000 I. U. polymyxin B sulphate, and was dissolved in 2 mL sterile distilled water. One vial of Supplement A plus one vial of Supplement B were added to each 500 mL of sterile Perfringens Agar Base. Medium was tempered to 48°C and 0.1 to 1 mL of sample (in duplicate) was pour plated with approximately 15 mL of medium.

Rogosa SL Agar (Difco) was used for the enumeration of *Lactobacillus*. Agar was mixed, steamed for 30 min, and supplemented with 1.32 mL/L 12 N glacial acetic acid (Sigma-Aldrich) and adjusted to a final pH of 5.4. Medium was tempered to 48°C and 0.1 to 1 mL of sample (in duplicate) was pour plated with approximately 15 mL of medium.

RCM was used for the enumeration of anaerobic bacteria to acquire a Total Anaerobe Count. Medium was tempered to 48°C and 0.1 to 1 mL of sample (in duplicate) was pour plated with approximately 15 mL of medium.

E.coli/Coliform Petrifilm (3M Microbiology Products, St. Paul, MN) was used for the enumeration of *E. coli* and Total Coliform counts. Samples (1 mL) were plated in duplicate. Blue colonies with gas were read as positive for *E. coli*. Red colonies with gas were counted as other coliforms. Blue colonies with gas plus red colonies with gas were considered to be a Total Coliform count.

Fecal samples. Swine feces were obtained from four Landrace X Yorkshire crossbred barrows. They were 5 months of age, weighed between 80 and 90 kg, and were housed at the

University of Kentucky Animal Laboratory. The diet consisted of 85% corn, 12% dehulled soybean meal, 1% dicalcium phosphate, and less than 1% of limestone, slat, vitamin and mineral mix. They were receiving no antibiotics. Pigs were kept in individual 5' x 10' pens with slatted floors, continuous lighting, and a constant temperature of approximately 25°C. Water was available *ad libitum.* Fresh stool samples were obtained, placed in four separate plastic bags, and were delivered into the anaerobic chamber within 10 min of collection for processing. Anaerobic chamber was under a CO_2 environment in the absence of O_2.

Equal weights of feces from each pig were pooled and blended with anaerobic dilution solution in a Waring blender for 60 seconds to achieve a 10% (w/v) fecal slurry. Aliquots of 50 mL fecal slurry were transferred into 125-mL serum bottles under a constant flow of CO_2.

***In vitro* treatments and inoculum.** Treatments were in a 4 x 2 factorial arrangement and included type of compound (factor A) and *C. perfringens* inoculum strain (Factor B). Treatment combinations are presented in Table 10. Each treatment had 3 replicates.

Carbadox (Sigma-Aldrich) was prepared under a sterile laminar flow hood (UV light for 15 min). A volume of 40 mL of 95% ethanol (0.2 μm filtered) was mixed with 0.12 g carbadox. The tubes were mixed and stored in 40 mL Fisherbrand (Fisher) plastic tubes surrounded by aluminum foil at 4°C.

CFA and CGA stock solutions contained 26 mg/mL and were solubilized in 70% ethanol. Stock solution (1 mL) was added to the appropriate serum bottle containing 50 mL fecal slurry and 1 mL of inoculum.

Serum bottles were prepared under a constant flow of CO_2 using gas jets. Each bottle was adjusted to contain 1.346% ethanol and was sealed by a butyl rubber stopper then crimp-sealed.

Clostridium cultures were transferred twice in Cooked Meat Medium and incubated anaerobically for 24 hr at 37°C. The cultures were then streaked onto RCM with 1.5% agar and incubated anaerobically for 24 hr at 37°C. Cultures were suspended in sterile dilution water to achieve 0.5 McFarland Standard, and 1 mL of the appropriate culture was anaerobically transferred into sealed serum bottles using sterile 1-mL syringes. After the addition of the compounds and inoculum, serum bottles were incubated in a shaking water bath at 125 rpm for 24 hr at 39°C.

Table 10. List of *in vitro* treatments and inoculum used in this study.

Treatment	Inoculum[a]	Compound
A	None	None
B	92D[b]	None
C	92G[c]	None
D	92D	CFA[d]
E	92G	CFA
F	92D	CGA[e]
G	92G	CGA
H	92D	Carbadox[f]
I	92G	Carbadox

[a] Inoculum level approximately 2 x 10^6 bacteria per mL
[b]Culture 92D = *Clostridium. perfringens* University of Kentucky isolate from poultry.
[c]Culture 92G= *C. perfringens* ATCC 13124.
[d]CFA = 500 ppm caffeic acid.
[e]CGA = 500 ppm chlorogenic acid.
[f]Carbadox = 15 ppm Carbadox.

Chemical analysis. Samples were taken at 0, 0.75, 1.5, 2.5, 4, 6, 12, and 24 hr. A volume of 1 mL was removed anaerobically from each serum bottle with a syringe and centrifuged for 5 min at 20,800 x *g* (Eppendorff Centrifuge 5417C, Brinkman Instruments, Inc., Westbury, NY). The supernatant was drawn off, placed in storage vials, and stored at a temperature of -20°C until further analysis.

Lactate was analyzed by an enzymatic method using lactate dehydrogenase (Hohorst, 1965). For every 50 assays, the lactate premix contained 1.42 g hydrazine sulfate (Nutritional Biochemicals Corporation, Cleveland, OH), 2.1 g glycine (Sigma-Aldrich), 0.056 g Na_2-EDTA•$2H_2O$ (Fisher) dissolved in 14 mL distilled water, and 20 mL 2 N NaOH. The pH of the premix was adjusted to 9.5 using 1 N NaOH, and 84 mg β-Nicotinamide Adenine Dinucleotide (β-NAD; Sigma-Aldrich) and 200 U lactate dehydrogenase (Sigma-Aldrich) were added. Samples were thawed and 50 μL dispensed into glass tubes (in duplicate). A standard curve was prepared using 4.44 mM lactate standard (Sigma-Aldrich). Premix (1 mL) was added, tubes were gently mixed and incubated 45 min at room temperature. Absorbance was read at 340 nm.

The maximum absorbances (λ_{max}) at pH 9.5 are 329 nm for CFA and 346 nm for CGA (Friedman and Jürgens, 2000). The λ_{max} of the compounds at pH 9.5 allows for interference with the lactate assay read at 340 nm. A standard curve was prepared using 500 ppm CFA and CGA stock solutions.

To confirm the presence or absence of lactate, high pressure liquid chromatography (HPLC) was used. A Sulpelcogel™ H HPLC Column (25 cm x 4.6 mm; Sulpelco, Bellefonte, PA) was run at 30°C using 0.1% phosphoric acid as the eluent at a rate of 0.17 mL/min on a Ranin HPXL solvent delivery system (Ranin Instrument Co., Inc., Woodburn, MA). A lactate standard (50 μL of 4.44 mM) was eluted to serve as a control.

Volatile fatty acids were analyzed by gas chromatography (Supelco, Inc., 1985; Gow Mac chromatograph model 580 equipped with a Supelco 1000 column [1% H_3PO_4, 100/120 mesh]). Samples were thawed, 400 μL dispensed into centrifuge tubes, and 125 μL of 25% meta-phosphoric acid (Fisher) was added. Samples were centrifuged for 7 min at 20,800 x *g*, and 500 μL of clarified sample was dispensed into Wheaton GC vials before analysis.

Initial slurry pH and final pH (time 0 and 24 hr) readings were recorded using Accumet pH Meter 930 (Fisher).

Differentiation of *C. jejuni*. In order to differentiate between *C. coli* and *C. jejuni*, the hippurate reaction was observed. Individual colonies were picked from Karmali agar. The colonies (18 total, 10 and 8 from each plate) were from the 10^{-5} dilution of the initial fecal slurry. They were each suspended in 5 mL of BB, streaked onto s-BBA, and incubated for 48 hr at 42°C in a microaerophilic incubator. The resulting colonies were then tested for the hippurate reaction (in duplicate) according to methods previously described (BAM, 1998). Approximately 2 mm loopfull of the culture was suspended in 0.4 mL of a 1% sodium hippurate solution and incubated for 2 hr in a 37°C water bath. Sodium hippurate (Sigma-Aldrich) solution was prepared by dissolving 0.1 g in 10 mL distilled water and filter sterilized (0.2 μm pore size, Millipore). After incubation, 0.2 mL of 3.5% ninhydrin reagent (1, 2, 3-triketohydrindene) was added, agitated, and incubated for an additional 20 min. Ninhydrin reagent (R47; Eastman Kodak) was prepared by dissolving 3.5 g in 100 mL of a 1:1 mixture of acetone (Fisher) and 1-butanol (Fisher). *Campylobacter jejuni* ATCC 33291 was used as the quality control organism. A color change to deep violet was considered a positive reaction.

Statistical analyses. Data were analyzed as a 4 x 2 factorial in a completely randomized design using PROC GLM of SAS (1988). Treatment combinations defined grouped, structured populations, and the Bonferroni t-test for nonorthogonal contrasts was used to determine differences between treatments. One serum bottle was the experimental unit, with 3 replicates per treatment. Bacterial counts were converted to logarithm units ($\log_{10}$) before statistical analysis. Bacterial counts were analyzed at 24 hr, while VFA concentrations were analyzed at each time point. Correlations between bacterial counts at 24 hr were analyzed using the PROC CORR procedure of SAS. Correlations between bacterial counts at 24 hr and VFA concentrations at 24 hr were analyzed using the PROC CORR procedure using the Pearson and Spearman options.

CHAPTER 5
Results and Discussion

Methods development

Some preliminary work was required to develop the methods to be used for the main studies. The National Committee for Clinical Laboratory Standards methods for macrobroth dilution technique (NCCLS, 2000), agar dilution technique (NCCLS, 2001), and the *in vitro* incubation were of concern. The results are discussed in more detail in Chapter 3, and will only be briefly summarized in the following paragraphs.

Two bacterial cultures were eliminated in Study 1. *Salmonella enteritidis* 108A and *Escherichia coli* ATCC 25404 were not able to survive in broth containing 3% ethanol. The alcohol interfered with the macrobroth dilution technique when trying to determine the MICs of alcohol soluble antimicrobials.

Study 2 determined that blood should not be used in agar dilution plates for determining MICs. Components in the blood interacted with CFA and CGA, preventing the compounds from inhibiting bacterial growth. This is a problem when attempting to determine MICs, giving false results. *Staphylococcus aureus* grew well on MHA with 7% ethanol. *Clostridium perfringens* grew well on all agars containing 7% ethanol except for RCM. This suggested that type of media influenced growth when agar contained ethanol.

Study 3 resolved the best combination of media to use for growth of cultures and for agar dilution plates used for testing MICs. Ethanol concentration is a concern when using an agar dilution method (compared to broth). Growth of *C. perfringens* was supported when RCM was used as the source agar then transferred to BA containing 3.5% ethanol. This determined type of media to be used for the MICs of the compounds on *C. perfringens* cultures. *Campylobacter jejuni* growth was inhibited on all agars containing 3.5 and 7% ethanol, and required further investigation.

Study 4 demonstrated that *C. jejuni* growth was supported on agar containing less than or equal to 1.5% ethanol. *C. jejuni* grew well on source agar s-BBA with no ethanol, and BA with 1.5% ethanol. For MIC testing, s-BBA was chosen as the source agar. Since hemin and vitamin K_1 do not interfere with determining the MICs, s-BA was chosen for agar dilution testing.

However, another limiting factor emerged when CFA would not solubilize. Despite the visibly insoluble compound, CFA inhibited *C. jejuni* at 480 ppm.

Study 5 further defined specific media formulations to be used in MIC testing of *C. jejuni*. It was determined that 0.5 mL of CFA and CGA stock solutions solubilized in 60% ethanol were to be used in agar dilution medium.

One final problem arose during a preliminary *in vitro* incubation. *Campylobacter* did not survive the incubation period when swine feces were introduced. This complication could not be addressed satisfactorily, indicating that this particular *in vitro* method was not adequate to study *Campylobacter* dynamics within the microbial ecology of swine feces.

Minimum inhibitory concentrations

A summary of results can be found in Table 11. While several studies exist on the antimicrobial nature of CFA, there are few that examine CGA. All Gram-positive organisms were affected by CFA, and all but one was affected by CGA. Three of the four *S. aureus* cultures were inhibited by 100 ppm CFA and 500 ppm CGA. The remaining *S. aureus* ATCC 43300 is a methicillin- and oxacillin-resistant strain that required 200 ppm CFA and 500 ppm CGA for inhibition. Both *C. perfringens* cultures were inhibited by 300 ppm CFA. However, the toxin producing strain *C. perfringens* ATCC 13124 was not susceptible to 1000 ppm CGA. Yet, the other *C. perfringens* strain isolated from poultry was inhibited by 400 ppm CGA. These results agree with other studies where *S. aureus* and *C. perfringens* were inhibited by CFA (Debrauwer et al., 1989; Tunçel and Nergiz, 1993; Binutu et al., 1996; Lee et al., 2001).

In this study, Gram-negative organisms (*E. coli, Salmonella,* and *Campylobacter*) were not susceptible to 1000 ppm CFA or CGA. Two other studies concurred with these findings. *E. coli* and *Pseudomonas aeruginosa* were not inhibited by 500 ppm CFA (Debrauwer et al., 1989). CFA was solubilized in DMSO, and a microplate method with nutrient broth was used. In another study, *E. coli* was not inhibited by CFA using methanol as a solvent (Lee et al., 2001). A disk diffusion assay on Eggerth-Gagnon (EG) agar was the method used.

Other studies that reported inhibition of Gram-negative organisms used different solvents, media, or MIC methods. When inhibition was successful, common methods between the studies included using water as the solvent (Aziz et al., 1998; Tsou et al., 2000), use of trypticase soy broth or agar (Herald and Davidson, 1983; Tunçel and Nergiz, 1993; Tsou et al.,

Table 11. Minimum inhibitory concentrations (μg/mL) of caffeic acid and chlorogenic acid.

Culture	Caffeic acid	Chlorogenic acid
Escherichia coli ATCC 11775	NI	NI
Escherichia coli ATCC 25922	NI	NI
Escherichia coli ATCC 43895	NI	NI
Salmonella typhimurium ATCC 13311	NI	NI
Salmonella typhimurium ATCC 700408	NI	NI
Salmonella spp. TT42	NI	NI
Campylobacter jejuni ATCC 33291	NI	NI
Staphylococcus aureus ATCC 12600	100	500
Staphylococcus aureus ATCC 25923	100	500
Staphylococcus aureus ATCC 27543	100	500
Staphylococcus aureus ATCC 43300	200	500
Clostridium perfringens ATCC 13124	300	NI
Clostridium perfringens 92D	300	400

[a]NI = No inhibition

2000), and a broth dilution method (Herald and Davidson, 1983; Tsou et al., 2000). Tsou et al. (2000) were also able to inhibit *Shigella* with CGA.

The pH of the media can also have an affect. Herald and Davidson (1983) found that *S. aureus* was 46% more inhibited by 1000 ppm CFA in broth at pH 7 compared to pH 6. In the same study, *E. coli* was 16% more inhibited at pH 6 compared to pH 7. The media used in this study for MIC testing were MHB (pH 7.3) and BA (pH 7), and could be a factor contributing to the susceptibility of Gram-positive bacteria and the resistance of the Gram-negative bacteria to the compounds. The pH determines the chemical nature of the compound, affecting polarity and solubility, along with ability to inhibit some microorganisms (Baranowski and Nagel, 1982).

One interesting observation occurred when *C. jejuni* initially appeared to be susceptible to 480 ppm CFA. This transpired during the methods development (Study 4, Chapter 3). Later, *C. jejuni* was completely insensitive to 1000 ppm CFA. The major difference was the percent ethanol used as a solvent. Initially, 28% ethanol was used, while 60% ethanol was used later (1.4 and 1.5% ethanol in agar, respectively). This suggests that water solubility played a role in inhibition for this particular microorganism. This agrees with the studies that used water as a solvent and were able to inhibit Gram-negative organisms (Aziz et al., 1998; Tsou et al., 2000). However, Tunçel and Nergiz (1993) solubilized CFA in 95% ethanol and were able to inhibit Gram-positive and Gram-negative bacteria, but used an agar dilution method with trypticase soy agar. As supported by the findings of Stern et al. (1988), type of media my play a critical role in determining susceptibility to antimicrobials.

In vitro incubation

Results from the MIC testing revealed that concentrations of 500 ppm of CFA and CGA were sufficient to inhibit most Gram-positive organisms. This level was then chosen for the CFA and CGA treatments in the *in vitro* incubation.

Upon statistical analysis, data were consistent with the absence of interactions between type of compound (factor A) and *C. perfringens* inoculum strain (factor B). Therefore, data were pooled according to type of compound and analyzed as a one-way classification of treatments. Treatment A was the control and contained no compound (A, B, and C pooled), treatment B contained 500 ppm CFA (D and E pooled), treatment C contained 500 ppm CGA (F and G pooled), and treatment D contained 15 ppm carbadox (H and I pooled).

Carbadox is used as a growth promoter in swine, and its use has been banned by the European Union (EC, 1998b). Many studies have been done on the ability of carbadox to improve growth performance and the prevention or treatment of swine dysentery (Yen and Pond, 1993; Raynaud et al., 1981). Carbadox is thought to promote growth by reducing the mass of the small intestines, thereby decreasing maintenance energy (Yen et al., 1987). Few studies are available on the effect of carbadox on the microbial ecology of the gut. White et al. (2002) studied microbial populations in the pig *in vivo*, but found no differences between the basal diet and carbadox supplementation on *Bifidobacteria,* lactobacilli, *E. coli*, coliforms, total aerotolerant anaerobes, *Salmonella*, or *C. perfringens* counts in feces.

Effect of treatment on bacterial counts. Counts from all populations except *Bifidobacteria* were reduced by approximately 1 log after 24 hr of incubation. This can be explained by the lack of substrate in the medium used for the fecal slurry and accumulation of VFAs that inhibit some bacteria. The analysis of variance for bacterial counts are presented in Figures 9-15.

Treatment containing CGA, CFA, and carbadox had lower total aerotolerant anaerobe counts than the control (9.3, 9.4, and 9.7 versus 10.3 log, $p<0.0001$; Figure 9). CFA, CGA, and Carbadox were not different from each other ($p>0.12$). Although there were statistical differences, a reduction of 1 log is required to be considered to microbiologically important. There was a 0.6 log reduction for Carbadox, 0.9 log reduction for CGA, and a 1 log reduction in the CFA treatment. There was a very high, positive correlation ($r >0.8$, $p<0.05$) between total anaerobe and *Bifidobacteria* counts in the control ($r=0.87605$), CFA treatment ($r=0.90116$), and carbadox treatment ($r=0.99514$). In other words, 77, 81, and 99% of the variation in total anaerobe counts can be explained by the variation in *Bifidobacteria* counts in the control, CFA, and carbadox treatments, respectively. However, this does not establish a cause and effect between the two counts.

Treatments containing CGA, CFA, and carbadox had lower *Bifidobacteria* counts than the control (9.2, 9.4, and 9.7 versus 10.3 log, $p<.0001$; Figure 10). However, the three treatments were not different from one another ($p>0.08$). The differences between the control and the treatments were 0.6 log reduction for carbadox, 0.9 log reduction for CFA, and 1.1 log reduction for CGA. This contradicts the findings of Lee et al. (2001) where *Bifidobacteria* were not

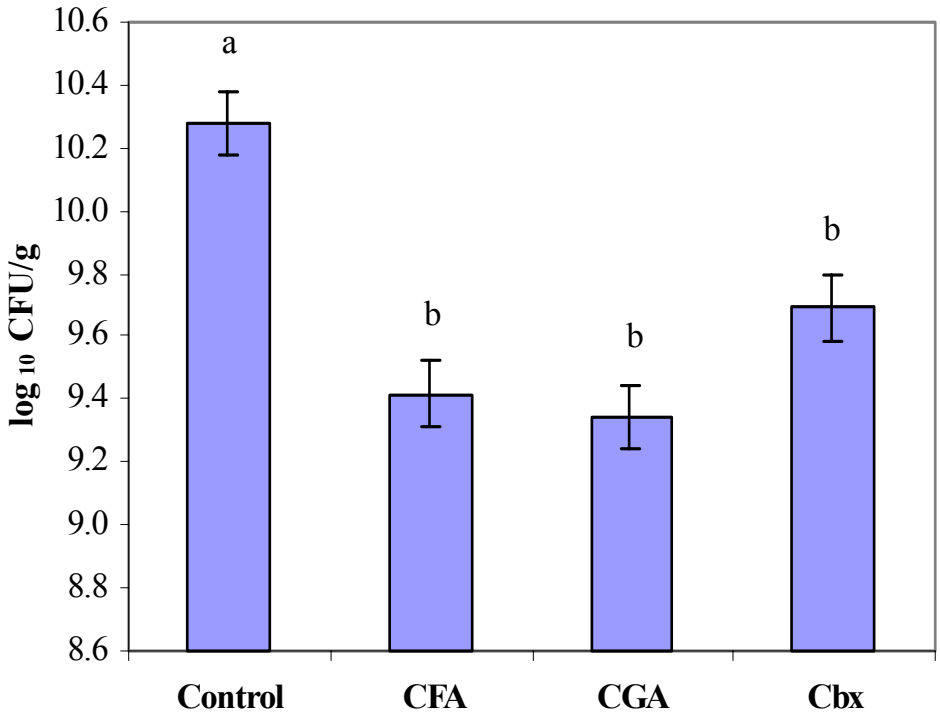


Figure 9. Total Aerotolerant Anaerobe counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with the unlike letters were different ($p<0.0001$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

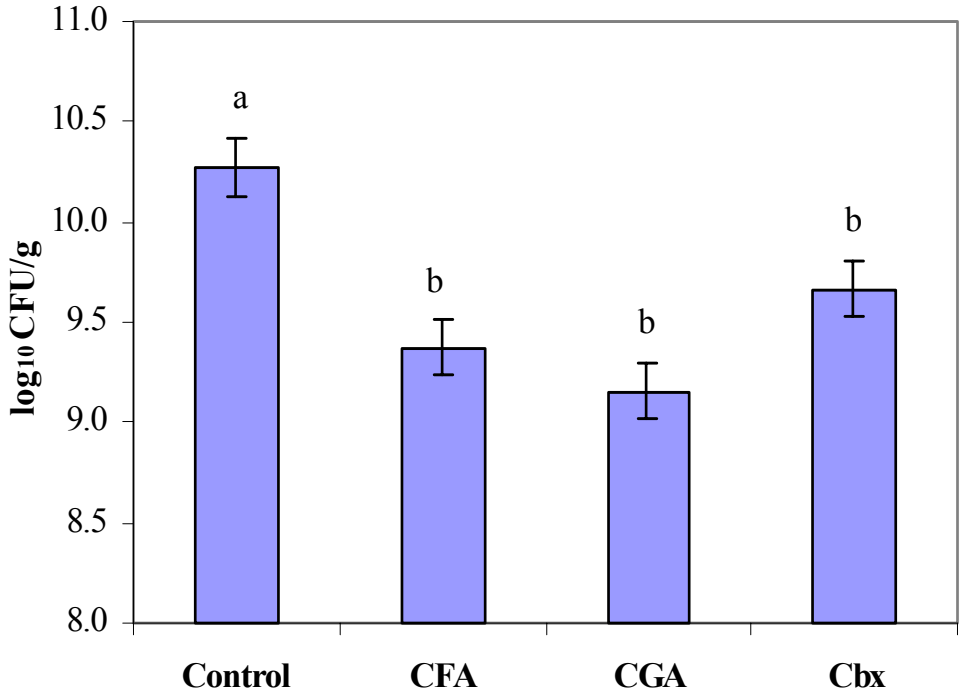


Figure 10. *Bifidobacteria* counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with the unlike letters were different ($p<0.0001$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

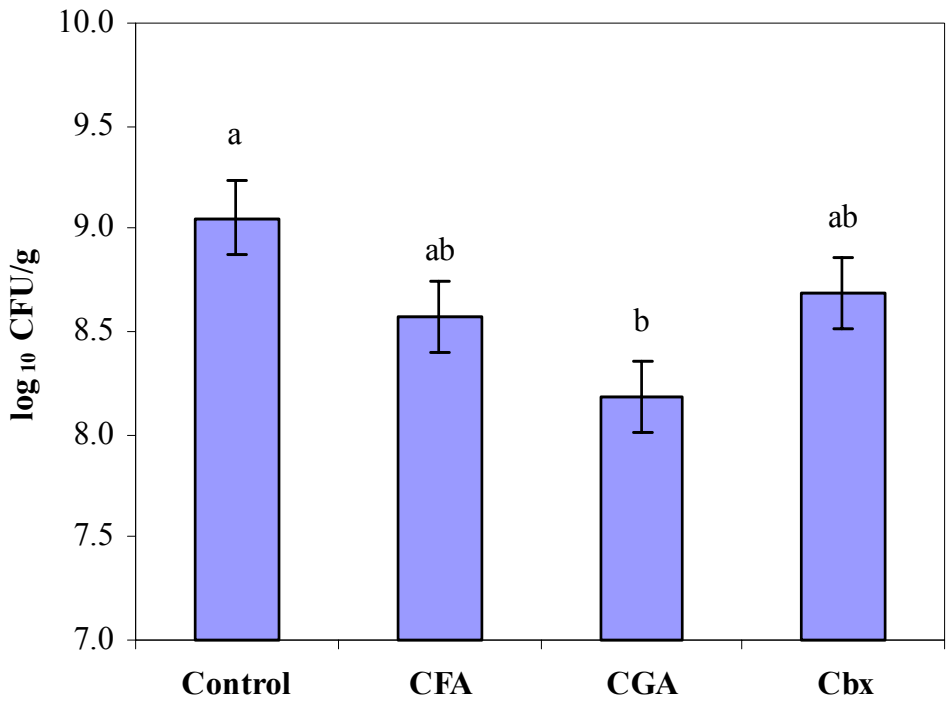


Figure 11. Lactobacilli counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with unlike letters were different ($p<0.01$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

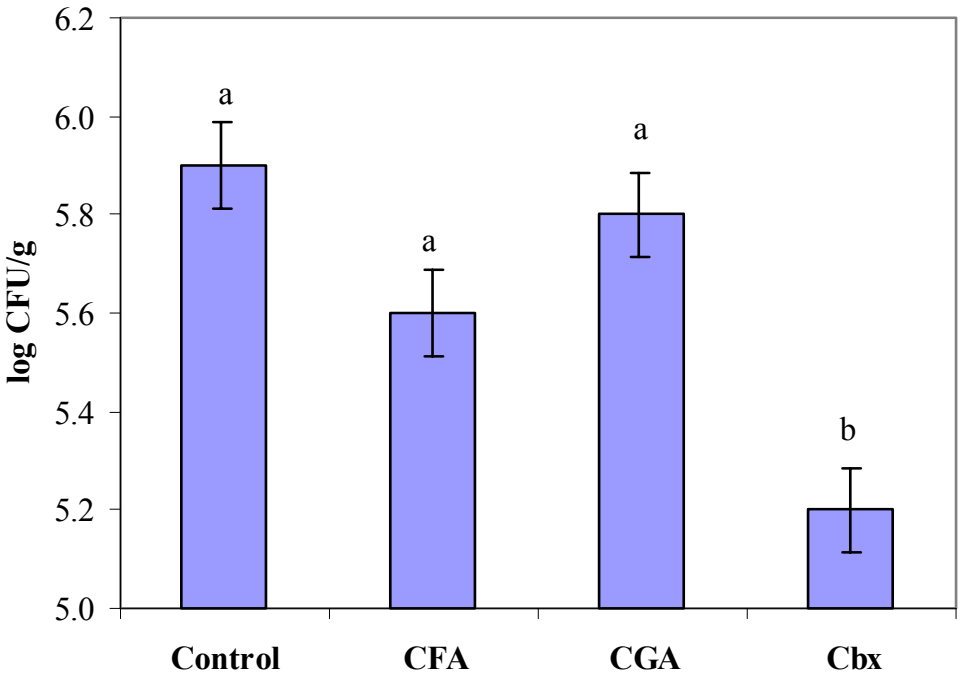


Figure 12. Total Coliform counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with the unlike letters were different ($p<0.0001$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

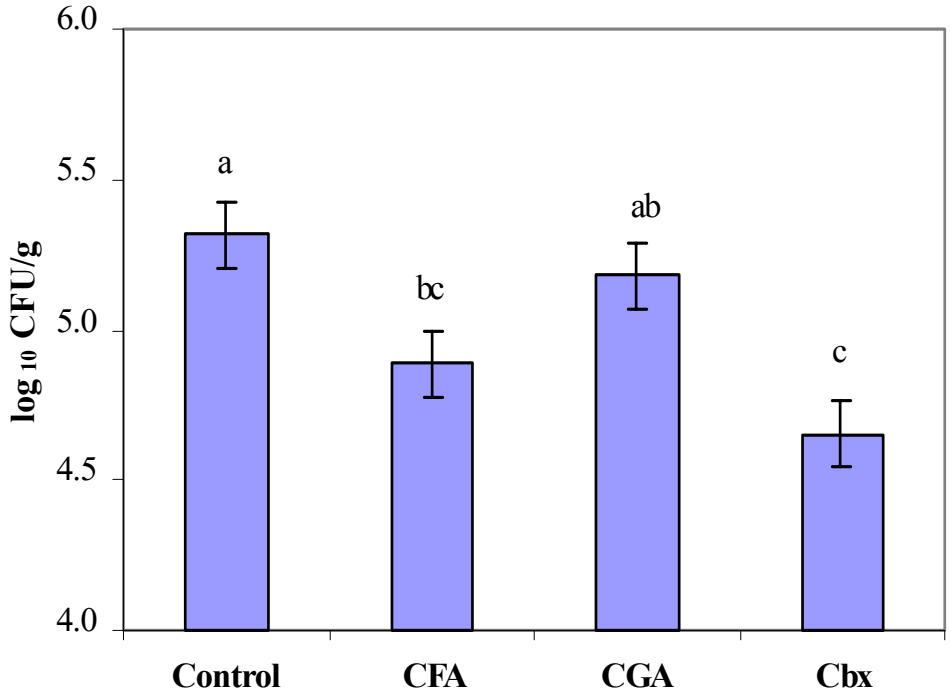


Figure 13. *Escherichia coli* counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with the unlike letters were different ($p<0.001$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

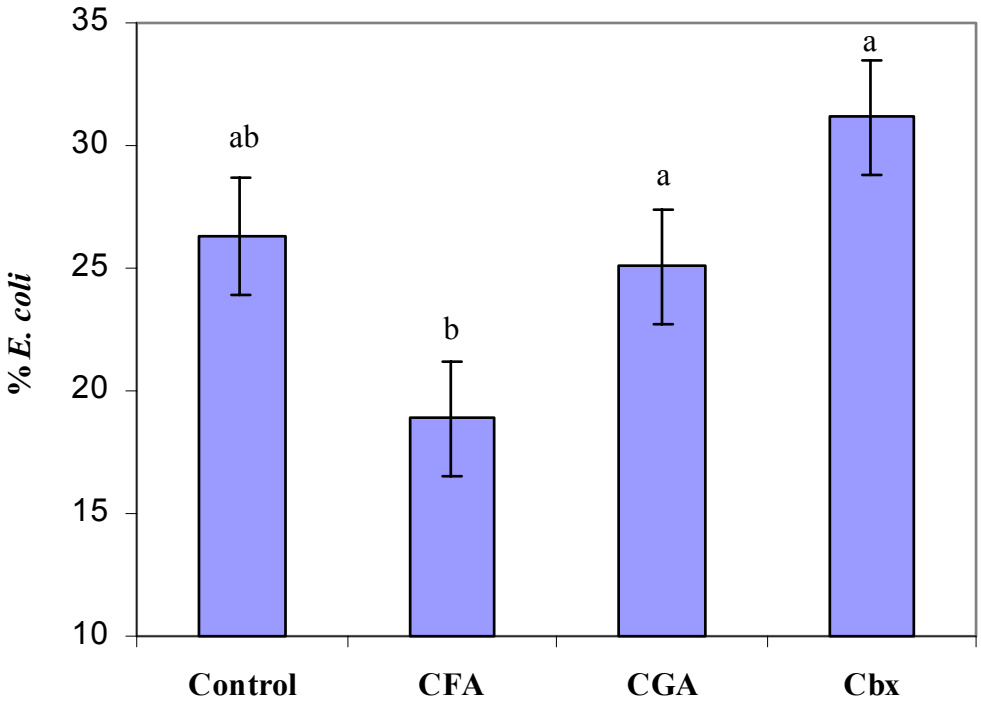


Figure 14. Percentage of *Escherichia coli* present in Total Coliform counts from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with the same letter are not different (p=0.16). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

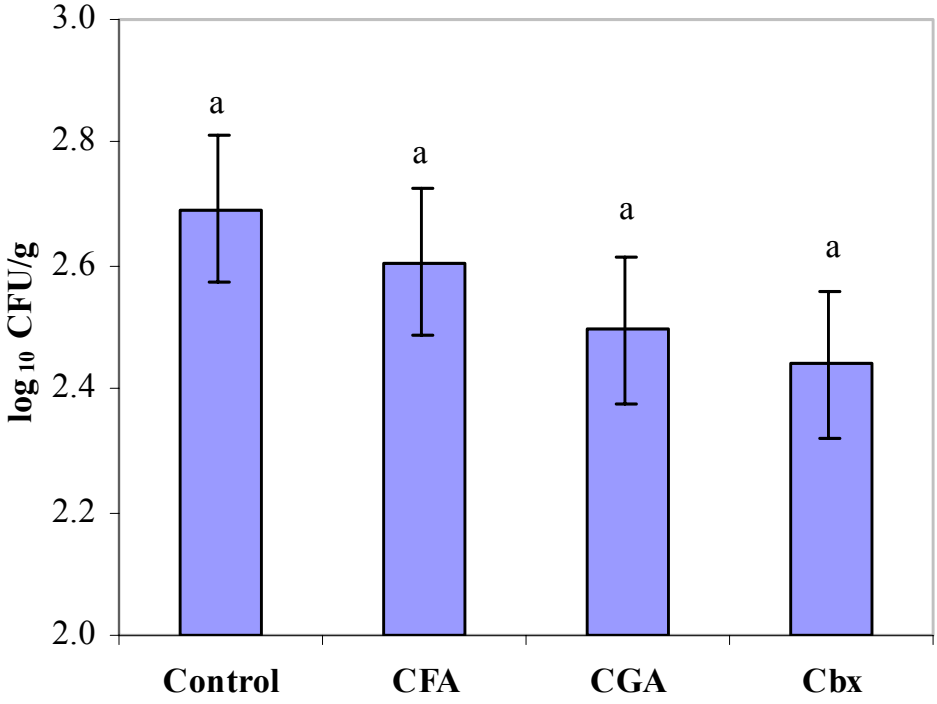


Figure 15. *Clostridium perfringens* counts (log_{10} CFU/g) from the *in vitro* incubation at 24 hours. Bars represent ± standard error (SE) of the mean. Treatments with like letters were not different ($p>0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

inhibited by CFA. Perhaps CFA and CGA metabolites created by other bacteria were toxic to the *Bifidobacteria* in this study.

For lactobacilli counts, there was one replicate from the control where no colonies grew. No growth was observed on the 10^{-7} dilution, and lower dilutions were not plated. Therefore, that replicate was excluded from analysis. The treatment containing CGA had lower lactobacilli counts than the control (8.2 versus 9.1 log, $p<0.01$; Figure 11). However, there were no differences between CGA, CFA, and the carbadox treatments (8.2, 8.6, and 8.7, $p>0.18$). Again, Lee et al. (2001) found that *Lactobacillus acidophilus* was not susceptible to CFA, suggesting that some lactobacilli may be susceptible to CFA and CGA metabolites produced by other bacteria.

Lactobacilli and *Bifidobacteria* are associated with beneficial effects on intestinal health. They interfere with adherence of pathogens to intestinal cells, elicit an immune response of the host, produce chemicals that inhibit the growth of pathogens, and restore the ecological balance of normal intestinal flora (Sullivan and Nord, 2002). Reduced counts of these bacteria have been related to sick animals (Robinson et al., 1984).

The treatment containing carbadox had lower total coliform counts than CFA, CGA, and the control (5.2 versus 5.6, 5.8, and 5.9 log, $p<0.0001$; Figure 12). However, there was only a 0.7 log reduction. White et al. (2002) were able to lower coliforms counts with carbadox in the jejunum and cecum of the pig by 0.9 and 1.3 logs, but did not have the same results when analyzing fecal swab samples. Coliforms are non-spore-forming, Gram-negative, rod-shaped bacteria that ferment lactose and produce gas and acid. They are often used as an indicator of the potential presence of pathogens (Rompré et al., 2002).

The treatments containing carbadox and CFA had lower *Escherichia coli* counts than CGA and the control (4.7 and 4.9 versus 5.2 and 5.3 log, $p<0.001$; Figure 13). The CFA and carbadox treatments were not different from each other ($p>0.35$). The CGA treatment and the control were not different from each other as well. In this study *E. coli* were not inhibited by the compounds during MIC testing, and responded differently to the *in vitro* incubation. This could be due to type of media used for MIC testing (previously discussed). There was a very high, positive correlation between *E. coli* and total coliform counts in the control ($r=0.89071$) and carbadox treatments ($r=0.88492$). *Escherichia coli* are thermotolerant bacteria and are a part of the natural flora of the intestine, and is known as a fecal coliform (Rompré et al., 2002). Many

different strains are associated with foodborne illnesses (Levine, 1987) and diarrheal diseases in swine (Shu et al., 2001).

The treatment containing CFA had lower percentages of *E. coli* in total coliform counts than CGA, the control, and carbadox (18.9 versus 25.1, 26.3, and 31.2%, $p<0.01$; Figure 14). The control, CGA, and carbadox treatments were not different from each other ($p>0.09$). Percentage of *E. coli* was used to study the dynamics of a section of the coliform population. A reduced percentage means that the proportion of other coliforms increased. The significance of this observation is beyond the scope of this study.

There were no differences in *Clostridium perfringens* counts between the control, CFA, CGA, and carbadox treatments (2.7, 2.6, 2.5, and 2.4 log, $p=0.37$; Figure 15). Despite the ability of CFA and CGA to inhibit growth of *C. perfringens* during the MIC testing in this study and by Debrauwer et al. (1989), *in vitro* there appeared to be no susceptibility to the compounds. This suggests that *C. perfringens* is not susceptible to the metabolic end products of CGA and CFA. *C. perfringens* produces toxins and causes foodborne illness in humans (Shandera et al, 1983) as well as depressing swine growth performance in livestock (McDonel, 1980; Strauch, 1987c).

Three colonies survived the *in vitro* incubation on Karmali Agar, selective for *C. jejuni* and *C. coli* at 42°C. After a Gram stain procedure, the surviving colonies were determined to be Gram-positive cocci. Since *Campylobacter* are Gram-negative rods, the surviving colonies were ruled out, concluding that *Campylobacter* did not survive the *in vitro* incubation. Because of the bacterium's fastidious nature, few studies have been done to determine the average number of *Campylobacter* found in feces. Weijtens et al. (1999) found a variation in counts between pigs, ranging from less than 630 CFU/g feces up to 3.6×10^6 CFU/g. In this study, it was determined that there were nearly 8×10^5 CFU/g in swine feces (fresh wet weight), but were not able to survive the *in vitro* incubation.

The hippurate reaction was used to differentiate *C. coli* from *C. jejuni* in the initial fecal slurry. The control strain produced a clear, deep violet reaction, testing positive as *C. jejuni*. Ten and 8 colonies were isolated from two 10^{-5} dilution plates. All eighteen colonies tested negative. Nielsen et al. (1997) found that 95% of thermophilic campylobacter in swine feces were identified as *C. coli*, where *C. jejuni* only accounted for 4%. Relative to this study, an estimated 3.2×10^4 CFU/g were *C. jejuni*.

Effect of treatment on volatile fatty acids. Volatile fatty acids (VFAs) are end products of microbial metabolism, and may provide an indication of microbial activity in the intestinal tract. They also serve as an energy source to the host animal. The analysis of variance for VFAs is presented in Figures 16-23.

Acetate concentrations remained lower for the carbadox treatment throughout the incubation (Figure 16). At 24 hr, the treatment containing CGA had higher acetate levels than the control and CFA treatment (40.3 versus 38.6 and 38.6 mM, $p<0.0001$), and the carbadox treatment had lower acetate levels than the control, CFA, and CGA treatments (31 versus 38.6, 38.6, and 40.3 mM, $p<0.0001$). There was no difference between the control and the CFA treatment ($p=1.0$)). One explanation for higher amounts in the CGA treatment could be that CGA was used as an electron sink for acetogenesis (Sunvold et al., 1995; Weimer, 1998). Excess H_2 can be deposited onto CGA metabolites (Imkamp and Müller, 2002). The reduction in available H_2 can cause a shift in fermentation, decreasing the carbon flow into succinate, ethanol, and lactate while increasing the flow to acetate (Wolin, 1975). Acetate can then transported for use by muscle as an energy substrate (Jensen, 2001).

At the end of the incubation, the treatment containing carbadox had lower propionate levels than the CFA and CGA treatments (8.9 versus 9.5 and 9.5 mM, $p<0.01$; Figure 17). However, the carbadox treatment was only 0.6 mM lower, and was not different from the control at 9.3 mM ($p>0.07$). Plus, there were no differences between the three treatments at any other time point. After absorption, propionate is transported to the liver and converted to glucose and used for energy. Increased amounts of propionate are associated with increased production of animal protein (Wolin, 1981).

At 24 hr, the treatment containing carbadox had lower acetate:propionate ratios than the control, CFA, and CGA treatments (3.5 versus 4.1, 4.1, and 4.2 units, $p<0.0001$; Figure 18). This was due to the reduced acetate levels in the carbadox treatment. CFA was not different from the control ($p=0.73$). However, the CGA had higher acetate:propionate ratios than the control and CFA treatment ($p<0.0001$). This was due to the increased acetate levels in the CGA treatment. Acetate to propionate ratio has been used as a measure of reduction of Gram-positive cocci that produce acetate in ruminants (Weimer, 1998). The change in ratio involves both reduction of acetate and increased propionate production. This was not observed in this study.

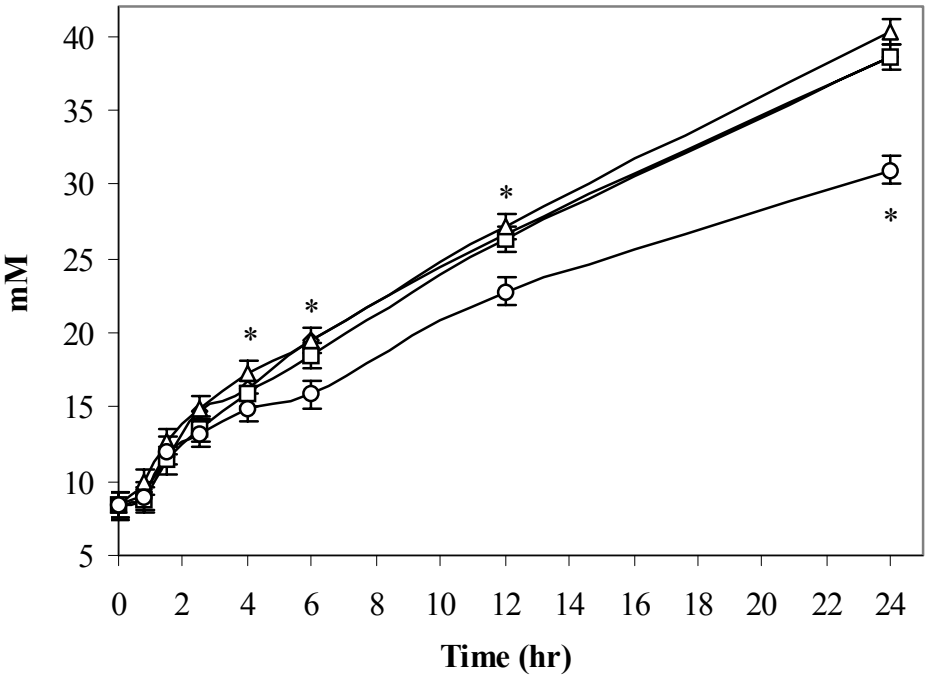


Figure 16. Acetate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

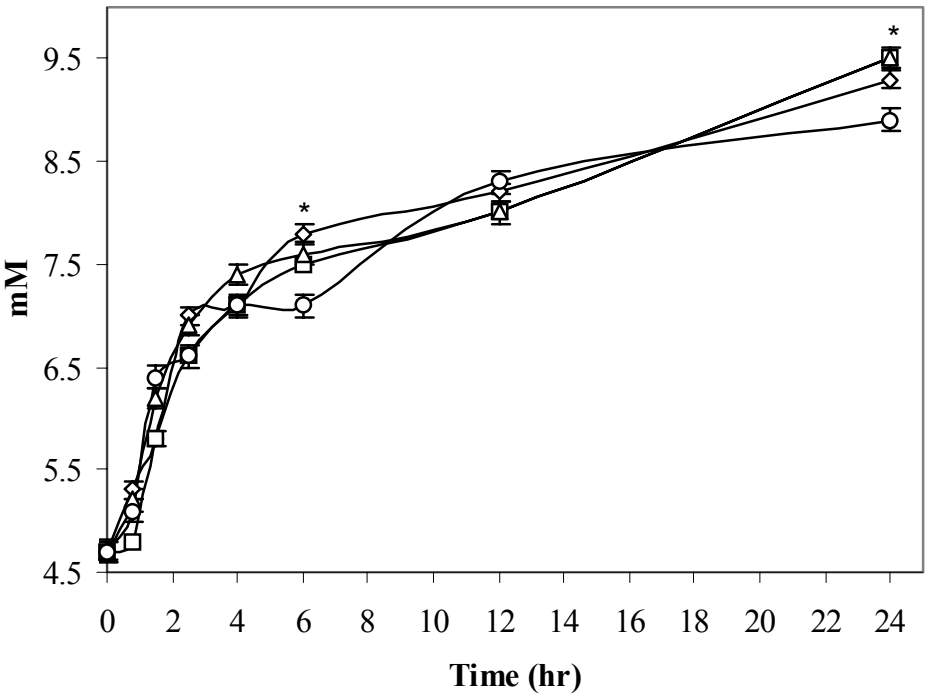


Figure 17. Propionate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

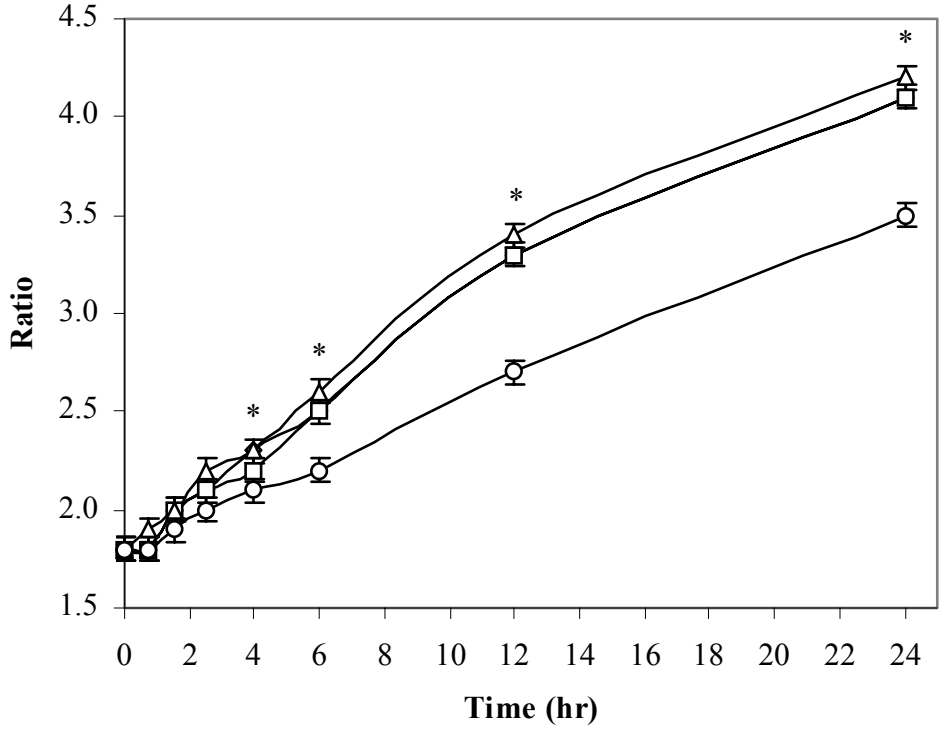


Figure 18. Acetate to propionate ratio trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

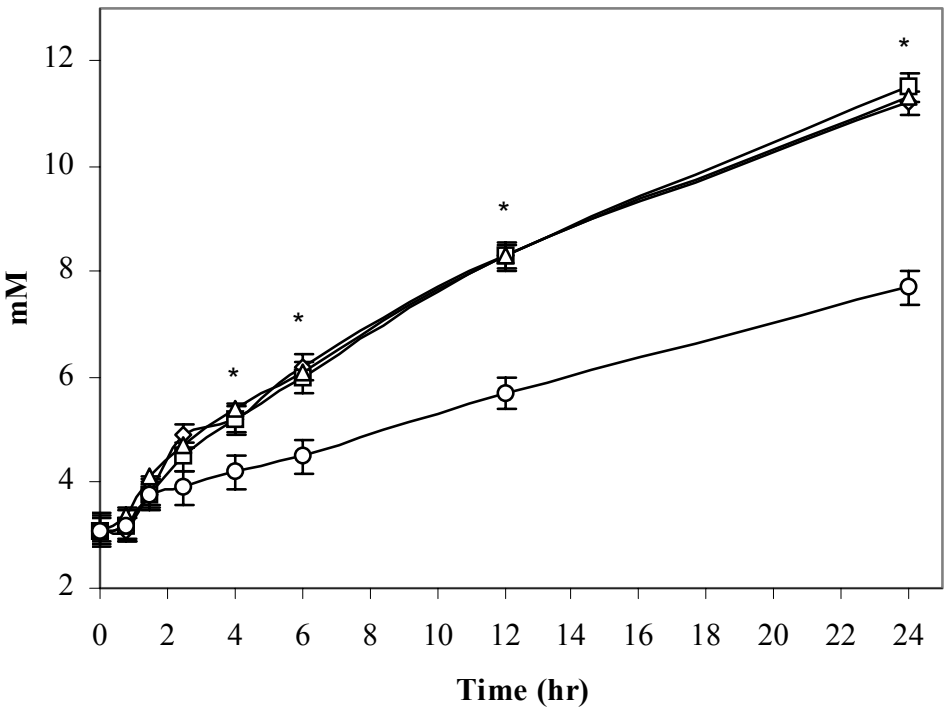


Figure 19. Butyrate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

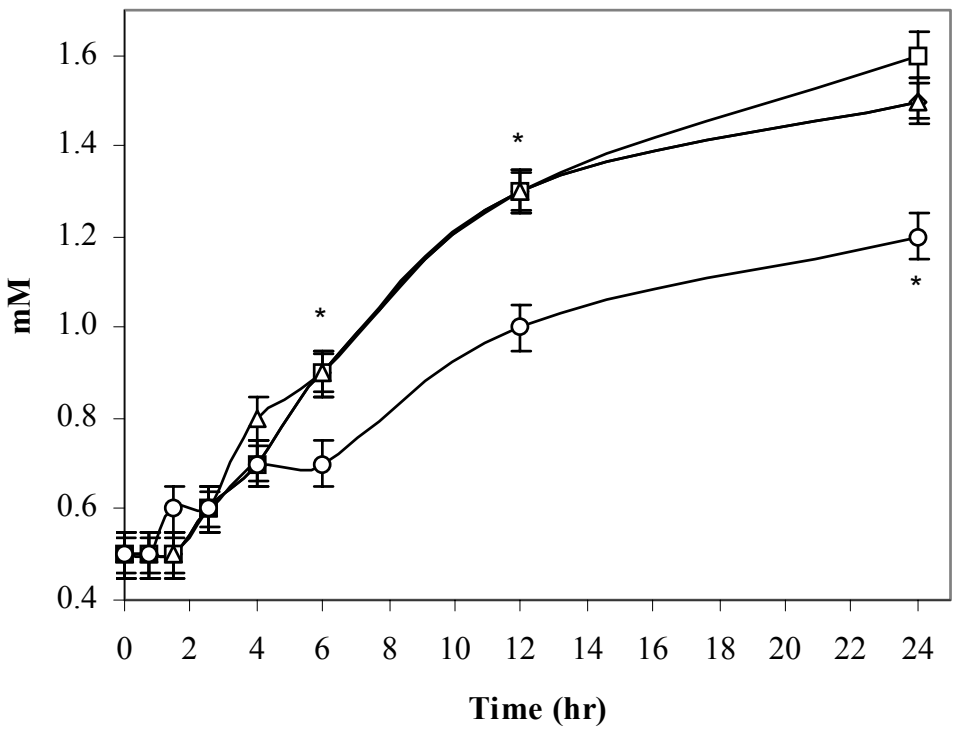


Figure 20. Valerate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

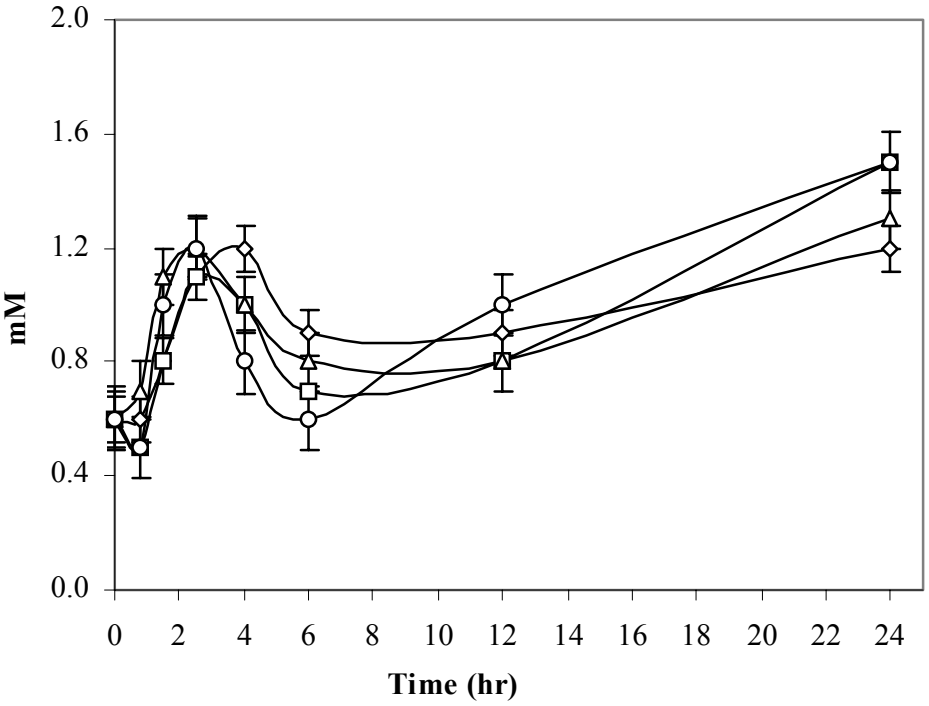


Figure 21. Isobutyrate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * p<.05.

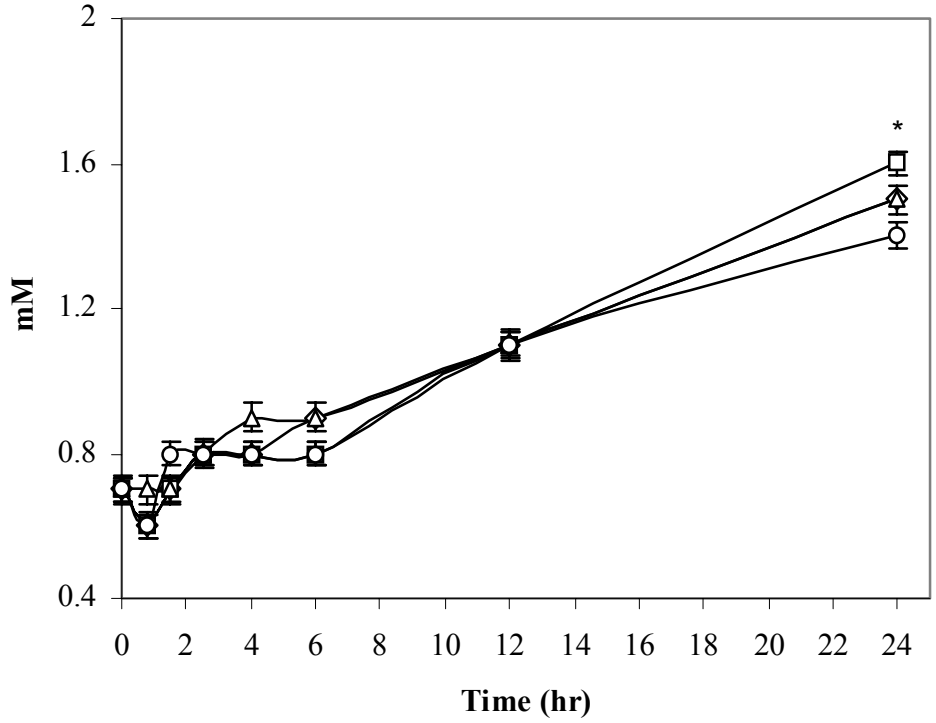


Figure 22. Isovalerate concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

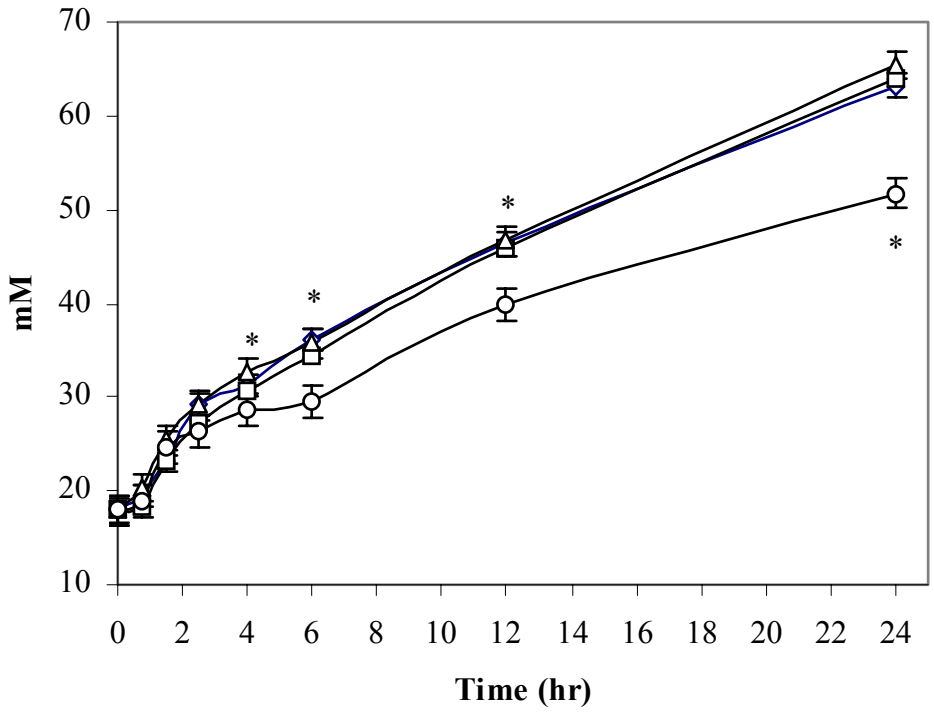


Figure 23. Total volatile fatty acid concentration (mM) trends over time during the *in vitro* incubation. Mean ± standard error (SE). ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox; * $p<0.05$.

Gram-negative as well as Gram-positive bacteria were reduced, and propionate production was not increased.

Butyrate concentrations remained lower for the carbadox treatment beginning at 2.5 hr (Figure 19). At the end of the incubation, the carbadox treatment had lower butyrate levels than the control, CGA, and CFA treatments (7.7 versus 11.2, 11.3, and 11.5, $p<0.0001$). The control, CFA, and CGA treatments were not different ($p>0.18$). The lower butyrate production resembles reduction of Gram-positive organisms in the rumen (Wolin, 1981). In this study, selected Gram-positive organisms were reduced, namely *Bifidobacteria* and lactobacilli. Other bacteria may be stimulated to produce butyrate in the presence of excess acetate (Russell, 2002). However, the transformation of excess acetate into butyrate in mixed cultures could also account for the increase in concentrations (Russell, 2002). Butyrate is the preferred energy source of energy for colonic epithelial cells (Sunvold et al., 1995). Reduced butyrate production by the carbadox treatment could help to explain the reduction of intestinal mass of swine.

At the end of the incubation, the carbadox treatment had lower valerate levels than the control, CGA, and CFA treatments (1.2 versus 1.5, 1.5, and 1.6 mM, $p<0.0001$; Figure 20). The control, CFA, and CGA treatments were not different ($p>0.14$).

There were no differences in isobutyrate levels between the control, CFA, CGA, and carbadox treatments (1.2, 1.3, 1.5, and 1.5 mM, $p=0.2689$; Figure 21).

The treatment containing carbadox had lower isovalerate levels than CGA and CFA treatments (1.4 versus 1.5 and 1.5 mM, $p<0.01$; Figure 22), and there was no difference between the control and carbadox treatment (1.5 and 1.4 mM, $p>0.58$). The control was also not different from the CGA and CFA treatments ($p>0.09$). Still, isovalerate levels in the carbadox treatment were merely 0.1 to 0.2 mM lower, and may not be biologically important.

Valerate, isobutyrate, and isovalerate are branched-chain fatty acids that are formed as a result of protein fermentation in the distal colon (Blaut, 2002). Increased levels of these three compounds may indicate an increase in protein metabolism (Sakata et al., 1999). Overall, valerate, isobutyrate and isovalerate concentrations increased through out the incubation, and was most likely due to microbial protein released from lysed cells.

The treatment containing carbadox had lower total VFA concentrations than the control, CFA, and CGA treatments (51.8 versus 63.2, 64.1, and 65.4 mM, $p<0.0001$; Figure 23). This was mostly due to the lower acetate and butyrate concentrations in the carbadox treatment.

There were no differences between the control, CFA, and CGA treatments ($p>0.06$). Total VFAs are a measure of overall microbial activity in the gut. In addition to the ability of CFA, CGA, and CGA to effect microbial populations, they were able to effect the chemical aspect of the microbial ecology of swine feces.

pH analysis. The pH at 0 and 24 hr are presented in Figure 24. The pH was 6.5 for the initial fecal slurry at 0 hr. At the end of the incubation, the pH of the control dropped by 0.1, while the CFA, CGA, and carbadox treatments were lowered by 0.2 units.

Lactate analysis. The control and the carbadox treatment showed a steady amount of lactate (0.3-0.6 mM) over the entire incubation (0 to 24 hr) as determined by the lactate assay. However, the CFA and CGA interfered with the assay, exhibiting higher amounts from 0.75 to12 hr. To determine the amount of lactate present without interference, HPLC was performed. Results demonstrated that lactate levels in the CFA and CGA treatments were consistently less than 1 mM for all time points (data not shown). It can then be assumed that lactate measurements obtained from CFA and CGA treatments were due to CFA and CGA.

The concentrations of CFA and CGA declined over time, and by 12 hr of incubation levels had diminished to below 1 mM for both compounds (Figure 25). Reduction of the compounds over time was caused by microbial metabolism. This is supported by several studies. Two strains of *Streptococcus gallolyticus* were able to decarboxylate caffeic acid (removal of COOH group) to produce the metabolite 4-vinylcatechol (Chamkha et al., 2002). The phenolic acid decarboxylase (PAD) enzyme has been found in *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Bacillus subtilis*, and *Bacillus pumilus* (Barthelmebs et al., 2001), and was able to decarboxylate CFA. The authors also suggest that PAD activity may be a stress response to avoid the toxicity of the compounds, though some species showed more enzyme activity than others did. The strictly anaerobic bacterium *Acetobacterium woodii* was able to reduce a double bond in CFA (caffeate) to produce hydrocaffeate (3,4-hydroxyphenyl propionic acid) (Imkamp and Müller, 2002). By using CFA as an electron acceptor, *A. woodii* was able to generate ATP. Besides decarboxylation and reduction, intestinal microflora can also dehydroxylate CFA (removal of OH group(s); Scheline, 1968; Chesson et al., 1999). Other metabolic products of CFA from hind-gut fermentation include 4-ethylcatechol, 3-hydroxyphenylpropionic acid (3HPP), and 3-phenylpropionic acid (3PPA; Scheline, 1968; Chesson et al., 1999).

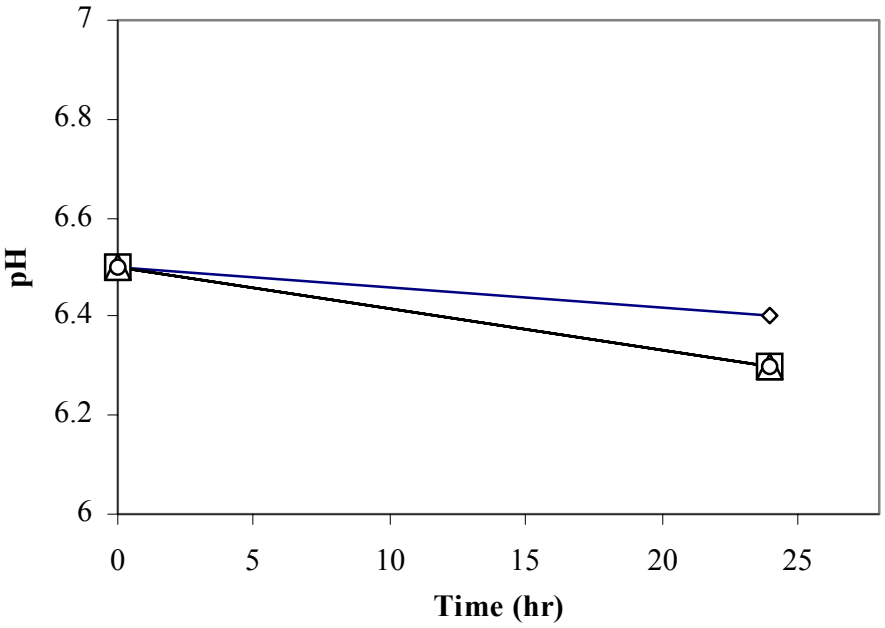


Figure 24. pH measurements during the *in vitro* incubation. ◇ Control; □ 500 ppm caffeic acid; Δ 500 ppm chlorogenic acid; ○ 15 ppm carbadox.

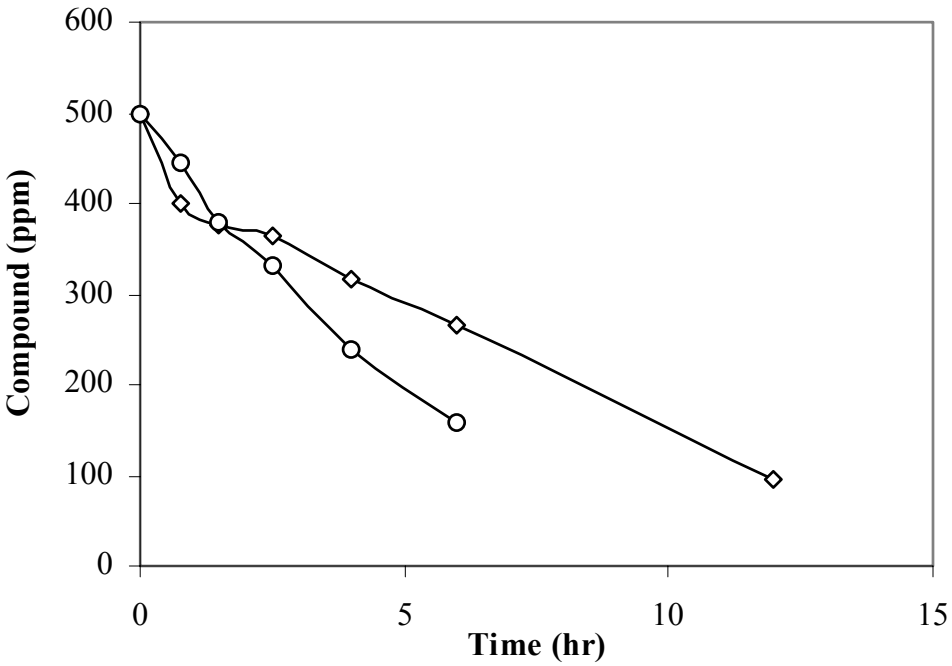


Figure 25. Disappearance of caffeic acid and chlorogenic acid for the *in vitro* incubation. Symbols represent caffeic acid (◇), and chlorogenic acid (○).

The metabolic products of CFA can be further used by other intestinal microbes (cross-feeding). Phenylpropionic acid (PPA) increased the adherence of *Ruminococcus albus* to cellulose (Pegden et al., 1998), which encourages the breakdown of plant material in the gut. There are several pathways in which *E. coli* can metabolize 3-hydroxycinnamic acid (3HCI), PPA, and 3HPP (Díaz et al., 2001). Metabolism involves breaking the aromatic (benzene) ring resulting in the production of succinic acid, acetyl-CoA, pyruvic acid, and fumaric acid. These end products can then enter other metabolic pathways including the citric acid cycle to generate reducing equivalents (NADH) and energy (ATP; Moran et al., 1994b).

Chlorogenic acid can also be metabolized by microbes. Couteau et al. (2001) isolated *Bifidobacterium lactis*, two strains of *Lactobacillus gasseri*, and three strains of *E. coli* from human feces. They demonstrated that these bacteria were able to hydrolyze CGA using the enzyme cinnamoyl esterase, producing CFA and quinnic acid. Once CGA is hydrolyzed, CFA is available to be further metabolized by other microbes.

CHAPTER 6

Summary

The objectives of this study were to determine the MICs of CFA and CGA against selected pathogens and to examine the effects of these compounds on the microbial ecology of swine feces. However, the standard or established methods proved inconsistent and these methods had to be modified.

The standard method for testing MICs for aerobic bacteria was not able to support the growth of two Gram-negative bacteria in the presence of a low level of ethanol, and were excluded from further testing. The standard method for testing the MIC for anaerobic and microaerophilic bacteria was used with the following adjustments: blood was not used in agar dilution plates, RCM was used as a source agar for *C. perfringens*, 1 mL of a 20x solution in 70% ethanol was used in agar dilution plates for *C. perfringens* testing, and 0.5 mL of a 40x solution in 60% ethanol was used in agar dilution plates for *C. jejuni* testing. For the *in vitro* incubation, the method used was unable to support the survival and growth of *C. coli* and *C. jejuni* in the presence of swine feces.

Upon completion of the methods development, the MICs were determined. CFA and CGA did not inhibit Gram-negative bacteria. CFA inhibited *Staphylococcus aureus* ATCC 12600, *S. aureus* ATCC 25923, and *S. aureus* ATCC 27543 at 100 ppm, *S. aureus* ATCC 43300 at 200 ppm, and *Clostridium perfringens* ATCC 13124 and *C. perfringens* 92D at 300 ppm. Chlorogenic acid inhibited all four *S. aureus* strains at 500 ppm, and *C. perfringens* ATCC 13124 at 400 ppm.

The concentrations of CFA and CGA to be used for the *in vitro* incubation were based on the MIC results at 500 ppm. The *in vitro* incubation examined specific bacterial populations and volatile fatty acid concentrations. CFA lowered the counts of *Bifidobacteria,* coliforms, *Escherichia coli*, total anaerobes, and percent *E. coli* ($p<0.01$). Percentage of *E. coli* was 12% lower than in the carbadox treatment. CGA lowered *Bifidobacteria*, lactobacilli, and total anaerobe counts ($p<0.01$). *E. coli* counts were 0.5 log higher than in the carbadox treatment, and percent *E. coli* was 6% higher than in the CFA treatment. CFA did not affect VFA production ($p>0.05$). CGA increased acetate concentrations by 1.7 mM ($p<0.05$). Carbadox lowered the concentrations of acetate, propionate, butyrate, isovalerate, and total VFAs ($p<0.01$). VFA

concentrations (excluding acetate) between caffeic and chlorogenic acid treatments were not different ($p>0.05$). Both compounds disappeared by the end of incubation.

CGA is present in many foods, and can travel to the large intestines where it can inhibit microorganisms or be metabolized into CFA and quinnic acid. CFA can then inhibit microbes or be metabolized by other gut microorganisms. CFA had similar actions to carbadox in inhibiting some of the same bacteria. A decreased number of pathogens may prevent disease, and a reduced bacterial load can lessen competition for nutrients with the animal. At the same time, VFA production for the CFA treatment did not decrease, mainly due to acetate and butyrate concentrations. Decreased pathogens and bacterial load without decreased VFA production could encourage the growth performance of swine.

APPENDIX

Appendix Table 1. Initial (0 hours) and final (24 hours) bacterial counts (log_{10} CFU/g†) from the *in vitro* incubation‡.

Treatment	*C. jejuni*	*Bifido-bacteria* (p<0.0001)	Lactobacilli (p=0.0064)	*Clostridium perfringens* (p=0..3693)	*Escherichia coli* (p=0.0005)	Total Coliforms (p=<0.0001)	%*E. coli* (p=0.0081)	Total Anaerobes (Aerotolerant) (p<0.0001)
Initial	5.9 + 0.04	10.1 + 0.22	10.1 + 0.33	3.7 + 0.06	6.5 + 0.04	7.1 + 0.73	27.7+ 1.8	11.5 + 0.04
Final								
Control	0	10.3 ± 0.15 [a]	9.1 ± 0.25 [a]	2.7 ± 0.21 [a]	5.3 ± 0.37 [a]	5.9 ± 0.28 [a]	26.3 ± 6.0 [ab]	10.3 ± 0.20 [a]
CFA	0	9.4 ± 0.14 [b]	8.6 ± 0.07 [ab]	2.6 ± 0.29 [a]	4.9 ± 0.07 [bc]	5.6 ± 0.05 [a]	18.9 ± 2.5 [b]	9.4 ± 0.12 [b]
CGA	0	9.2 ± 0.43 [b]	8.2 ± 0.60 [b]	2.5 ± 0.31 [a]	5.2 ± 0.09 [ab]	5.8 ± 0.10 [a]	25.1 ± 5.8 [a]	9.3 ± 0.04 [b]
Cbx	0	9.7 ± 0.45 [b]	8.7 ± 0.40 [ab]	2.4 ± 0.35 [a]	4.7 ± 0.17 [c]	5.2 ± 0.14 [b]	31.2 ± 0.1 [a]	9.7 ± 0.42 [b]

†CFU/g = colony forming units per grams of feces (wet weight) ± standard deviation (SD).
‡Treatments with unlike letters were different (p<0.05). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 2. Acetate concentrations (mM †) at various time points from the *in vitro* incubation‡.

Treatment	0 hr	0.75 hr (p=0.056)	1.5 hr (p=0.1425)	2.5 hr (p=0.0056)	4 hr (p=0.004)	6 hr (p<0.0001)	12 hr (p=0.0123)	24 hr (p<0.0001)
Control	8.4 ± 0.9	9.3 ± 0.8 [a]	11.6 ± 1.0 [a]	14.8 ± 1.2 [a]	16.3 ± 0.7 [ab]	19.5 ± 0.9 [a]	26.6 ± 1.4 [a]	38.6 ± 0.7 [a]
CFA	8.4 ± 0.9	8.7 ± 0.1 [a]	11.4 ± 0.9 [a]	13.6 ± 0.8 [ab]	15.9 ± 0.9 [ac]	18.5 ± 0.5 [a]	26.3 ± 1.2 [a]	38.6 ± 0.7 [a]
CGA	8.4 ± 0.9	10.0 ± 1.0 [a]	12.7 ± 0.2 [a]	14.9 ± 0.2 [a]	17.2 ± 0.6 [b]	19.5 ± 1.2 [a]	27.1 ± 3.5 [a]	40.3 ± 1.0 [b]
Cbx	8.4 ± 0.9	9.0 ± 0.7 [a]	12.0 ± 1.2 [a]	13.2 ± 0.3 [b]	14.9 ± 0.4 [c]	15.9 ± 0.4 [b]	22.8 ± 0.6 [b]	31.0 ± 0.5 [c]

†Mean ± standard deviation (SD).

‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 3. Propionate concentrations (mM †) at various time points from the *in vitro* incubation‡.

Treatment	0 hr	0.75 hr (p=0.6383)	1.5 hr (p=0.0207)	2.5 hr (p=0.083)	4 hr (p=0.0827)	6 hr (p=0.0027)	12 hr (p=0.843)	24 hr (p=0.0076)
Control	4.7 ± 0.2	5.3 ± 1.3 [a]	5.8 ± 0.3 [a]	7 ± 0.5 [a]	7.1 ± 0.3 [a]	7.8 ± 0.2 [a]	8.2 ± 0.5 [a]	9.3 ± 0.2 [ab]
CFA	4.7 ± 0.3	4.8 ± 0.04 [a]	5.8 ± 0.3 [a]	6.6 ± 0.2 [a]	7.1 ± 0.3 [a]	7.5 ± 0.2 [ab]	8.0 ± 0.3 [a]	9.5 ± 0.2 [a]
CGA	4.7 ± 0.4	5.2 ± 0.3 [a]	6.2 ± 0.1 [a]	6.9 ± 0.1 [a]	7.4 ± 0.2 [a]	7.6 ± 0.4 [ab]	8.0 ± 1.0 [a]	9.5 ± 0.3 [a]
Cbx	4.7 ± 0.5	5.1 ± 0.3 [a]	6.4 ± 0.5 [a]	6.6 ± 0.2 [a]	7.1 ± 0.1 [a]	7.1 ± 0.2 [b]	8.3 ± 0.2 [a]	8.9 ± 0.3 [b]

†Mean ± standard deviation (SD).

‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 4. Acetate to Propionate Ratio[†] at various time points from the *in vitro* incubation[‡].

Treatment	0 hr	0.75 hr (p=0.601)	1.5 hr (p=0.0025)	2.5 hr (p=0.0001)	4 hr (p<0.0001)	6 hr (p<0.0001)	12 hr (p<0.0001)	24 hr (p<0.0001)
Control	1.8 ± 0.1	1.8 ± 0.3 [a]	2.0 ± 0.06 [a]	2.1 ± 0.04 [ab]	2.3 ± 0.01 [a]	2.5 ± 0.04 [a]	3.3 ± 0.04 [a]	4.1 ± 0.07 [a]
CFA	1.8 ± 0.2	1.8 ± 0.01 [a]	2.0 ± 0.05 [ab]	2.1 ± 0.07 [ac]	2.2 ± 0.04 [b]	2.5 ± 0.01 [a]	3.3 ± 0.02 [a]	4.1 ± 0.01 [a]
CGA	1.8 ± 0.3	1.9 ± 0.08 [a]	2.0 ± 0.01 [a]	2.2 ± 0.02 [b]	2.3 ± 0.02 [a]	2.6 ± 0.04 [b]	3.4 ± 0.02 [b]	4.2 ± 0.06 [b]
Cbx	1.8 ± 0.4	1.8 ± 0.04 [a]	1.9 ± 0.06 [b]	2.0 ± 0.01 [c]	2.1 ± 0.03 [c]	2.2 ± 0.02 [c]	2.7 ± 0.03 [c]	3.5 ± 0.06 [c]

†Mean ± standard deviation (SD).

‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 5. Butyrate concentrations (mM †) at various time points from the *in vitro* incubation‡.

Treatment	0 hr	0.75 hr (p=0.0078)	1.5 hr (p=0.0692)	2.5 hr (p<0.0001)	4 hr (p<0.0001)	6 hr (p<0.0001)	12 hr (p<0.0001)	24 hr (p<0.0001)
Control	3.1 ± 0.1	3.1 ± 0.03 [a]	3.8 ± 0.2 [a]	4.9 ± 0.3 [a]	5.2 ± 0.2 [a]	6.2 ± 0.2 [a]	8.3 ± 0.5 [a]	11.2 ± 0.2 [a]
CFA	3.1 ± 0.1	3.2 ± 0.03 [ab]	3.8 ± 0.2 [a]	4.5 ± 0.2 [a]	5.2 ± 0.2 [a]	6.0 ± 0.2 [a]	8.3 ± 0.4 [a]	11.5 ± 0.2 [a]
CGA	3.1 ± 0.1	3.4 ± 0.1 [b]	4.1 ± 0.1 [a]	4.7 ± 0.1 [a]	5.4 ± 0.1 [a]	6.1 ± 0.3 [a]	8.3 ± 1.1 [a]	11.3 ± 0.3 [a]
Cbx	3.1 ± 0.1	3.2 ± 0.2 [ab]	3.8 ± 0.3 [a]	3.9 ±0.1 [b]	4.2 ± 0.1 [b]	4.5 ± 0.1 [b]	5.7 ± 0.2 [b]	7.7 ± 0.2 [b]

†Mean ± standard deviation (SD).
‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 6. Valerate concentrations (mM[†]) at various time points from the *in vitro* incubation[‡].

Treatment	0 hr	0.75 hr (p=0.429)	1.5 hr (p=0.0012)	2.5 hr (p=0.0074)	4 hr (p=0.0107)	6 hr (p<0.0001)	12 hr (p=0.0002)	24 hr (p<0.0001)
Control	0.5 ± 0.03	0.5 ± 0.04 [a]	0.5 ± 0.02 [a]	0.6 ± 0.03 [a]	0.7 ± 0.05 [a]	0.9 ± 0.04 [a]	1.3 ± 0.09 [a]	1.5 ± 0.06 [a]
CFA	0.5 ± 0.03	0.5 ± 0.04 [a]	0.5 ± 0.02 [a]	0.6 ± 0.04 [ab]	0.7 ± 0.02 [ab]	0.9 ± 0.03 [a]	1.3 ± 0.06 [a]	1.6 ± 0.08 [a]
CGA	0.5 ± 0.03	0.5 ± 0.02 [a]	0.5 ± 0.01 [a]	0.6 ± 0.01 [ab]	0.8 ± 0.05 [b]	0.9 ± 0.06 [a]	1.3 ± 0.16 [a]	1.5 ± 0.04 [a]
Cbx	0.5 ± 0.03	0.5 ± 0.04 [a]	0.6 ± 0.02 [b]	0.6 ± 0.02 [c]	0.7 ± 0.07 [a]	0.7 ± 0.02 [b]	1.0 ± 0.08 [b]	1.2 ± 0.06 [b]

†Mean ± standard deviation (SD).
‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 7. Isobutyrate concentrations (mM †) at various time points from the *in vitro* incubation‡.

Treatment	0 hr	0.75 hr (p=0.0277)	1.5 hr (p=0.122)	2.5 hr (p=0.4019)	4 hr (p=0.0074)	6 hr (p=0.2178)	12 hr (p=0.3798)	24 hr (p=0.2689)
Control	0.6 ± 0.4	0.6 ± 0.1 ^{ab}	0.8 ± 0.3 ^{a}	1.1 ± 0.2 ^{a}	1.2 ± 0.1 ^{a}	0.9 ± 0.3 ^{a}	0.9 ± 0.2 ^{a}	1.2 ± 0.2 ^{a}
CFA	0.6 ± 0.4	0.5 ± 0.01 ^{a}	0.8 ± 0.3 ^{a}	1.1 ± 0.2 ^{a}	1.0 ± 0.2 ^{ab}	0.7 ± 0.1 ^{a}	0.8 ± 0.1 ^{a}	1.5 ± 0.1 ^{a}
CGA	0.6 ± 0.4	0.7 ± 0.1 ^{b}	1.1 ± 0.01 ^{a}	1.2 ± 0.1 ^{a}	1.0 ± 0.2 ^{a}	0.8 ± 0.3 ^{a}	0.8 ± 0.2 ^{a}	1.3 ± 0.2 ^{a}
Cbx	0.6 ± 0.4	0.5 ± 0.02 ^{ab}	1.0 ± 0.3 ^{a}	1.2 ± 0.03 ^{a}	0.8 ± 0.2 ^{b}	0.6 ± 0.02 ^{a}	1.0 ± 0.2 ^{a}	1.5 ± 0.4 ^{a}

†Mean ± standard deviation (SD).
‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 8. Isovalerate concentrations (mM [†]) at various time points from the *in vitro* incubation[‡].

Treatment	0 hr	0.75 hr (p=0.001)	1.5 hr (p=0.0096)	2.5 hr (p=0.1726)	4 hr (p=0.088)	6 hr (p=0.0362)	12 hr (p=0.9899)	24 hr (p=0.0029)
Control	0.7 ± 0.05	0.6 ± 0.004 [a]	0.7 ± 0.06 [a]	0.8 ± 0.06 [a]	0.8 ± 0.06 [a]	0.9 ± 0.06 [a]	1.1 ± 0.09 [a]	1.5 ± 0.05 [ab]
CFA	0.7 ± 0.05	0.6 ± 0.01 [a]	0.7 ± 0.04 [a]	0.8 ± 0.04 [a]	0.8 ± 0.03 [a]	0.8 ± 0.03 [ab]	1.1 ± 0.07 [a]	1.6 ± 0.06 [a]
CGA	0.7 ± 0.05	0.7 ± 0.03 [b]	0.7 ± 0.01 [ab]	0.8 ± 0.01 [a]	0.9 ± 0.02 [a]	0.9 ± 0.08 [ab]	1.1 ± 0.14 [a]	1.5 ± 0.07 [a]
Cbx	0.7 ± 0.05	0.6 ± 0.03 [ab]	0.8 ± 0.06 [b]	0.8 ± 0.03 [a]	0.8 ± 0.06 [a]	0.8 ± 0.02 [b]	1.1 ± 0.06 [a]	1.4 ± 0.12 [b]

[†]Mean ± standard deviation (SD).
[‡]Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 9. Lactate and phenolic compound concentration (mM[†]) trends at various time points from the *in vitro* incubation[‡].

Treatment	0 hr	0.75 hr (p=0.429)	1.5 hr (p=0.0012)	2.5 hr (p=0.0074)	4 hr (p=0.0107)	6 hr (p<0.0001)	12 hr (p=0.0002)	24 hr (p<0.0001)
Control	0.5 ± 0.04	0.3 ± 0.3 [a]	0.4 ± 0.02 [a]	0.3 ± 0.03 [a]	0.4 ± 0.01 [a]	0.4 ± 0.02 [a]	0.5 ± 0.04 [a]	0.6 ± 0.06 [ac]
CFA	0.5 ± 0.04	5.5 ± 0.2 [b]	4.9 ± 0.1 [b]	4.6 ± 0.1 [b]	3.6 ± 0.1 [b]	2.7 ± 0.1 [b]	0.7 ± 0.05 [b]	0.7 ± 0.2 [ac]
CGA	0.5 ± 0.04	2.6 ± 0.2 [c]	2.0 ± 0.2 [c]	1.6 ± 0.1 [c]	1.0 ± 0.1 [c]	0.6 ± 0.05 [c]	0.7 ± 0.03 [b]	1.0 ± 0.08 [b]
Cbx	0.5 ± 0.04	0.4 ± 0.3 [a]	0.4 ± 0.03 [a]	0.4 ± 0.03 [a]	0.4 ± 0.03 [a]	0.4 ± 0.01 [a]	0.4 ± 0.02 [a]	0.5 ± 0.1 [c]

†Mean ± standard deviation (SD).

‡Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 10. Total volatile fatty acid concentrations (mM [†]) at various time points from the *in vitro* incubation[‡].

Treatment	0 hr	0.75 hr (p=0.0718)	1.5 hr (p=0.0952)	2.5 hr (p=0.0113)	4 hr (p=0.0006)	6 hr (p<0.0001)	12 hr (p=0.0225)	24 hr (p<0.0001)
Control	17.9 ± 1.4	19.4 ± 1.4 [a]	23.3 ± 1.8 [a]	29.3 ± 2.3 [a]	31.3 ± 1.4 [a]	36.1 ± 1.5 [a]	46.4 ± 2.6 [a]	63.2 ± 1.2 [a]
CFA	17.9 ± 1.4	18.2 ± 0.2 [a]	23.1 ± 1.7 [a]	27.1 ± 1.4 [a]	30.6 ± 1.5 [ab]	34.5 ± 0.9 [a]	45.8 ± 2.0 [ab]	64.1 ± 1.3 [a]
CGA	17.9 ± 1.4	20.4 ± 1.5 [a]	25.4 ± 0.4 [a]	29.1 ± 0.5 [a]	32.6 ± 1.0 [a]	35.7 ± 2.1 [a]	46.6 ± 6.1 [a]	65.4 ± 1.8 [a]
Cbx	17.9 ± 1.4	18.8 ± 1.2 [a]	24.6 ± 2.3 [a]	26.4 ± .05 [b]	28.5 ± 0.9 [b]	29.6 ± 0.7 [b]	40.0 ± 1.2 [b]	51.8 ± 1.3 [b]

[†]Mean ± standard deviation (SD).
[‡]Treatments with unlike letters were different ($p<0.05$). Treatments included control with no compound (Control), 500 ppm caffeic acid (CFA), 500 ppm chlorogenic acid (CGA), and 15 ppm carbadox (Cbx).

Appendix Table 11. Bacterial correlation coefficients[a] (p<0.05) for the *in vitro* incubation at 24 hours.

	Bifido-bacteria	Lactobacilli	*C. perfringens*	Total Anaerobes	*E. coli*	Total Coliforms
Control[b]						
Lactobacilli	NS[e]					
C. perfringens	NS	NS				
Anaerobes	**0.87605**	NS	NS			
E. coli	NS	NS	NS	NS		
Coliforms	NS	NS	NS	NS	**0.89071**	
% *E. coli*	NS	**0.74869**	NS	NS	NS	NS
Caffeic acid[c]						
Lactobacilli	NS					
C. perfringens	NS	NS				
Anaerobes	**0.90116**	NS	NS			
E. coli	NS	NS	NS	NS		
Coliforms	NS	NS	NS	NS	NS	
% *E. coli*	NS	NS	NS	NS	NS	NS
Chlorogenic acid[c]						
Lactobacilli	NS					
C. perfringens	NS	NS				
Anaerobes	NS	NS	NS			
E. coli	NS	**0.88866**	NS	NS		
Coliforms	NS	NS	NS	NS	NS	
% *E. coli*	NS	NS	NS	NS	NS	NS
Carbadox[d]						
Lactobacilli	**0.88604**					
C. perfringens	NS	NS				
Anaerobes	**0.99514**	NS	NS			
E. coli	NS	NS	NS	NS		
Coliforms	NS	NS	NS	NS	**0.88492**	
% *E. coli*	NS	NS	NS	NS	NS	NS

[a] $r \geq 0.8$, very high correlation; $0.8 > r \geq 0.6$, substantial correlation.
[b] df=7, r_{crit}=0.666.
[c] df=4, r_{crit}=0.811
[d] df=3, r_{crit}=0.878.
[e] NS, Not Significant, p>0.05.

Appendix Table 12. Correlation coefficients[a] ($p<0.05$, df=10, $r_{criet}=0.576$) between bacterial counts and volatile fatty acid concentrations for the *in vitro* incubation at 24 hours[d].

	Bifido-bacteria	Lacto-bacilli	*C. perfringens*	*E. coli*	Total Coliform	*% E. coli*	Total Anaerobe
Caffeic acid							
Acetate	NS[b]	NS	NS	NS	**0.77253**	NS	NS
Propionate	NS	NS	NS	NS	**0.70268**	NS	NS
Acetate: Propionate	NS	NS	NS	NS	NS	NS	NS
Butyrate	NS	**0.69464**	NS	NS	**0.70009**	NS	**0.68571**[c]
Valerate	NS	NS	NS	NS	**0.66853**	NS	NS
Isovalerate	NS	**0.62462**	NS	NS	**0.75822**	NS	**0.68571**[c]
Isobutyrate	NS	NS	NS	NS	**0.75685**	NS	NS
Total VFA	NS	NS	NS	NS	**0.75533**	NS	**0.68571**[c]
Chlorogenic acid							
Acetate	NS	NS	NS	**-0.60152**	**-0.69209**	NS	**0.66288**
Propionate	NS	NS	NS	**-0.68571**[c]	NS	NS	**0.65714**[c]
Acetate: Propionate	NS	NS	NS	NS	NS	NS	NS
Butyrate	NS	NS	NS	**-0.68571**[c]	**-0.61283**	NS	**0.61339**
Valerate	NS	NS	NS	NS	NS	NS	NS
Isovalerate	NS	NS	NS	**-0.65600**	**-0.66278**	NS	**0.59427**[c]
Isobutyrate	NS	NS	NS	**-0.70389**	**-0.71831**	NS	**0.59210**
Total VFA	NS	NS	NS	**-0.61253**	**-0.69597**	NS	**0.65740**

[a] $r \geq 0.8$, very high correlation; $0.8 > r \geq 0.6$, substantial correlation; $0.6 > r \geq 0.4$, moderate correlation.
[b]NS, not significant ($p>0.05$).
[c]Non-linear correlation using Spearman option.
[d]None of the correlations from the control or the carbadox treatment were significant ($p>0.05$).

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