MICROFLORAL PROFILE OF THE BROILER MIDGUT INTESTINAL TRACT AND CECUM AFTER ADDITION OF LUPULONE (HOP β-ACID) THROUGH DRINKING WATER AND CHALLENGE WITH *CLOSTRIDIUM PERFRINGENS*

by

GLENN EDWARD TILLMAN

(Under the Direction of Mary Alice Smith)

ABSTRACT

The use of antibiotic growth promotants in poultry rearing is a public health concern due to antibiotic resistance in bacteria and the harborage of resistance genes. Presently, lupulone, a hop β-acid from *Humulus lupulus*, was evaluated as a feed antibiotic alternative. The intestinal microflora of broilers was quantified after addition of lupulone to water and challenge with *Clostridium perfringens*. Microbial DNA was extracted from the broiler midgut and cecal sections and bacterial groups were quantified using real-time PCR. The predominant cecal bacterial groups were *Clostridium leptum, C. coccoides* and *Bacteroides*, whereas *Lactobacillus, Enterobacteriacea* and *Enterococcus* dominated the midgut. Lupulone at 125 ppm significantly decreased the *C. perfringens* subgroup in both the midgut and cecum and *Lactobacillus* in the midgut. Overall, no significant changes were noted in the microbial profile for the cecum or the midgut. Lupulone could be further evaluated as a measure against *C. perfringens* in poultry.

INDEX WORDS: Antibiotic Alternative, Necrotic Enteritis, Microfloral Profile, Hops Extract, *Humulus lupulus*, lupulone, *Clostridium perfringens*

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DEDICATION

I would like to dedicate my thesis to Tamara Corwin who provided encouragement along the way. I would also like to dedicate this to my parents for all their support over the years.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The use of antibiotics at subtherapeutic levels in livestock feed has been a common practice in the United States since the 1950s. Subtherapeutic antibiotics, also known as antibiotic growth promotants (AGP), improve feed efficiency and conversion in food animals, lower morbidity and mortality and improve the general health of food animals (Jukes and Williams, 1953). AGPs are thought to exert their growth-promoting effect on the intestinal microbiota of the animal. Proposed mechanisms of AGP's effects on the intestinal microbiota include: protection of nutrients from microbial utilization, improved absorption of nutrients due to thinner intestinal barrier, decrease of toxin production by intestinal bacteria and reduction of subclinical intestinal infections (Gaskins *et. al*, 2002).

Despite the benefits from AGP use in livestock feed, members of the public health community are concerned that antibiotics in feed cause antibiotic resistance and harborage of resistance genes in pathogenic bacteria found in humans. Sweden, in 1986, became one of the first nations to eliminate the use of AGPs in feed. The European Union banned avoparcin in feed in 1997 and Denmark banned the use of virginiamycin in feed in 1998 (Dibner and Richards, 2005). Removing AGPs from broiler diets can result in an increase of poultry necrotic enteritis, which can subsequently lead to flock condemnation, production losses or therapeutic use of antibiotics to treat clinical symptoms (Baba *et al.*, 1997). Necrotic enteritis (NE) is generally characterized by a decreased growth rate, enlarged liver and intestinal lesions. Poultry exhibiting NE have one or several disposing factors, including diets high in wheat or barley, diets with protein derived from animal sources or infection with a coccoidia protozoan, *Eimeria* (Baba *et*

al., 1997; Dahiya *et al.*, 2005; Drew *et al.*, 2004). Coccidiostat drugs, such as salinomycin, generally help control outbreaks of NE. These drugs also have an antibacterial effect on *Clostridium perfringens*, which is known to be the causative agent of NE (Immerseel *et al.*, 2004).

One of the challenges facing the food animal industry and the public health community is to effectively limit the use of AGP in feed while producing food animals humanely, efficiently and safely. A number of AGP alternatives with little relationship to traditional human antimicrobial therapy have been evaluated. Among these are bacteriocins, antimicrobial peptides, bacteriophages, probiotics, competitive exclusion cultures and plant products (Chen and Stern, 2001; Joerger, 2003; Nurmi and Rantala, 1973; Teuber and Schmalreck, 1973). Nurmi and Rantala (1973) described the concept of competitive exclusion (CE) as the administration of probiotics as defined or undefined bacterial cultures in feed. Competitive exclusion cultures have since been shown to affect the normal and pathogenic flora in the GI tract and have growth promoting effects similar to those of AGPs (Chen and Stern, 2001; Hofacre *et al.*, 2003; La Ragione *et al.*, 2004; Smirnov *et al.*, 2005). Extracts from the hop plant, *Humulus lupulus*, used in beer brewing for centuries, possesses antimicrobial effects on gram-positive bacteria (Srinivasan *et al.*, 2004; Teuber and Schmalreck, 1973). Cornelison *et al.* (2006) demonstrated the efficacy of whole hop flowers as growth promotants in chicken feed. As with other AGPs in feed, little is known about the effective mechanisms of hop extracts on growth promotion. Hanske *et al.* (2005) showed that xanthohumol, a constituent of hop, had no effect on the diversity of the resident microflora of the rat GI tract.

The diversity of microflora found in the chicken gastrointestinal tract has made understanding the ecology challenging. Cultural and molecular techniques, such as 16S rDNA

analysis and G+C% profiling, have helped to elucidate a qualitative microflora representation of the chicken GI tract. The small intestine has been shown to be dominated by *Lactobacillus* species; whereas, the cecum has been shown to contain mostly low G+C content, gram-positive bacteria, such as *Clostridium* species, *Fusobacterium* species, *Bacteroides* and *Lactobacillus* (Barnes *et al.*, 1972; Dumonceaux *et al.*, 2006; Lan *et al.*, 2002; Lu *et al.*, 2003; Salanitro *et al.*, 1974). To understand how AGPs or the alternatives positively affect growth and performance, it is important to determine both the normal intestinal microflora of the chicken gastrointestinal (GI) tract and the microflora of the GI tract after AGP use (Dumonceaux, 2006).

In this study, we quantified the GI microflora of broilers fed lupulone, a β-acid derived from hops, and challenged with necrotic enteritis-associated *Clostridium perfringens* strains (Wise and Siragusa, 2006). The aims of this study were to show the efficacy of lupulone as an antimicrobial against *C. perfringens* in the chicken GI tract and to ascertain its effects on the GI microflora of the broiler midgut and cecum. We tested two hypotheses: first, lupulone would have an antimicrobial effect on *C. perfringens*; second, lupulone would affect the overall microbial profile of the broiler midgut and cecum.

CHAPTER 2

LITERATURE REVIEW

Antimicrobial Growth Promotants in Animal Feed

For over 50 years, the practice of feeding subtherapeutic antibiotics to livestock has been common in the United States and other countries. Jukes and Williams (1953) reviewed the nutritional effects of antibiotics stating "that adding small quantities of antibiotics to the diets of young animals not only served to control certain obvious disturbances such as diarrhea and mild respiratory infections but also produced unexpected increases in the growth rate even when the animals were asymptomatic". Subtherapeutic antibiotics have been used in feed to encourage increased animal growth, increased meat production and illness avoidance in food animals. Subtherapeutic antibiotics in feed have also been named antibiotic growth promotants, or AGPs, due to their growth-promoting effects. In the United States, the sustained use of feed AGPs in domestic animals is currently a common practice, and although the actual amount of antibiotics administered annually in U.S. agriculture is unknown, the majority of agricultural use is subtherapeutic (Anderson *et al.*, 2006). The Union of Concerned Scientists (2001) estimated approximately 24.6 million pounds of subtherapeutic antibiotics are used annually. The estimate, which does not include therapeutic use, encompasses use in swine, cattle and poultry, which accounts for 10.5 million pounds according to their estimate. Chapman and Johnson (2002) showed that antibiotic use in poultry starter feed, grower feed and withdrawal feed decreased by 29.5%, 31.3% and 27.0% respectively from 1995-2000 in the U.S. However, the

number of poultry farms using ionophores and roxarsone, an arsenical drug, increased during the same time period.

Beneficial effects of AGP use in feed include growth promotion through improved feed conversion and feed efficiency in farm animals. AGP use also reduces morbidity and mortality associated with clinical and subclinical diseases (Butaye *et. al*, 2003). Although no single mechanisms of AGP action has been identified, germ-free, or gnotobiotic, animal studies have indicated that growth may be enhanced by an antibacterial effect on the microflora of the chicken gastrointestinal (GI) tract. Germ-free chickens grow faster than conventionally-raised chickens and do not respond to AGPs in feed. Conventionally raised chickens given AGPs in feed approach the growth efficiency of germ-free chickens (Feighner and Dashkevicz, 1987).

Researchers have proposed several mechanisms for AGP's effect on the microflora of the GI tract (Gaskins, *et al.*, 2002). AGPs exert their growth promoting effect through action on the microflora thereby allowing protection of nutrients from microbial utilization. Bacteria present in the GI tract needing sugars as an energy source use the host's available nutrients for metabolic energy. AGPs improve absorption of nutrients due to thinner intestinal barrier. Germ-free animals have thinner small intestine walls, a more slender villus structure with a thinner lamina propia and a slower rate of renewal of epithelial cells. AGPs can lead to a decrease in toxin production through their antimicrobial effects on toxin-producing bacteria. AGP use reduces the incidence of clinical and subclinical intestinal infections caused by pathogenic and opportunistic bacterial pathogens. In the Gaskins review (2002), it is noted that all the proposed mechanisms of AGPs assume that intestinal bacteria, commensal or pathogenic, depress animal growth directly or indirectly. The use of AGPs encourages host organism growth through absorption of available nutrients across a thinner intestinal barrier. The host organism expends less energy in

epithelial cells turnover in a reduced microbial GI environment. In the absence of GI microbiota, less mucus is secreted by intestinal goblet cells and the inflammatory response of the immune system is not activated through IgA production (Dibner and Richards, 2005). The host expends energy in mucin and IgA production rather than using the energy for growth.

Public Health Actions Regarding Antimicrobial Growth Promotants in Livestock Feed

With the continued use of AGPs in modern food animal rearing, members of the public health community have voiced concern over antibiotic resistance and harborage of resistance genes in zoonotic pathogens leading to human infections. In response to regulatory pressure, the European Union adopted the "precautionary principle", which called for discontinuation of in-feed antibiotics before the problem of antibiotic resistance emerged (Turnidge, 2004). In 1986, Sweden eliminated the use of antimicrobial agents as growth promoters in feed (Dibner and Richards, 2005). In the United Kingdom, the Swann Report (1969) argued that antibiotics should not be used unless the antibiotic makes an economic impact on the raising of livestock; has no significance as therapeutic agents in humans or animals and does not create bacterial strains resistant to prescribed therapeutic drugs (Butaye *et al.*, 2003). The Swann Report also recommended the discontinuation of penicillin, tetracyclines, tylosin and sulfonamides as growth promoters in livestock feed.

The European Union banned avoparcin in feed in 1997, and Denmark banned the use of virginiamycin in feed in 1998 (Dibner and Richards, 2005). Avoparcin is used as a growth promoter in pigs and chickens but has cross-reactivity with a medical equivalent, vancomycin. Researchers have isolated vancomycin-resistant *Enterococcus faecium* (VREF) from pigs and poultry (Aarestrup *et al.,* 1995). VREF poses a nosocomial problem and had little recourse until the FDA approved Synercid for use in treating VREF (McDermott *et al.*, 2005). Due to

Synercid-resistant strains of *E. faecium* found in retail poultry, there has been concern over the transfer of genetic elements of resistance to Synercid (Dumonceaux, 2006).

The agricultural use of antibiotics including subtherapeutic and therapeutic has decreased since the bans were implemented in the European communities. Opponents of the European ban on feed antibiotics suggest that such bans have increased the use of therapeutic antibiotics to treat infections in farm animals (Philips *et al.*, 2004). The use of veterinary therapeutic antibiotics with analogs to those of human medicine can select for bacteria with resistance genes (Casewell *et al.*, 2003). Veterinary therapeutic antibiotics, including tetracyclines, penicillins, sulfonamides, macrolides and aminoglycosides, have been thought to contribute to antibiotic resistance in bacteria found in human illnesses (Philips *et al.*, 2004).

Necrotic Enteritis in Broiler Flocks

The removal of AGPs from broiler diets can lead to an increased incidence of poultry necrotic enteritis (NE), though a sporadic disease is economically important (Baba *et al.,* 1997). Necrotic enteritis is associated with increased morbidity and mortality in birds and leads to condemnation of the bird or an entire flock. The clinical signs of the disease can generally be characterized by a severe growth rate decrease, mucosal lesions and enlarged livers. The disease is generally seen in broilers at 2-5 weeks of age but can be seen also in older layers (Collier *et al.,* 2003). Considered to be the causative agent of NE, *Clostridium perfringens* is normally present in low amounts in the GI tract of healthy chickens but can grow to excessive amounts in birds suffering from NE (Baba *et al.*, 1997).

In a review by Van Immerseel *et al.* (2004), the authors list dietary factors and coinfection with coccidial pathogens as predisposing elements correlating *C. perfringens* colonization with NE. The increased incidence of NE seen in Europe can be linked to certain

poultry diets containing the small grains rye, wheat and barley as the major carbohydrate energy source. These feed types increase reduce intestinal viscosity through enhanced mucous production with a concurrent decrease in intestinal motility. Dahiya *et al.* (2005) found that diets derived from animal sources, which are high in glycine, could be a predisposing factor to NE. Chickens fed high glycine diets had increased mortality, higher lesion scores, increased levels of *C. perfringens* and decreased levels of lactobacilli. The source type and amount of protein in poultry feed were shown by Drew *et al.* (2004) to significantly increase *C. perfringens* growth in the intestines. The authors found that birds fed fish meal had significantly higher counts of *C. perfringens* compared to birds fed a non-animal derived soy protein diet.

Baba *et al.* (1997) showed in experimental systems that *C. perfringens* alone was not sufficient to cause NE symptoms but acted in conjunction with *Eimeria necratix*, a coccidial pathogen, to produce mortality and NE-associated problems. The highest mortality rates were seen in groups given both *Eimeria* oocytes and *C. perfringens.* Interestingly, the authors found similar intestinal lesion scores in the *Eimeria* only group and the *Eimeria* plus *C. perfringens* group. Coccidial pathogens can cause a leakage of plasma proteins into the lumen of the intestinal tract providing growth factors for *C. perfringens* or can cause intestinal lesions for *C. perfringens* colonization.

Coccidial pathogens found in most *C. perfringens*-associated NE outbreaks can be controlled using coccidial vaccines and coccidiostatic drugs (Immerseel *et al.*, 2004). Salinomycin, an anti-coccidial and anti-clostridial drug shown to inhibit the growth of *C. perfringens*, has been widely used in Europe (Knarreborg *et al.*, 2002). Brennan *et al.* (2001) evaluated the efficacy of narasin, an anti-coccidial agent, in prevention of NE. The authors found that groups given narasin-containing feed had significantly higher mean body weight and

feed efficiency at day 21 and lower NE mortality at day 41. Surprisingly, the coccidiosis lesion scores for all treatment groups on day 17 were the same, indicating that narasin did not prevent coccidiosis but possibly did prevent colonization of *C. perfringens*. Jackson *et al.* (2003) showed that β-mannanase enzyme had an effect similar to narasin on the performance of chickens in a NE challenge model. The authors found that β-mannanase enzyme improved feed conversion and body weight but had no effect on lesion scores indicating that it also does not prevent coccidiosis.

Microbial Competitive Exclusion as an Alternative to AGP

Due to concern of AGP use in the United States and total bans in the European Union, various replacements for antibiotics in feed have been evaluated. The challenge is to achieve the current growth rate and feed efficiency of chickens in the USA without using in-feed antimicrobials. Nurmi and Rantala (1973) described a concept termed competitive exclusion (CE), which has the potential to control pathogens in poultry through the administration of probiotics as defined or undefined cultures. La Ragione and Woodward (2003) demonstrated the efficacy of *Bacillus subtilis* spores in controlling *Salmonella enterica* serotype Enteriditis and *Clostridium perfringens* in young chickens. A single oral inoculum of 10^9 *B. subtilis* spores given a day before challenge significantly suppressed colonization and persistence of the pathogens. Chen and Stern (2001) used chicken-derived *Campylobacter jejuni* isolates as defined cultures for controlling human-pathogenic *C. jejuni* strains in poultry. The authors found the non-pathogenic *C. jejuni* strains to be effective at preventing colonization of human-pathogenic *C. jejuni* in poultry when orally challenged at a 1:1 ratio.

La Ragione *et al.* (2004) administered *Lactobacillus johnsonii* FI9785 as a defined probiotic culture to control the colonization of *Salmonella enteriditis*, *E. coli* O78:K80 and *C.*

perfringens in poultry. The probiotic culture had no significant effect on *S. enteriditis* colonization in the small intestine or ceca but had an effect on *E. coli* O78:K80 colonization of the ceca and significantly reduced the colonization of *C. perfringens*. Estrada *et al.* (2001) found that administering *Bifidobacterium bifidum* as a defined probiotic had no significant effect on growth performance but reduced the number of condemnations of birds due to cellulitis, or loose subcutaneous tissue inflammation often associated with pathogenic *E. coli*. Possibly related to the lowered incidence of cellulitis, the authors found that *B. bifidum*-treated groups showed a reduction in total aerobic bacteria, coliforms and clostridia in chicken feces.

Smirnov *et al.* (2005) administered either a defined probiotic mix containing *L. acidophilus, L. casei, Bfidobacterium bifidum* and *E. faecium* or AGP to broiler diets. The authors found the AGP-fed group compared to the probiotic group had higher counts of *Bifidobacterium* in the duodenum, increased villous surface area in jejunum and low levels of mucin glycoprotein in the duodenum. The probiotic-fed group had increased proportions of *Lactobacillus* species in the ileum but not the jejunum and duodenum, enlarged goblet cell area and increased mucin glycoprotein levels in the jejunum. The authors concluded that probiotics and AGP administered in feed alter mucin biosynthesis through changes in the bacterial community. Mucin production improves gut function and health through protection from pathogenic bacterial attachment, lubrication and transport between luminal contents and epithelial cells.

Hofacre *et al.* (2003) compared the effectiveness of five non-antibiotic food additives and two competitive exclusion (CE) cultures in a necrotic enteritis model using *C. perfringens*. The CE cultures represented both undefined and defined cultures, consisting of *Lactobacillus acidophilus, Enterococcus faecium, Lactobacillus plantarum* and *Pediococcus acidilactici*. The

treatment group given the defined CE culture showed lower mortality than the control groups and had increased feed efficiency and weight gain. Also, the group given both the defined CE culture and mannan-oligosaccharide also had increased feed efficiency and weight gain. The undefined CE culture as well as the other additives administered alone had no significant effects on feed efficiency, weight gain or mortality.

Netherwood *et al.* (1999) used molecular and culture techniques to examine changes in the chicken GI tract microflora following the inclusion of probiotics in the feed. Using a genetically modified (GM) *Enterococcus faecium* CE strain and the *E. faecium* parent CE strain, the authors found no difference in community structure with the cultural method but a significant response using 16S rDNA community analysis. There was significant decrease of *E. faecalis* in the birds given the wild-type *E. faecium* probiotic and a significant increase of *E. faecalis* in the groups given the GM strain of *E. faecium.* The authors suggested that the *E. faecium* probiotics occupied the same niche as *E. faecalis*.

Hop Extracts and the Possible Use as an Alternative to AGP in Poultry Rearing

Hops (*Humulus lupulus*) have been used as an antimicrobial and flavor enhancer in beer brewing for centuries. The constituents of hop, grouped into *alpha* and *beta* acids, act as antimicrobial compounds on gram-positive bacteria in in vitro systems (Gerhauser, 2005). The *beta*-acids have stronger antimicrobial properties due to their hydrophobic nature allowing interactions with bacterial membranes, specifically gram-positive bacteria. The hop constituents lupulone, humulone, isohumulone and humulinic acid were shown by Teuber and Schmalreck (1973) to control *Bacillus subtilis* through bacterial membrane leakage. Hop extracts and their alpha and beta acids have been shown to inhibit *Listeria monocytogenes* and other gram-positive bacteria in foods with low acidity (Larson *et al.,* 1996). Srinivasan *et al.* (2004) found that hops

acids have an antimicrobial effect on gram-positive bacteria and protozoa but have no effect on gram-negative bacteria. Hanske *et al.* (2005) examined the bioavailability of xanthohumol, a prenylated chalcone flavonoid found in hops, and the effects on the intestinal microbiota of Sprague-Dawley rats. The authors recovered intact xanthohumol in the feces; additionally, they found that the compound had no significant effect on the intestinal microbiota diversity according to PCR-DGGE analyses. As an alternative to AGPs, Cornelison *et al.* (2006) administered ground hop instead of AGPs to the diets of broilers and assessed weight gain, feed conversion and feed efficiency. They found that hop added at 0.5 lb per ton of feed improved feed conversion and efficiency throughout the grow-out period and increased body weight at day 14 of growth compared to the control group.

To effectively understand how AGPs or alternatives positively affect growth performance, researchers must determine both the normal intestinal microflora of the chicken gastrointestinal (GI) tract and the microflora of the GI tract after AGP use (Dumonceaux, 2006). Much of the data regarding microflora has been collected via cultural methods but molecular methods are used increasingly as technology has progressed.

Culture Method Studies of the Chicken Gut Microbial Ecology

Early data on the microbiota of the chicken gut was based on evidence gathered from cultural methodology. Barnes *et al.* (1972) assessed the intestinal flora of broilers in the age range of 2 to 6.5 weeks old. The authors found that in the small intestine *Lactobacilli* was the only group of organisms to exceed 10^4 cfu/g. They also showed the cecal content contained high anaerobic counts of around 10^{11} cfu/g but did not identify all of the groups. Anaerobic streptococci had predominated at 2 weeks at around 30% but had decreased to 9% of the flora by the middle of the sixth week of growth. Many of the gram-negative anaerobes only appeared

between the fourth and sixth week. This data suggested a microbial community succession in chickens over time. The initial cecal colonizers are anaerobic and utilize oxygen creating an environment in which obligate anaerobes can thrive.

Salanitro *et al.* (1974) used rumen fluid medium to enhance recovery and identification of anaerobic bacteria in the chicken cecum. The data from this study indicated the presence of anaerobic gram-negative cocci, facultatively anaerobic cocci and streptococci, *Peptostreptococcus*, *Propionibacterium*, *Eubacterium*, *Bacteroides*, and *Clostridium*. Using anaerobic roll tubes and aerobic plating, Salanitro *et al.* (1978) found the facultatively anaerobic groups *Streptococcus, Staphylococcus, Lactobacillus,* and *Escherichia coli* to be the predominant flora detected in the duodenum and ileum of the chicken at 14 days. The authors also determined that gram-positive, strict anaerobes *Eubacterium, Clostridium, Gemmiger, Fusobacterium,* and *Bacteroides* populated the cecum. The study indicated that strict anaerobes dominated the cecal environment.

Molecular-Based Methods for Chicken Gut Microbial Ecology Determination

Culture method studies have limits in the recovery and identification of intestinal microbes. These methods tend to be labor and time intensive and require an a priori basis of selective media for certain bacteria types (Amit-Romach *et al.,* 2004). The use of molecular techniques such as G+C% profiling and 16S rDNA analysis has allowed for characterization of previously uncultured microbes and assessment of molecular diversity in the chicken intestine. The genome of a complex biological system, such as a chicken intestine, can be analyzed by fractionation of the genome according to G+C content of the component populations. Percent G+C profiling gives a representation of the bacteria present in an ecosystem based on the differential density of fractionated DNA. Analysis of 16S rDNA sequences also gives a

representation of bacterial groups present in a complex ecosystem. The 16S rRNA genes, which encode for the 16S subunit of the bacterial ribosome, are highly conserved and specific for distinct phylogenetic groups. Genes for 16S rDNA isolated from bacteria are sequenced and can be used for future identification of the bacterial group (Apajalahti *et al*., 2001).

Lan *et al.* (2002) assessed the cecal microbiota using both strict anaerobe culture-based methodology and 16S rDNA clone library analysis. The study results indicated that 90% of the bacteria observable by microscopic examination was uncultivable. The culture method was able to detect 19 isolated strains of which 11 yielded distinct operational taxonomic units, a measure of relatedness. Eleven of the 19 isolates (58%) were classified into a low G+C Gram-positive group, 5 isolates (26%) belonged to the *Bacteroides* group and the other 3 isolates belonged to *Proteobacteria* group, which includes *Enterobacteriaceae*. Clone sequence analysis revealed that 38% of the clone sequences belong to *Clostridium* subcluster XIVa, 13% of the clone sequences belong to *Clostridium* cluster IV, 24% of the sequences belong to the *Lactobacillus* group and 4% of the sequences belong to *Bacteroides* group.

Amit-Romach *et al.* (2004) used 16S rDNA analysis to focus on 6 groups representing GI bacteria that are either beneficial or pathogenic to poultry or humans. Using densometric analysis of PCR products on gel agarose, they determined *Lactobacilli* and *Bifidobacterium* constituted 40% of the total bacteria at Day 14 of growth and 50% at Day 25. *Salmonella* was detected at 40% on Day 4, 30% on Day 14 and decreased to 20% by Day 25. They also found *E. coli* and *Clostridium* species at 33% of total bacteria on Day 4 and remaining static through Days 14 and 25.

Gong *et al.* (2002) used molecular analysis of 16S rDNA and culture-dependant methods to examine the diversity of the bacterial community in chicken cecal mucosa. They found the

cecal mucosa to contain mainly low G+C content, gram-positive bacteria. Butyrate-producing bacteria, such as *Fusobacterium prausnitzii*, comprised the largest groups with 25% of the 116 cloned sequences. The authors highlighted the significance butyrate has in animal health through regulation and turnover of colonic epithelial cells. Zhu *et* al. (2002) found approximately 89% of 16S rRNA gene clonal sequences in the cecum represented four phylogenetic groups including: *Clostridium leptum* (20.2% of cloned sequences), *C. coccoides* (27.1%), *Sporomusa* (21.2%) and enterics and relatives (20.8%). Minor groups they found included *Atopobium* (3.6%), *Bacteroides* (1.9%), and *Bacillus-Lactobacillus-Streptococcus* subdivision (1.5%).

Using 16S rDNA cloning and sequence analysis, Bjerrum *et al.* (2006) compared the GI microbial flora of conventionally and organically-raised chickens. They found the ileums from both the organic and conventional flocks to be dominated by *Lactobacillus* spp. with 93.8% of the cloned sequences. The cecum had a greater diversity of bacteria with conventional flocks having 15.5% of cloned sequences belonging to *Eubacterium* and around 60% of the sequences belonging to the *Clostridium* group including *Faecalibacterium prausnitzii*. In the organic flock, 35.5% of the clones belonged to the *Eubacterium* group and 43% of the clone sequences belonged to the *Clostridium* group. The authors noted that the role of *F. prausnitzii* in chicken gut health is unknown and needs to be elucidated but ostensibly has beneficial effects such as butyric and lactic acid production.

Lu *et al.* (2003) used a qualitative 16S rRNA gene sequence library analysis to illustrate the diversity of bacterial flora in chicken fed a vegetarian corn-soy diet. The low G+C gram-positive group, including *Lactobacillus, Bacillus, Clostridia* and streptococci, was the most prevalent in both the ileum and cecum. The authors found the following bacterial groups in the ileum,: *Lactobacillus* species, 67% of the clone sequences; *Clostridiaceae*, 11%; *Streptococcus,*

6.5% and *Enterococcus,* 6.5%. They found the following bacterial groups in the cecum: *Clostridiaceae,* 65% of the clone sequences; *Fusobacterium*, 14%; *Lactobacillus*, 8% and *Bacteroides,* 5%. The authors also showed that a community succession occurred in the ileum and the cecum as the birds aged. Each compartment had a unique bacterial community on day 3, day 7 and day 49 of grow-out but had a stable community structure from days 7-21 and days 21-28 for the ileum. The cecal community was stable for days 14-28.

Diversity of bacteria has been demonstrated among the different compartments of the chicken GI tract during growth. Van der Wielen *et al.* (2002) amplified the V6-V8 regions of 16S rDNA of intestinal contents and used denaturing gradient gel electrophoresis (DGGE) to describe diversity in the banding patterns. They observed that the number of bands in intestinal compartments increased as broilers grew in age indicating a higher level of microbial complexity with age. Even though the chickens were reared under the same conditions in the study, each compartment had its own banding pattern regardless of age suggesting that host-related factors affect the establishment of a bacterial community. This suggested that each compartment had its own specific factors that play a role in bacterial development of the compartment. Pedroso *et al.* (2006) conducted a study using DGGE analysis of 16S rDNA to assess the effects of AGPs on the intestinal microbiota of chickens raised in floor pens or battery cages. The antibiotics used in the study improved the performance of the broilers in the floor pens and induced changes in the bacterial community of the GI tract. The authors indicated that the induced changes might have led to improvement in growth performance.

Dumonceaux *et al.* (2006) studied the intestinal microbiota and the response to virginamycin in the broiler diet. The authors characterized the microbial ecology of five GI tract positions including the duodenal loop, mid-jejunum, proximal ileum, ileocecal junction and the

cecum in 47-day old birds given diets with or without virginiamycin. The clonal library analysis of chaperonin 60 (*cpn60*) gene sequences indicated that approximately 90% of sequences in the small intestine were related to *Lactobacillus*, whereas clostridial sequences made up 68% of the cecal sequences along with *Lactobacillus* (25%) and *Bacteroidetes* (6%). Using quantitative, real-time PCR, they found that virginiamycin increased the abundance of bacterial group targets in the proximal GI tract but had no effect on the ileocecum junction or the cecum.

CHAPTER 3

MATERIALS AND METHODS

Experimental Group Design and Sample Collection

All animal and bird experiments were conducted in compliance with Agricultural Research Service-USDA Laboratory Animal Care and Use Committee standards for care, feeding, euthanasia and disposal. This present study was run in duplicate and samples from both trials were combined to obtain the data. Newly-hatched broiler chickens (*Gallus gallus* domesticus) were assigned to one of 5 groups (n=4) as shown in Table 1. Broilers were fed diets consisting of non-medicated broiler starter diet feed (corn-soy based 23% crude protein, 6% fat, 2.5% fiber, 1.0 % calcium and 0.48 available phosphorous; 3100 kcal/kg estimated metabolic energy) obtained from the University of Georgia, Department of Poultry Science feed mill. On Day 13-22, broilers in groups 3 and 5 (lupulone treatment groups) were given 125 ppm (307 μM) lupulone (see Figure 2 for structure) through cage watering systems. The midrange level of 125 ppm lupulone was chosen based on prior laboratory studies (data not shown) showing no significant difference in the administered level of lupulone ranging from 62.5 to 250 ppm. Beginning on Day 14, broilers in the challenge groups 4 and 5 were administered 0.1 ml of a three strain *C. perfringens* cocktail (per bird dosage of ~$\log_{10}$ 7) per os for three consecutive days (Days 14-16 of growth). The *C. perfringens* cocktail consisted of three *C.* perfringens Type A strains obtained from commercially-reared birds from a NE outbreak as described in Wise and Siragusa (2006). Challenge with *C. perfringens* was meant as a colonization model rather than a necrotic enteritis model. The strains were propagated in pre-reduced brain heart infusion broth

under anaerobic conditions for 16 h at 37°C in screw cap tubes prior to challenge dosage preparation. Birds from Group 1 were sacrificed by cervical dislocation on Day 14, and birds from Groups 2-5 were sacrificed on Day 22. Cecal and midgut sections (Figure 1) were removed from the bird gastrointestinal tracts and placed in individual, sterile Whirl-Pack bags.

DNA Extraction from Intestinal Samples

Midgut and cecal samples for DNA extraction were removed by squeezing the luminal contents into 15 ml conical tubes. The samples were diluted 1:10 with phosphate-buffered saline (PBS) and vortexed vigorously for 1 min. Microbial DNA was extracted from the mixture following manufacturer's instructions for MoBio UltraClean™ Fecal DNA Kit (Solana Beach, California). Briefly, 1 ml of each mixture was transferred to the Bead Tubes provided in the kit. Samples were centrifuged at 10,000 *x*g for 5 minutes and the supernatant was discarded. Five hundred microliters of Bead Solution (guanidine thiocyanate), 60 µl of S1 Solution (sodium dodocyl sulfate) and 200 µl of IRS solution (proprietary inhibitor removal solution) were added to each tube and bacterial cell walls mechanically lysed at maximum speed on the MoBio Vortex Adaptor™ for 10 min (Solana Beach, California). The tubes were centrifuged for 3 min at 10,000 *x*g and the supernatant was transferred to labeled, sterile microcentrifuge tubes. The supernatant was mixed with 250 µl of Solution 2 (proprietary acetate solution), vortexed and placed on ice for 5 min. The tubes were centrifuged at 12,000 x g for 3 minutes, supernatants removed and mixed with 1 ml of Solution 3 (guanidine HCl and isopropanol). The mixture was added to spin filter columns, washed with 300 µl of Solution 4 (ethyl alcohol) and purified DNA was eluted with 50 µl of Solution 5 (Tris aminomethane/hydrochloride). Purified DNA was stored at -20°C until use as template in PCR.

Quantitative Real-Time PCR

All PCR reactions consisted of 20 µl volumes in a 96 well format for the Applied Biosystems 7300 instrument. Reaction volumes, as described in Wise and Siragusa (2006), included 10 µl 2X SYBR Green MasterMix (Applied Biosystems, Foster City, California), 1 µl of each group-specific forward primer at 10 µM (final concentration of 0.5 µM), 1 µl of group-specific reverse primer at 10 µM (final concentration of 0.5 µM), 1 µl of BSA at 2.5 mg/ml (final concentration 125 µl/ml), 5 µl of nuclease-free water and 2 µl of purified DNA template. Due to the presence of inhibitors in intestinal samples, all templates were diluted 1:10 in MoBio Elution Buffer before adding to PCR reactions (Wise and Siragusa, 2005). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, primer-specific annealing temperatures (Table 2) for 1 minute and 78°C for 30 sec. A dissociation step was included after amplification to analyze the melting curves of amplified product. Data was obtained at the 78°C step to ensure primer-dimers were not measured as amplification of the target.

Quantification of Bacterial Groups in Intestinal Samples

Major and minor groups of the midgut and ceca were quantified using standard curve assays as described in Wise and Siragusa (2006). Standard curves were constructed using 16S rDNA fragments from the representative isolates from each group shown in Table 2. Amplified gene products from each 16S rDNA region were purified and cloned into pCR4-TOPO using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids containing the insert for each group were purified and quantified using a spectrophotometer. The number of target gene copies was calculated from the mass of DNA and the number of base pairs in the insert and plasmid. The gene copies were serially diluted from $2x10^9$ to $2x10^1$. Standards for each bacterial group

were amplified along with the unknowns. The cycle threshold, the point at which the fluorescence passed the threshold, was determined for the standards, and unknowns were extrapolated from the curve. Gene copy numbers were multiplied by 100 to account for the dilution of original sample (1:10 of GI material in PBS) and dilution of DNA template (1:10) for PCR. Relative gene copy numbers for the unknowns were converted to $\log_{10}$ for normal distribution and reported per gram intestinal material.

Statistical Analyses

Average $\log_{10}$ gene copy numbers and standard deviations were determined for the bacterial groups in all experimental groups. Additionally, bacterial groups were considered relative to total eubacteria determined in the midgut and ceca and percentage of gene copies was determined. Microfloral comparisons were made between lupulone treatment groups and control groups, challenge groups with non-challenge groups and interactions of challenge and lupulone. Presence-absence analyses were performed on all groups using Fisher's exact test. For non zero counts in each group, a profile analysis using Analysis of Variance with SAS® 9.1 was performed at the Biostatistics Consulting Service in College of Public Health, The University of Georgia.

CHAPTER 4

RESULTS

Average bacterial group counts and standard deviations for the cecum are shown in Table 3 and average group counts for the midgut are shown in Table 4. For the consideration of the effect of lupulone on the individual bacterial groups of the GI tract, data for groups two and four (0 ppm lupulone) were aggregated and called the control group. Individual bacterial group data for groups three and five (125 ppm lupulone) were aggregated and called the lupulone treatment group. The bacterial groups *Enterobacteriacea*, *C. coccoides, Atopobium, Bacteroides, Enterococcus, C. leptum, Lactobacillus* and *Bifidobacterium* were detected in 100% of the control group samples taken in the cecum, and *C. perfringens* was detected in 7/8 samples. *Campylobacter* and *Veillonella* were undetected in the cecum and the midgut for all of the experimental group samples. All other bacterial groups were detected in 100% of the lupulone treatment group samples except *C. perfringens* and *C. leptum* which were both detected in 7/8 samples.

In the midgut, *Enterobacteriacea, C. coccoides* and *Enterococcus* was detected in 100% of the samples taken for all groups. *Lactobacillus* was undetected in one sample from the lupulone treatment group and one sample from the control group. *C. leptum* was undetected in two samples for the lupulone treatment group and twice for the control group. The presence analysis for *C. leptum* showed a significant difference ($p=0.0163$) in presence for the challenge groups versus the baseline day 14 group. In the midgut, *C. perfringens* was detected in 7/8 for the control group and 6/8 for the lupulone treatment group. *Atopobium* was detected in 2/8 for

the midgut samples for the lupulone treatment group but was undetected in the control group. *Bacteroides* was detected in 4/8 for both the lupulone treatment group and the control group. *Bifidobacteria* were not detected in the midgut for the control group but was detected in 1/8 for the lupulone treatment group.

Since there was no statistical difference (p=0.46) between region (i.e. cecum or midgut), we were able to aggregate data for all gram positive bacteria, regardless of region, and found lupulone had no significant effect (p=0.15). There was no statistically significant effect of lupulone on the aggregated total gram negative bacteria assayed as well (p=0.51). However, two bacterial groups showed a significant effect when considered individually. The counts for *C. perfringens* in both the cecum (p=0.0253) and the midgut (p=0.0179) were significantly lower in the lupulone treatment group than in the control group as shown in Figure 3 and Figure 4, respectively. Lupulone treatment groups had significantly lower (p=0.0275) *Lactobacillus* counts in the midgut than did the control groups.

Other statistical observations of the effects of hops-by-challenge interaction and challenge effect were made. There was a significant increase (p=0.0257) in *C. coccoides* from day 14 baseline to all day 22 groups including lupulone and control groups. Counts for *C. coccoides* in the cecum were significantly higher (p=0.0030) in birds that were challenged. The challenge group had significantly (p=0.0008) higher counts for *C. leptum* in the cecum but not the midgut. The lupulone treatment and challenge interaction also had significantly higher (p=0.0110) *C. leptum* counts in the cecum. The baseline day 14 group had significantly lower (p=0.0289) counts of *C. leptum* than any of the day 22 groups.

The predominant groups of the cecum including *C. coccoides*, *C. leptum*, *Bacteroides* and *Enterobacteriacea* were considered relative to the total bacteria assayed and their frequencies are shown in Figure 5. *Enterobacteriacea, Enterococcus* and *Lactobacillus* counts dominated the midgut and their frequencies relative to total bacteria are shown in Figure 6. The bacterial profile for the midgut is shown in Figure 7 and the profile for the cecum is shown in Figure 8.

CHAPTER 5

DISCUSSION

In this study, we used quantitative real-time PCR to quantify the major bacterial groups of the broiler cecum and midgut with or without the addition of 125 ppm lupulone (hop β-acids) to the broiler diet. Our study design included quantifying bacterial groups at day 14 and day 22. We quantified the microflora at day 14 to assess any temporal succession in the bacterial community by day 22, which represents the point at which necrotic enteritis is generally observed in the field. The purpose of the study was to determine if lupulone would have an effect on the microbial profile of the GI tract and to determine if lupulone would have an antimicrobial effect on *C. perfringens* in the broiler GI tract. For our first hypothesis, we found that the addition of lupulone to the broiler diet did not affect the overall profile of the cecum or the midgut but did have a significant effect on the populations of several individual groups of the midgut and cecum. Both *Lactobacillus* and *C. perfringens* counts were lower in the midgut for the hops extract treatment groups than in the zero hops treatment group. We also found that *C. perfringens* was significantly decreased in the cecum for the hops treatment groups as predicted in our second hypothesis. *Lactobacillus* and *C. perfringens* are gram positive bacteria, and we had expected that hops extract would have an antimicrobial effect as found previously (Srinivasan *et al.*, 2004; Teuber and Schmalreck, 1973). Interestingly, *Lactobacillus* counts did not significantly decrease with hops exposure in the cecum. Dumonceaux *et al.* (2006) found that virginamycin had no effect on the microbiota of the distal portion of the GI tract including the ileo-cecal junction and the cecum.

We found that *C. coccoides, C. leptum, Bacteroides* and *Enterobacteriacea* dominated the cecal environment as shown in both culture and molecular studies (Barnes *et al.*, 1972; Dumonceaux *et al.*, 2006; Lan *et al.*, 2002; Lu *et al.*, 2003; Salanitro *et al.*, 1974). *Enterobacteriacea, Enterococcus* and *Lactobacillus* dominated the midgut in all experimental groups. There was not a significant shift in the microbial profile with lupulone treatment. Hanske (2005) found no significant changes in the microbial diversity of the rat GI tract exposed to xanthohumol, a hop extracts. Although not significant, *Enterobacteriacea* appeared to increase in the midgut with lupulone exposure as *Lactobacillus* counts decreased. *Lactobacillus* is considered to be part of a normal gut and possibly occupies a niche that prohibits pathogenic organisms from colonizing the GI tract. In our study, lupulone had an antimicrobial effect on *Lactobacillus* which possibly allowed the proliferation of bacteria from the *Enterobacteriacea* family including *Salmonella* and *E. coli*. This interaction was not statistically significant possibly due to high variation inherent in the small data set.

One of the main goals of this study was to elucidate a quantitative profile of the broiler GI tract with and without an AGP alternative. Previous studies have provided the basis for a qualitative analysis of the resident microflora but few studies have quantified the bacterial groups. In this study, we used gene sequences from highly conserved regions of 16S rDNA to develop standard curve assays for real-time PCR. The early quantitative and qualitative work was done through culture methods (Barnes *et al.* 1972; Salanitro *et al.* 1978) but had limitations in its scope. As molecular methods have progressed, previously uncultured microbes have been found to be an integral part of the microflora of the broiler GI tract. However, quantitative molecular methods for detecting microbes in a natural ecology do have limitations. Problems arise from PCR bias, primer design, extraction efficiency and PCR inhibitors.

The bacterial groups assayed were found in the literature to be the predominant groups in the broiler GI tract microflora based on cultural methods and clonal sequence libraries. Each group-specific primer set amplifies a myriad of bacterial species. The *Clostridium perfringens* subgroup Cluster I consists of *C. perfringens*, *C. butyricum*, *C. botulinum* and several *Eubacterium* spp. In this study, we saw a reduction in Cluster I *Clostridium*, a group in which many of the known pathogenic clostridial species are found. The *Clostridum leptum* subgroup, Cluster IV, contains *C. leptum*, *Ruminococcus*, *Eubacterium* and *Fusobacterium prausnutzii*. Many of the species found within Cluster IV are mesophilic and cellulolytic and can impart the benefit of cellulose degradation for the host. The *Clostridium* coccoides subgroup Cluster XIVa and XIVb consists of *C. coccoides*, *Eubacterium rectale*, and other butyrate-producing bacteria. Butyrate promotes gut health through cell proliferation and colonic cell turnover (Gong *et al.,* 2002).

Lactobacillus group, consisting of *Lactobacillus* spp., *Leuconostoc*¸ *Pediococcus*, *Aerococcus* and *Weissella*, are thought to be beneficial for gut health and are often used as probiotics (Hofacre *et al.* 2003; La Ragione *et al.* 2004; Smirnov *et al.* 2005). The reduction of the *Lactobacillus* subgroup in the midgut of the lupulone-treated birds could be a deleterious effect allowing colonization of pathogenic species and needs to be further investigated. The *Enterococcus* group, including *E. faecalis*, did not appear to have a significant change, which could be an important point for future studies. In a review, Sullivan *et al.* (2001) reported findings on antimicrobial use correlating with an increase in antibiotic resistance in enterococci species. Enterococci species have been shown to obtain antibiotic resistance and could lead to nosocomial infections in human medicine. *Campylobacter* and *Veillonella* had no observable counts but were expected to have a presence in the cecum. The absence of these two groups

could be attributable to experimental error or PCR inhibitors rather than the true absence of the organisms. The midgut also possessed several bacterial groups that had less than 100% presence. The absence of these groups was likely attributable to experimental error. An explanation for the high variability seen, especially in the midgut, was the inconsistent rate at which groups of birds defecate and feed. Unpredictability of defecation and gut peristalsis could make midgut profile assays highly variable. A quantitative bacterial group assay in a complex system also has another limitation. The number of *rrn* gene copies is not always one copy per genome. The actual number varies due to the number of *rrn* operons per genome and the number of genomes if the cell is in exponential growth (Wise and Siragusa, 2006).

CHAPTER 6

CONCLUSIONS

This study was unique in its goal to quantify bacterial groups in the broiler GI tract following lupulone exposure. A better understanding of the GI bacterial groups and their interactions with the host organism will facilitate development of intervention methods that continue the current feed efficiency and conversion in conventionally-raised poultry yet do not create or select bacteria that are antibiotic resistant. Cornelison *et al.* (2006) found that hops administered to feed could have a growth promoting effect similar to that of penicillin. In the present study, we administered lupulone, a hop β-acid, to the drinking water of broilers to assess the effect on the bacterial flora of the midgut and cecum and to assess its anti-clostridial potential. We found that lupulone had no overall effect on the profile of the intestinal bacterial groups, but its use corresponded with a reduction in the *Lactobacillus* group and the *Clostridium perfringens* subgroup. The reduction of *Lactobacillus* in the midgut could be a negative effect leading to proliferation of pathogenic organisms in the *Enterobacteriacea* family, including *Salmonella* and pathogenic *E. coli*. Members of the *Lactobacillus* group have been used in probiotic studies for their ability to inhibit the colonization of pathogenic bacteria. The reduction of the *C. perfringens* subgroup was a promising finding for lupulone use as an AGP. The *C. perfringens* subgroup Cluster I contains most of the pathogenic clostridial species including *C. perfringens, C. botulinum* and *C. tetani.* Since excessive growth of *C. perfringens* is associated with necrotic enteritis in poultry, its control has relevance to the poultry industry.

Future studies with hop acids could further explore the interaction of lupulone and the resident microflora of the GI tract at time points beyond 3 weeks into a typical grow-out period. This current study was conducted at 3 weeks, a time period in which necrotic enteritis is typically seen in broilers. We found that lupulone had an antibacterial effect of on *C. perfringens* in our colonization model. However, another study could omit the *C.* perfringens challenge and solely examine the effect of lupulone on the host organism feed conversion, feed efficiency, weight gain and changes in the GI microbial flora at weeks 5-6 when the grow-out period is near completion. The fate and uptake of lupulone in a complex biological system like the chicken intestine would be an important and relevant to the effect on the microbial flora of the intestines. Appropriate drug design and delivery would include study of the fate of the compound. Also, study of the delivery would address the host organism's metabolic inactivation the compound making it unavailable to exert its effect on the bacterial flora.

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Table 1. Experimental group design with days of treatment, challenge and sacrifice

Group	Code	Challenge	Treatment with 125 ppm lupulone	Sacrifice
1	Baseline[A]	None	None	Day 14
2	NCL0[B]	None	None	Day 22
3	NCL125[C]	None	Day 13-22	Day 22
4	CL0[D]	Day 14-16	None	Day 22
5	CL125[E]	Day 14-16	Day 13-22	Day 22

[A] Day 14 Baseline No challenge/0 ppm lupulone
[B] Day 22 No challenge/0 ppm lupulone
[C] Day 22 No challenge/125 ppm lupulone
[D] Day 22 Challenge with *C. perfringens*/0 ppm lupulone
[E] Day 22 Challenge with *C. perfringens*/125 ppm lupulone

Table 2. Bacterial groups assayed and their primer annealing temperatures

Target Group	Representative Isolate	Primer Annealing Temp
domain *Bacteria*	*Escherichia coli*	60º C
Clostridium 16S rRNA Cluster IV	*C. leptum* ATTC 29065	60º C
Clostridium 16S rRNA Cluster XIVa and XIVb	*C. coccoides* ATTC 29236	50º C
Bacteroides group	*B. fragilis* ATTC 25285	59º C
Bifidobacterium group	*Bifidobacterium* sp. wild type	60º C
Enterobacteriaceae family	*E. coli* TOP10	63º C
Lactobacillus group	*Lactobacillus* sp. wild type	58º C
Clostridium 16S rRNA Cluster I	*C. perfringens* ATTC 13124	60º C
Enterococcus Genus	*Enterococcus faecalis* ATTC 19433	61º C
Veillonella Genus	*Veillonella parvula* ATTC 10790	62º C
Atopobium Genus	*Atopobium minutum* ATTC 33267	61º C
Campylobacter Genus	*Campylobacter jejuni* NCTC 11168	61º C

Table 3. Mean counts of bacterial groups in the cecum expressed as LOG_{10} 16S rDNA gene copies per gram of intestinal material

	Total Bacteria	*Enterobacteriacea*	*C.coccoides*	*C.leptum*	*Atopobium*	*Bacteroides*	*Enterococcus*	*Lactobacillus*	*C. perfringens*	*Bifidobacterium*
Baseline[A]	9.94	7.42	9.28	9.20	5.28	8.36	6.57	5.44	3.40	3.87
(S.D.)	(0.04)	(0.44)	(0.27)	(0.18)	(0.40)	(0.08)	(0.44)	(0.94)	(0.38)	(0.09)
NCL0[B]	9.91	7.24	9.19	9.51	5.26	8.13	6.28	5.63	4.95	3.82
(S.D.)	(0.12)	(0.18)	(0.25)	(0.06)	(0.56)	(0.29)	(0.55)	(0.67)	(1.47)	(0.28)
NCL125[C]	9.68	7.17	9.01	8.99	5.43	7.85	6.39	5.93	3.21	3.76
(S.D.)	(0.21)	(0.36)	(0.51)	(0.14)	(0.20)	(0.26)	(0.44)	(1.17)	(0.27)	(0.17)
CL0[D]	10.0	6.82	9.47	9.65	5.62	8.14	6.09	6.16	4.46	4.07
(S.D.)	(0.14)	(0.43)	(0.47)	(0.35)	(0.45)	(0.20)	(0.54)	(0.78)	(1.22)	(0.49)
CL125[E]	10.01	6.68	9.48	9.75	5.74	8.20	5.86	5.72	3.67	4.10
(S.D.)	(0.06)	(0.83)	(0.35)	(0.15)	(0.19)	(0.43)	(0.40)	(1.26)	(0.13)	(0.33)

[A] Day 14 Baseline No challenge/0 ppm lupulone
[B] Day 22 No challenge/0 ppm lupulone
[C] Day 22 No challenge/125 ppm lupulone
[D] Day 22 Challenge with *C. perfringens*/0 ppm lupulone
[E] Day 22 Challenge with *C. perfringens*/125 ppm lupulone

Table 4. Mean counts of bacterial groups in the midgut expressed as LOG_{10} 16S rDNA gene copies per gram of intestinal material

	Total Bacteria	*Enterobacteriacea*	*C.coccoides*	*C.leptum*	*Atopobium*	*Bacteroides*	*Enterococcus*	*Lactobacillus*	*C. perfringens*
Baseline[A]	7.35	6.52	3.05	ND	ND	ND	5.39	4.67	3.20
(Std. Deviation)	(0.86)	(0.80)	(0.31)	ND	ND	ND	(0.74)	(1.10)	(0.48)
NCL0[B]	7.30	6.84	3.27	4.04	ND	1.31	5.97	6.19	3.76
(Std. Deviation)	(0.60)	(0.77)	(0.78)	(0.30)	ND	(2.61)	(1.37)	(0.88)	(0.61)
NCL125[C]	7.40	7.15	3.73	4.19	1.95	1.51	6.23	4.78	2.68
(Std. Deviation)	(1.00)	(0.63)	(0.31)	(0.19)	(2.76)	(1.77)	(1.01)	(0.87)	(0.64)
CL0[D]	7.33	6.26	3.87	4.54	ND	2.76	5.36	6.93	3.43
(Std. Deviation)	(0.50)	(1.46)	(0.48)	(0.24)	ND	(1.90)	(1.99)	(1.43)	(0.55)
CL125[E]	6.91	7.13	4.31	4.72	1.84	1.55	6.27	4.93	2.78
(Std. Deviation)	(1.34)	(0.64)	(0.34)	(0.48)	(2.60)	(1.79)	(1.18)	(1.06)	(0.58)

[A] Day 14 Baseline No challenge/0 ppm lupulone
[B] Day 22 No challenge/0 ppm lupulone
[C] Day 22 No challenge/125 ppm lupulone
[D] Day 22 Challenge with *C. perfringens*/0 ppm lupulone
[E] Day 22 Challenge with *C. perfringens*/125 ppm lupulone

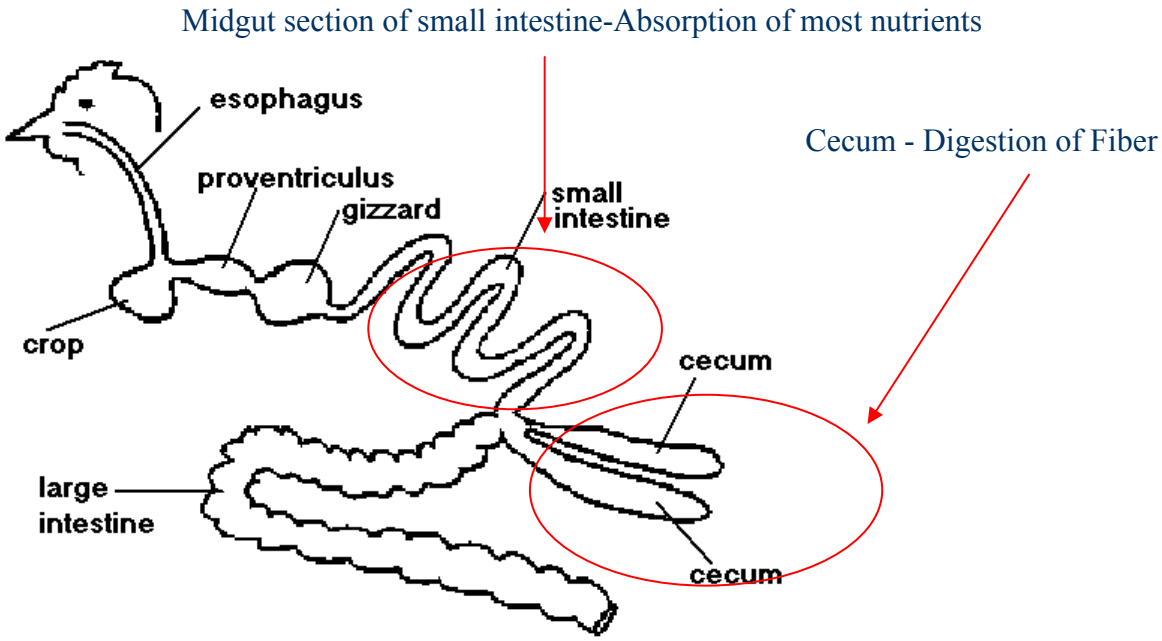
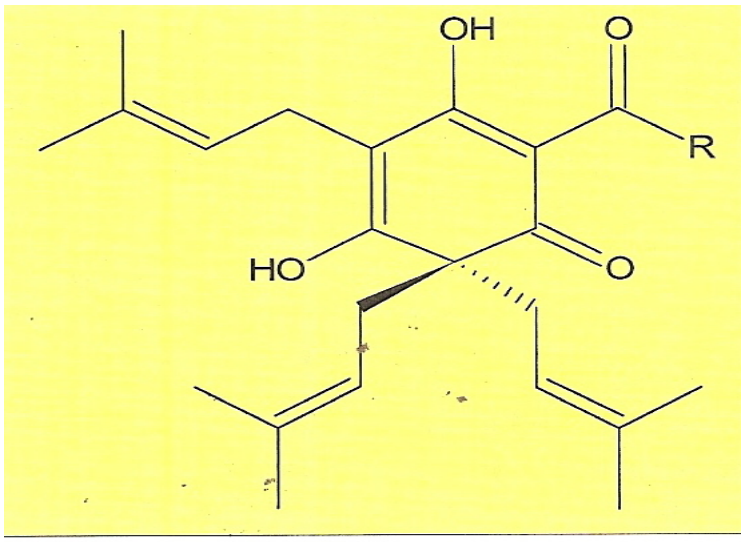


Figure 1. Location of the cecum and the midgut section of the small intestine in the broiler digestive tract.



R = -CH-$(CH_3)_2$ (Colupulone)
R = -CH_2-CH-$(CH_3)_2$ (Lupulone)
R = -CH(CH_3)-CH_2–CH_3 (Adlupulone)

Figure 2. Structure of the β-acids fraction of the hops plant with side chains.

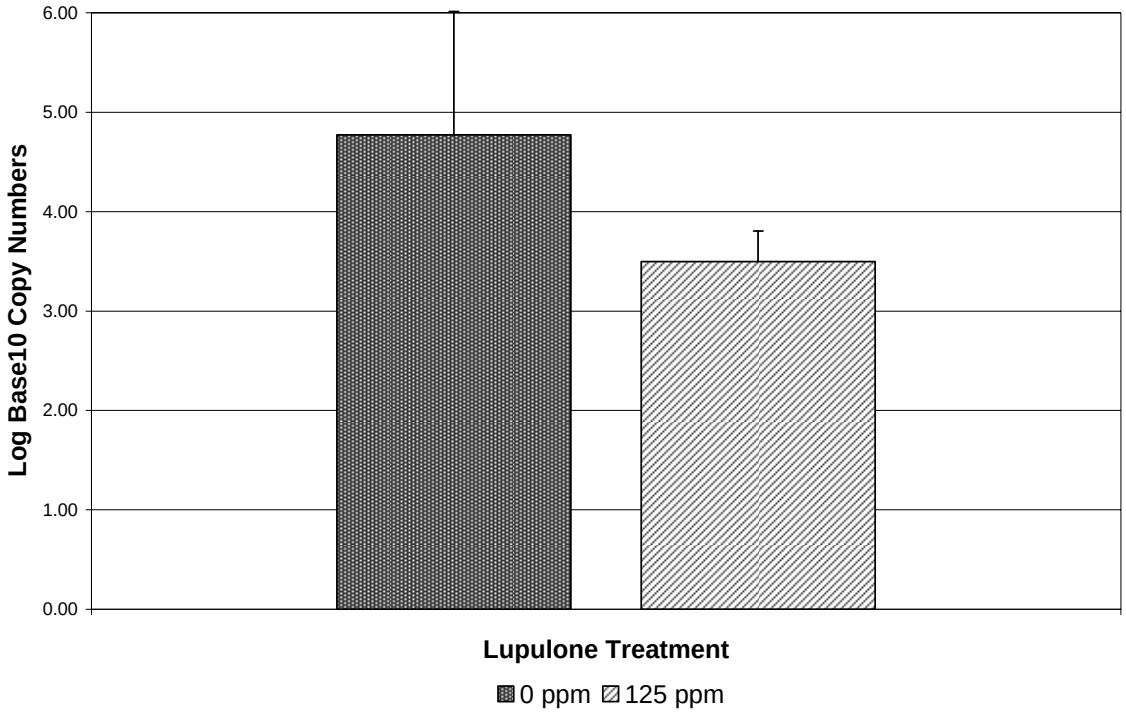


Figure 3. Reduction (p=0.0253) of gene copies for the *Clostridium perfringens* subgroup in the cecum following lupulone treatment at 125 ppm.

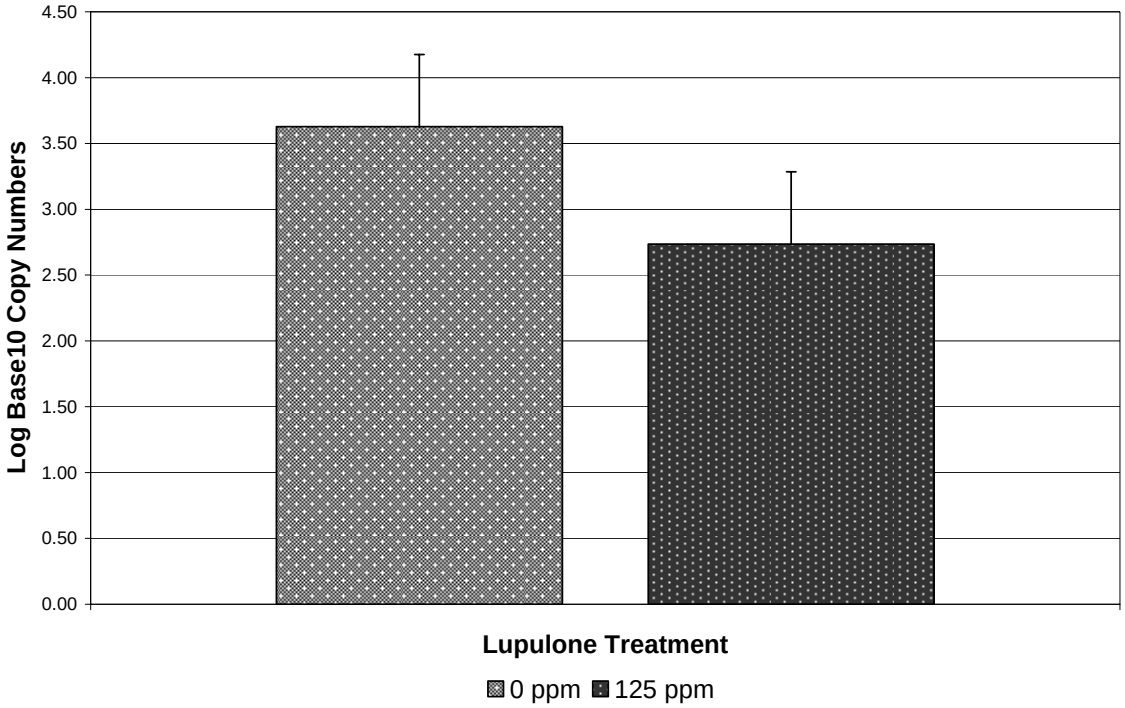


Figure 4. Reduction (p=0.0179) of gene copies for the *Clostridium perfringens* subgroup in the midgut following lupulone treatment at 125 ppm.

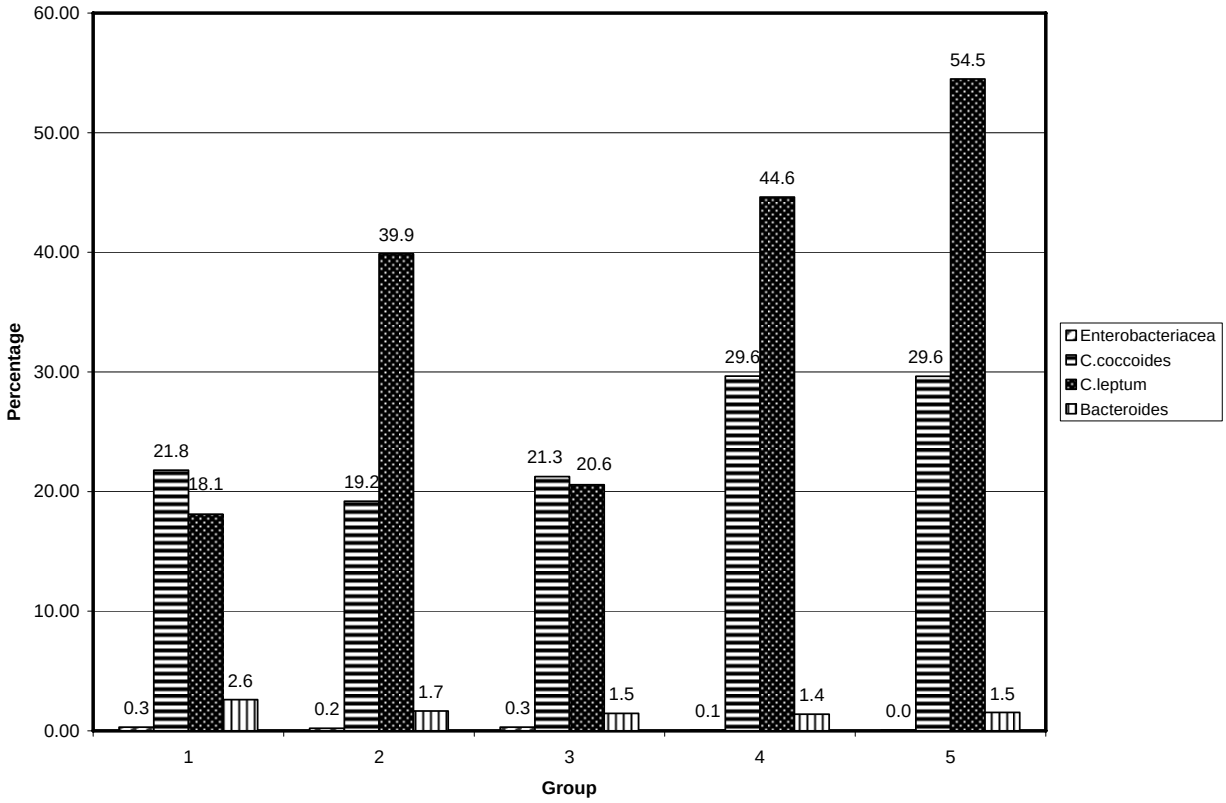


Figure 5. Percentage of major bacterial groups relative to total bacteria assayed in the cecum. Experimental groups are identified as follows: Group 1-Baseline Day 14 Group 2-No challenge with *C. perfringens*/0 ppm lupulone Group3- No challenge with *C. perfringens*/Treatment with 125 ppm lupulone Group 4- Challenge with *C. perfringens*/0 ppm lupulone Group 5- Challenge with *C. perfringens*/Treatment with 125 ppm lupulone.

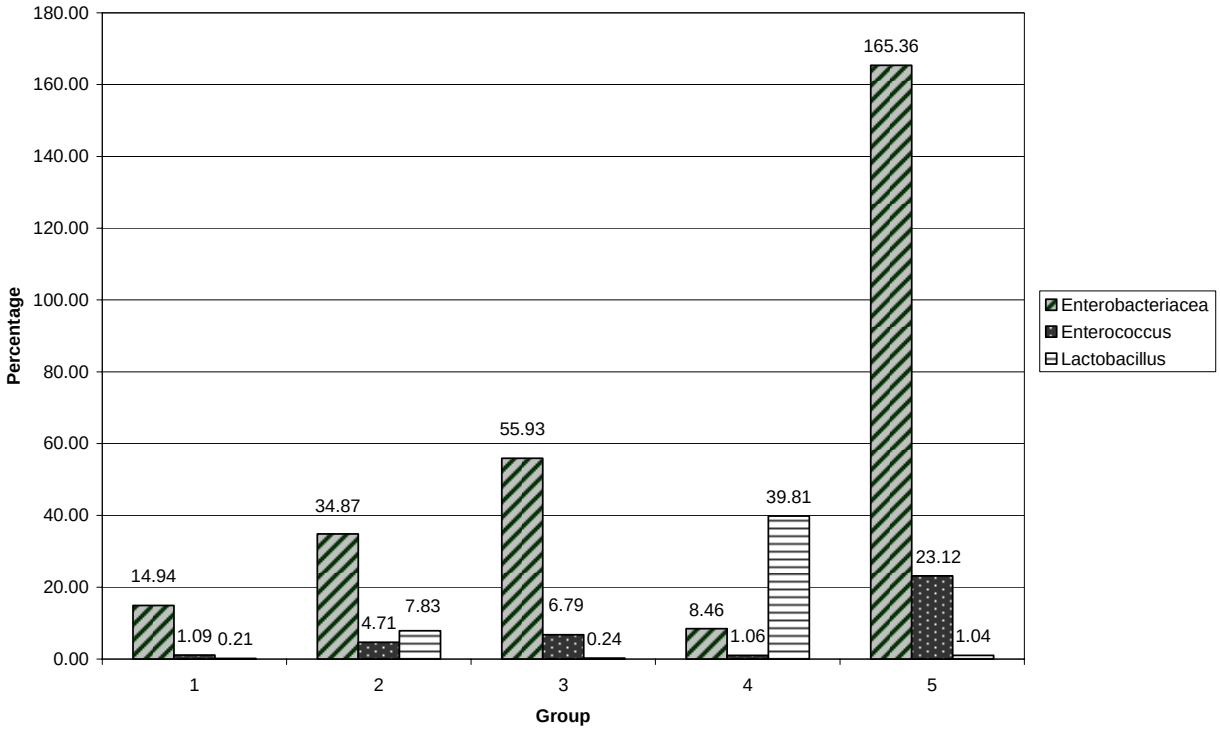


Figure 6. Percentage of major bacterial groups relative to total bacteria in the midgut. Experimental groups are identified as follows: Group 1-Baseline Day 14 Group 2-No challenge with *C. perfringens*/0 ppm lupulone Group3- No challenge with *C. perfringens*/Treatment with 125 ppm lupulone Group 4- Challenge with *C. perfringens*/0 ppm lupulone Group 5- Challenge with *C. perfringens*/Treatment with 125 ppm lupulone.

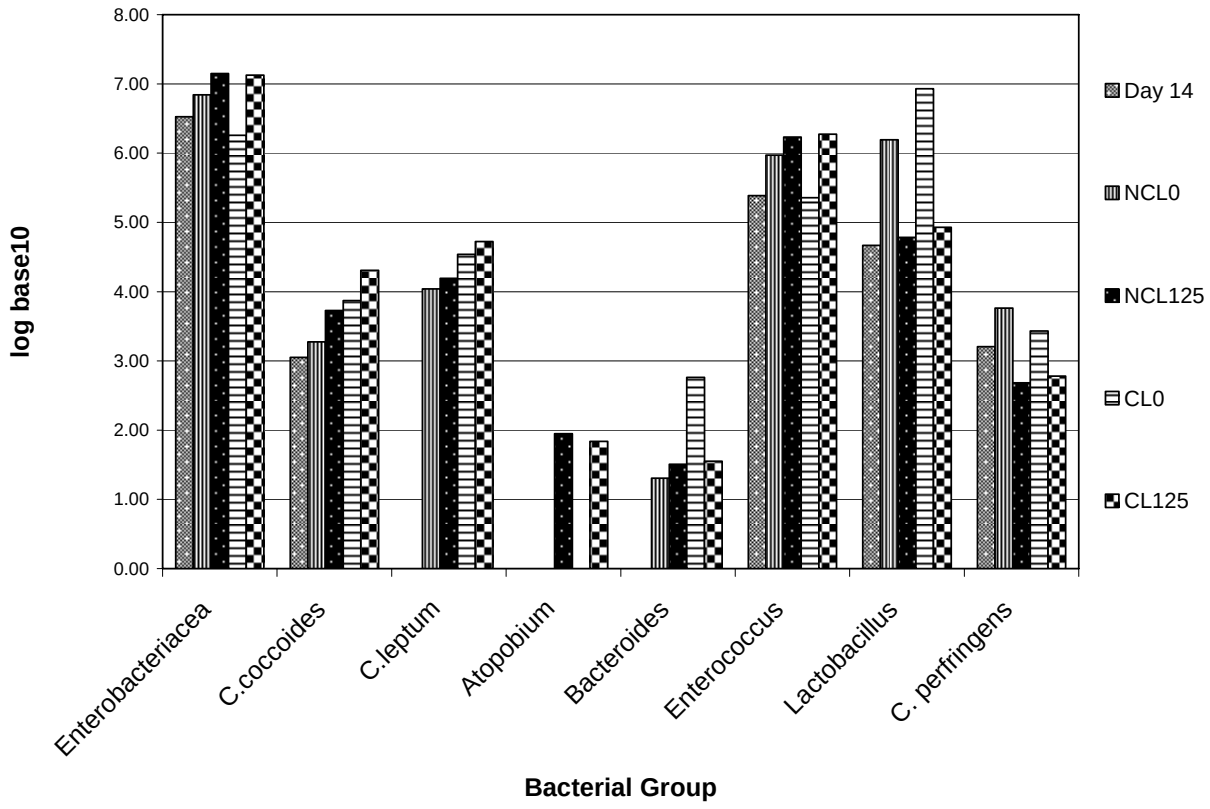


Figure 7. Microbial profile for the midgut with bacterial groups expressed as log base10 gene copies per gram. The experimental groups are identified as follows: Day 14-Baseline Day 14 NCL0-No challenge with *C. perfringens*/0 ppm lupulone NCL125- No challenge with *C. perfringens*/Treatment with 125 ppm lupulone CL0- Challenge with *C. perfringens*/0 ppm lupulone CL125- Challenge with *C. perfringens*/Treatment with 125 ppm lupulone.

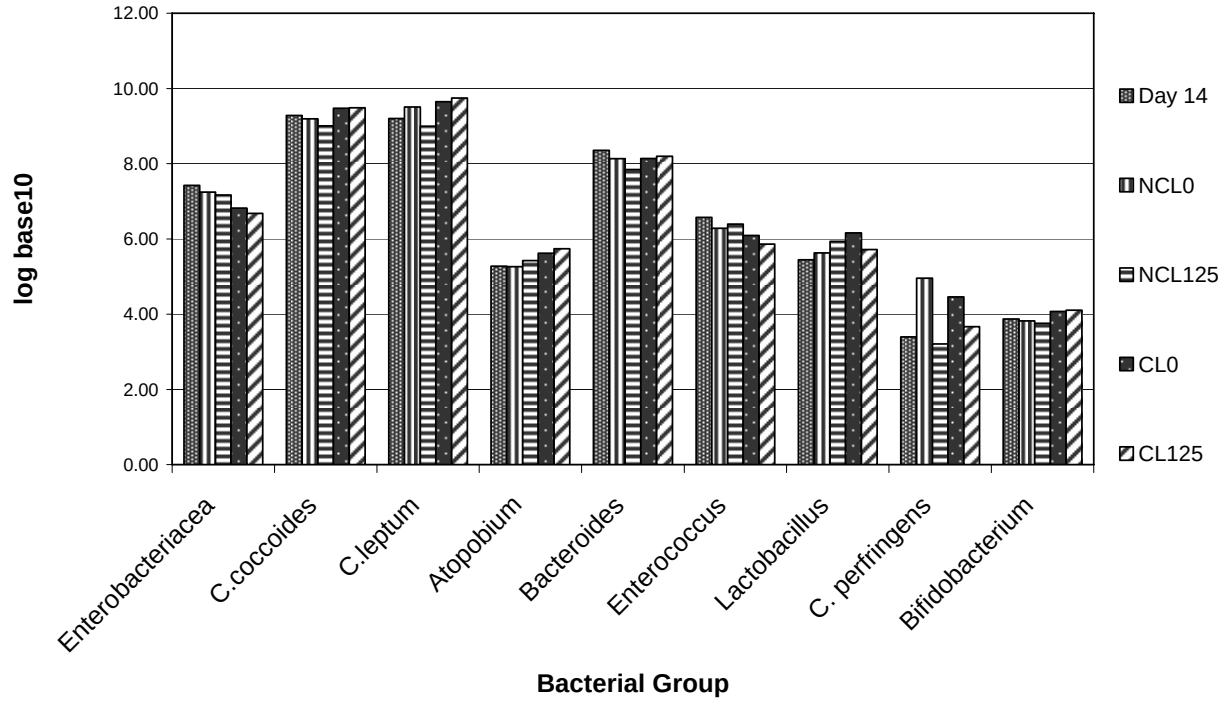


Figure 8. Microbial profile for the cecum with bacterial groups expressed as log base10 gene copies per gram. The experimental groups are identified as follows: Day 14-Baseline Day 14 NCL0-No challenge with *C. perfringens*/0 ppm lupulone NCL125- No challenge with *C. perfringens*/Treatment with 125 ppm lupulone CL0- Challenge with *C. perfringens*/0 ppm lupulone CL125- Challenge with *C. perfringens*/Treatment with 125 ppm lupulone.