THE EFFECTS OF AN EXPERIMENTAL CHLORATE PRODUCT ON THE

MICROBIAL ECOLOGY IN *GALLUS GALLUS* VAR. *DOMESTICUS*

A Dissertation

by

JACKSON LEE MCREYNOLDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Poultry Science

THE EFFECTS OF AN EXPERIMENTAL CHLORATE PRODUCT ON THE

MICROBIAL ECOLOGY IN *GALLUS GALLUS* VAR. *DOMESTICUS*

A Dissertation

by

JACKSON LEE MCREYNOLDS

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

J. A. Byrd (Co-Chair of Committee)	R. C. Anderson (Member)
S. D. Pillai (Co-Chair of Committee)	T. L. Poole (Member)
L. F. Kubena (Member)	A. R. Sams (Head of Department)

May 2004

Major Subject: Poultry Science

ABSTRACT

The Effects of an Experimental Chlorate Product on the Microbial Ecology in *Gallus*

Gallus Var. *Domesticus*. (May 2004)

Jackson Lee McReynolds, B.S., Texas A&M University;

M.S. Texas A&M University

Co-Chairs of Advisory Committee: Dr. J. A. Byrd
Dr. S. D. Pillai

Previous reports have shown that some bacteria utilize a dissimilatory nitrate reductase enzyme (NR) in anaerobic environments. This enzyme reduces nitrate to nitrite and also has been shown to co-metabolize chlorate to cytotoxic chlorite. A commercially available competitive exclusion (CE) product was evaluated for its nitrate reductase activity and therefore its experimental chlorate product (ECP) sensitivity. Of the 29 constituent bacteria of the CE culture, 11 had slight utilization of NR, 3 had moderate utilization of NR; the remaining were NR negative (with slight and moderate utilization: >0.1 to < 1.0 mM and > 1.0 mM nitrate utilized within 6 h, respectively). In vivo studies utilizing CE and ECP showed significant reductions in *Salmonella.* Although some of the bacteria were affected by ECP, the combined effect of the CE culture and ECP were effective in reducing *Salmonella.*

Clostridium perfringens (CP) is a pathogen in the commercial poultry industry, which is the etiologic agent of necrotic enteritis (NE). Day-of-hatch broilers were fed a wheat diet and assigned to the following groups: control, commercial coccidia vaccine, commercial bursal disease vaccine, or the combination of the two, and challenged with

CP in order to develop a disease model. Broilers in each treatment group had significant increases (P≤ 0.05) in lesion scores, mortality, and CP incidence. As pressure mounts for discontinuing the use of antibiotics in the agriculture industry, it is important to develop new strategies to combat these costly enteric pathogens. In vitro investigations evaluated a mixed gut culture with CP and the ECP at 5 mM or a 10 mM concentrations, over time. By 3 h there was a reduction (P≤ 0.05) in the 5 mM ECP and 10 mM ECP treatment groups. In vivo studies showed significant reductions in the incidence of CP and populations of intrinsic *E. coli* in all of the chickens provided ECP in the drinking water. Birds administered ECP in the feed showed significant reductions in lesion scores, incidence of CP and also had reduced *E. coli* $\log_{10}$ values. These results show that an ECP could provide the industry with a new management tool for controlling NE.

DEDICATION

I would like to dedicate this dissertation in the loving memory of my little brother Jonathan Taylor McReynolds. His death has been one of the hardest things that I have dealt with in my entire life, but it has also been the centerpiece to my life and the way I live it. My devotion to God, my wife, my family and my friends is based on one of my little brother's greatest attributes Love. Although Jon's life was cut short, he left behind a lifetime of memories. Losing my brother has taught me to live life, and I write this small poem as a token to his death.

The sun will rise and the sun will set,

With or without you, Let us never forget.

When the sun doesn't rise in the early dawn,

Have you lived, your life all along?

Be grateful for the time you have and enjoy your life everyday,

Because God makes no promise the sun will rise another day.

ACKNOWLEDGEMENTS

I would like to thank my mentor and major professor, Dr. James Allen Byrd II, for the guidance and support during my college career. Allen, I have been through some tough times throughout my tenure as a graduate student, and I would like to thank you for your support through all of them. Thanks for all the time you have given to me over the last several years and for allowing me the opportunity to do my research in your laboratory. I am very proud of the friendship that we have developed and hope that we can work together for many years to come. I would also like to thank my graduate committee for all the support and knowledge that you have provided me. Thanks for the help with research ideas and for guiding me in the right direction when I was headed down the wrong path.

I would also like to thank my loving wife for her support and love for the past thirteen years. Jan without you none of this would have been possible; you are my life and everything I accomplish is made easier because of you. Thanks for the words of encouragement and for not allowing me to quit when times where tough. I know that I will never find another person in this world that I love more than you. I hope that you and I have many wonderful years together, and I look forward to spending the rest of them with you. To my Mother and Father, thanks for giving me all the appropriate ingredients to make my life a success. The opportunities that I had as a child have made me the person I am today, there are too many to mention but each and every one of them would not have been possible without your sacrifice. I hope one day I will be able to give your grandchildren the same opportunities you gave me. To my brother Jeff, I

would like to thank you for all the good times we had as kids and for the friendship we have today. I know that we have had some hard times but now that we have both matured I see how special you are to me. I want you to know that I love you and think the world of you and your lovely wife Betty. I must mention the fantastic family of in-laws that I acquired with the marriage of my wife. Larry and Sandra the both of you have served as foster parents for the past decade, I really appreciate the love and support you have given me. Larry, thank you for all the opportunities you have given me, and for accepting me as you own son. Sandra, thanks for all the meals and laughs you have provided, I want you to know how much I have enjoyed you as a mother in-law. I would also like to thank my brothers and sisters in-law for all the laughs and good time we have shared with each other. Finally I would like to mention John and Francis Smith who have been two of the most gracious people in the world to my family. I would like to thank them for their friendship and for providing Jan and me a place to live in the country. The Smiths have given their lives to helping young Aggies and should be commended for their generosity over the years. John and Francis I want you to know that Jan and I love you very much, and we thank you again for all that you have done.

To my extended family at the UDSA in College Station, Texas, I would like to thank each and every one of you for your friendship and support over the last two years. I have been very blessed to share this experience in my life with you, and I can honestly say "they have been the best years of my college career". I would like to thank all of the Research Scientists at this great establishment, your ideas and constructive criticisms have helped me develop into the scientist that I have become. I will forever be indebted

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

One of the keys to developing preventative measures for reducing foodborne

illness in the United States is to understand the complexity of the microorganism's that

cause disease. The commercial poultry industry has been linked to many of the top

pathogens associated with foodborne illness. There are many different bacteria such as

Salmonella, Escherichia coli, Clostridium perfringens, and *Listeria monocytogenes* that

contaminate poultry food products. There has been an enormous amount of research

conducted on the post-harvest decontamination of meat products in the food industry.

Many of the intervention strategies that have been implemented have had a dramatic

effect on reducing foodborne pathogens from food products. One way to even further

reduce foodborne illness is to focus on the pre-harvest reduction of pathogens.

Commercial poultry are reared in large flocks consisting of approximately 20,000

birds per rearing facility. This environment provides bacteria, parasites, viruses, and

protozoa a perfect habitat to flourish. Reducing pathogens in these environmental

settings has been managed with a wide variety of management tools including

antibiotics, vaccines, and competitive exclusion cultures. These products have been very

successful in reducing these pathogens for many years. Although these products have

_____

This dissertation follows the style of Poultry Science.

been successful, new management strategies must be evaluated to preempt the problems associated with resistance. In many cases antibiotic resistance has been observed and is a real issue in both human and animal medicines. Increasing public concerns are beginning to put pressure on the agricultural community to reduce the use of many antibiotic products. Removing these useful antibiotics from the industry will have significant effects on animal health. Many of the disease problems that have been controlled with these products will once again become a major issue in the industry. Most pathogens seen in the poultry industry only produce subclinical infections. These infections cause immunosuppression and give opportunistic pathogens a chance to attack weakened animals. One such pathogen is *Clostridium perfringens*. This pathogen has been controlled with growth promoting antibiotics, which target many of the Gram-positive organisms in the digestive tract. This bacterium is one of the etiologic agents of necrotic enteritis, a disease that is usually managed very well with present day management tools. However, there has been an increase of this disease condition in recent years in antibiotic free flocks and some flocks that use antibiotics. If the public demands persist on the removal of these antibiotic tools from the agricultural setting, this disease condition could become a significant problem in the poultry industry.

Understanding the disease progression of NE has been very difficult due to its complexity and several predisposing factors (dietary components, immuno-suppression, and mechanical irritation of the gut) that appear to contribute to this syndrome. Research in this dissertation is dedicated to the development and control of a disease

model that evaluates many of the parameters involved in necrotic enteritis. Currently,

there are very few disease models being used that accurately describe the condition.

CHAPTER II

REVIEW OF LITERATURE

Background

Microbial Ecology

Microorganisms are found everywhere in our environment. They are commonly found in the soil, air, water, and influence many aspects of our lives. However, contrary to many beliefs, most microorganisms are not harmful to us and generally do not cause illness unless they are pathogenic. In fact, bacteria are used in a wide variety of ways that are very beneficial to us in our everyday lives. Bacteria put the tang in yogurt, or the sour in sourdough bread, and are used in the processing of many cheeses, and wines. In the agricultural setting, bacteria are essential for composting; restoring nitrogen to the soil, and help reduce pathogens in the commercial livestock and poultry industries. Without the presence of these beneficial bacteria we simply could not live.

It has long been known that the gastrointestinal tract is composed of a wide array of bacteria that play a crucial role in animal health and performance. The gastrointestinal microbial community is a sophisticated complex of many different species of bacteria and this flora differs from host to host. There are many different factors that play a vital role in the development of a microbial population. Some of these factors include geographical location, age, health status, body niche, diet, and type of animal reservoir (Savage, 1977). The normal microbial populations develop on the

mucosal surfaces which line the nose, mouth, stomach, intestinal tract, respiratory tract, urinary tract, vagina, and the skin. These bacteria can be classified as commensal bacteria and start to develop at birth. It has been shown that neonatal children can shed facultative anaerobes reaching concentrations of 10^8 to 10^{10}/g of feces within 2 days of life (Hentges, 1993). As the child develops throughout the stages of life the microbial populations will change. The adult human body contains 10^{14} cells and of these only 10% make up the body and 90% make up the microbial population. There are several criteria that have been developed to describe the indigenous microflora: bacterial organisms should be able to grow anaerobically, they should be found in normal adults, be able to colonize particular areas of their respective tracts, be able to colonize their niche during succession in infant animals, maintain stable population levels, and be able to have complex interactions with the mucosal epithelium (Savage, 1977).

Our current knowledge of gut microbial diversity and ecology is largely based on classical anaerobic culture techniques, phenotypic characterization of culturable isolates, and light and electron microscopy. These studies demonstrate that all major groups of microbes (bacteria, protozoa, fungi, yeasts and bacteriophage) are represented in the gastrointestinal tract (Raskin et al., 1997). However, these techniques are limited to culturable bacteria and only represent 1 % of the total microbial population (MP) in the gastrointestinal tract (Hugenholtz et al., 1998). With the development of new molecular technologies, the MP can be studied in a culture-independent manner. New molecular methods identify bacteria based upon differences in their 16s, 23s, and 5s rRNA (Schmidt, 1994). These methods generate a fingerprint that can be used to classify

bacteria the genus and the species level. DNA is extracted from a colony of bacteria and then restricted into discrete-sized fragments using specific restriction enzymes such as PUV II, PST I, and ECHO RI (Pillai, 2002; Schmidt, 1994). The DNA is then transferred to a membrane and probed with a region of the rRNA to reveal the pattern of rRNA genes. This pattern is known as the fingerprint of the bacterium. The pattern is then recorded, and stored in a database that can be used by researchers worldwide. The databases contain over 185 different genera and many different species of bacteria. Some examples of bacteria associated with foodborne illness in the database are as follows: *Listeria* (105 pattern types), *Salmonella* (605 pattern types), *Escherichia* (158 pattern types) and *Staphylococcus* (611 pattern types). These have all been established in the database (Dupont, 2002). In a recent study, Franks and coworkers (1998) showed that about two thirds of the known microbial population could be identified using specific molecular probes. The results from their study showed that 20 % of the fecal flora was *Bacteroides,* 29% came from the *Clostridium coccoides-Eubacterium rectale* species, 12% were Gram positive bacteria, and 3% of the bacteria enumerated were *bifidobacteria* (Franks et al., 1998). Utilizing these relatively new techniques will allow research microbiologists an opportunity to better understand the intricate populations of the gastrointestinal tract.

The beneficial bacteria in the gastrointestinal tract play several very important roles in animal health. There are several key reasons to develop a normal microflora: these bacteria aid in colonization resistance, competition for intestinal attachment sites, and aid in the stimulation of the immune system. The first line of defense against

pathogens is the normal gut microflora. These bacteria have many different mechanisms which they can use to defend the host's gastrointestinal tract. One member of the normal microflora are *Lactobacillus* bacteria, which are capable of producing several compounds that aid in pathogen reduction. Many of the commensal bacteria produce compounds known as bacteriocins that effect both Gram-positive and Gram-negative bacteria. Reuterin, a bacteriocin produced by *Lactobacilli*, has been shown in vitro to be inhibitory against *Salmonella*, *Shigella*, *Clostridium* and *Listeria* (Naido et al., 1999). *Lactobacilli* also produce lactic acid which has been shown to have inhibitory effects on *Salmonella* in the crops of broiler chickens. Corrier and coworkers (1999) showed the effects of feed withdrawal on crop pH, lactic acid concentrations, and *Salmonella* concentrations in broiler chickens. Their results showed that the lactic acid concentrations decreased and pH levels increased during an 8 h withdrawal period; conversely, *Salmonella* levels in the crop also increased significantly during this withdrawal time (Corrier et al., 1999). These results show the importance of the normal microflora in the host animal. Other products such as hydrogen peroxide are produced by commensal bacteria. Hydrogen peroxide product results in the peroxidation of lipid membranes, and increased bacterial membrane permeability. Other protective products are short chain fatty acids, which are created by the commensal bacteria as an end product of microbial fermentation. These compounds are predominately the volatile fatty acids (VFA), acetic, propionic, and butyric acids, products shown to be biological indicators of a healthy microbial ecosystem, as well as having inhibitory effects on *Salmonella* colonization in chickens (Nisbet et al., 1996).

The native microfloras not only protect the host by producing bactericidal compounds but also stimulate the maturation of the gut associated lymphoid tissue (GALT). The contents of the flora present a number of different antigenic stimulants to the GALT, which affects both local and systemic immunity (Franks et al., 1998). The microbial flora of the intestinal tract has also been shown to protect against intestinal colonization by pathogens and to stimulate the immune response (Mead, 1989). A study was performed comparing germ free mice and mice with an established intestinal microflora. The results showed that the mice with an established microflora had an increase in the production of epithelial cells lining the gastrointestinal tract, as seen by a thickening of the small intestine. This study also compared the transit time of chyme through the intestine and found that an established microflora significantly increased transit time (Rolfe, 1984). This increase in transit time has been shown to be involved with increased orad peristalsis, which is controlled by the enteric nervous system (Guandalini, 1997).

The microflora also plays a role in the production of vitamins. Some examples of the vitamins produced by the flora are panthothenic acid, biotin, pyridoxine, and menaquinone. In the absence of these normal flora, these vitamins are either not produced or not broken down into a usable form. The intestinal microflora can usually produce enough vitamins to meet the daily minimum requirement for much of the animals needs (Silverman et al., 1999). There are multiple contributions that a healthy microflora can provide to the host animal.

Certain microorganisms cause disease and are know as pathogens; these pathogens come from many different ranges of microorganisms, including parasites, viruses, and bacteria. Several bacterial pathogens come from the genus *Clostridia*, and a few of these organisms such as *Clostridium botulinum, C.chauvoei, C. tetani,* and *C. perfringens* cause serious illness. Many of these organisms are opportunistic pathogens, and given the appropriate conditions these bacteria will grow and flourish. The normal intestinal microflora protects the host from these pathogens. However, if the ecology of the gut is disturbed, these pathogens can grow and cause disease such as necrotic enteritis.

Competitive Exclusion

The concept of competitive exclusion (CE) has been known since the beginning of the 20th century, when Russian biologist I. Metchnikoff, was convinced of the importance of intestinal microflora in human health (Nurmi et al., 1996). In the early 1950's, Milner and Schaffer (1952) reported that the development of intestinal flora increased the resistance of neonatal chicks to *Salmonella*. However, the concept of CE was not seriously investigated until the early 1970's. A *Salmonella infantis* outbreak occurred in Finland's broiler flocks in 1971 which resulted in 277 human illnesses that were found to be caused by the same serotype as was found in the infected poultry flocks (Nurmi and Rantala, 1973). This initiated research at Finland's National Veterinary Institute in 1972 that was very instrumental in developing the modern concept of CE that is practiced today. In these experiments, CE was used to administer intestinal microflora from healthy adult chickens to neonatal chickens for the successful prevention of

intestinal colonization by *Salmonella infantis*. Subsequent studies by Nurmi and coworkers (1992) demonstrated that the minimum infectious dose of *Salmonella* for chickens was surprisingly low during the first few days of neonatal life. Between one and ten bacteria cells administered into the crop was sufficient to infect chicks with *Salmonella infantitis*, confirming that chicks were most susceptible during the first week of life (Nurmi et al., 1992).

The precise mechanisms used by CE cultures to exclude *Salmonella* are not fully understood. It has been postulated that CE cultures provide protection against pathogens in several ways. One mechanism involves competition for intestinal attachment sites on the mucosa of the intestine (Nurmi et al., 1992). If the microorganisms of the CE culture can fill intestinal attachment sites prior to challenge with a pathogen, it is believed that the pathogen will not have a site to bind, and thus will pass through the animal. Another method of excluding pathogens involves the competition for nutrients in the intestine of the animal. If a pathogen doesn't have the appropriate nutrients available for growth, it will not establish in the host (Nurmi et al., 1992). Another postulated method to prevent pathogen establishment is through the production of compounds, which may be toxic to invading pathogens. These compounds are predominantly the volatile fatty acids (VFA), acetic acid, propionic acid, and butyric acid (Nisbet et al., 1996). Under low intestinal pH conditions, VFA's are in non-dissociated forms, which allow them to pass across the lipophilic bilayer of the bacterial cell wall. Once inside the bacterial cell, VFA's dissociate due to the increased pH, resulting in an increase in protons in the cytosol of the cell. This will disrupt the equilibrium of ions and cause the cell to actively pump out

the extra protons using a proton pump. However, this process requires the expenditure

of ATP, resulting in a net loss of energy in the cell, which ultimately lead to cell

exhaustion or cell death.

Competitive exclusion products have been available commercially to producers

in the United Kingdom since the late 1980's. Broilact™, a commercial CE product that

is mainly used in breeder flocks, is an undefined mixture of anaerobic intestinal

microflora (Hirn et al., 1992). When using undefined cultures it is very important to

maintain clean donor flocks in order to prevent the unintentional spread of disease into

the recipient flock or into the human population. Despite the dangers of the

unintentional spread of pathogens which may be present with the use of undefined

cultures, undefined cultures are allowed to be sold commercially in the UK. In the US,

there is only one CE culture, Preempt™, on the market for commercial use (Corrier et

al., 1998; Corrier et al., 1993; Hume et al., 1998). This CE culture is a characterized

culture that was developed using continuous flow culture methodologies. Preempt™ is

a CE culture that contains 29 bacterial isolates of which 15 are facultative anaerobes and

14 strains are obligate anaerobes from 10 different genera (Corrier and Nisbet, 1999).

There are several methods in which CE cultures can be administered to provide

protection against pathogens. These include oral gavage, drinking water, cloacal, gel,

and spray application (Cox et al., 1991; Stavric et al., 1991). In the poultry industry, it is

crucial to administer the CE culture in the most cost and labor effective manner. Spray

and drinking water application are two methods in which the CE cultures can be

administered to commercial flocks and satisfy these criteria. Currently in the United

States both methods of administration are being used to provide protection against *Salmonella* contamination of commercial poultry flocks.

Clostridium

When the topic of food safety arises, many people think of foodborne disease and the microorganisms that are associated with this common illness. There are a variety of food borne, organisms that cause disease in humans; including pathogens such as *Salmonella, Escherichia coli, Clostridium perfringens* (CP), *Campylobacter, Shigella* and *Staphylococcus aureus*. All of these pathogens are capable of producing serious illness in the infected host; however, the primary focus of this discussion will be on the pathogen *Clostridium perfringens* and how it relates to foodborne illness. *Clostridium perfringens* is a spore-forming, gram positive rod-shaped bacterium that grows in anaerobic conditions; these bacteria can be found in many areas of our environment and are very ubiquitous. Some of the primary host reservoirs of these bacteria include humans, cats, cows, pigs, sheep, and chickens (Maier et al., 2000). In the surveillance for foodborne disease outbreaks, *Clostridium perfringens* was reported to be the third leading cause of foodborne disease behind *Salmonella*, and *E. coli* in the United States with an estimated 32,610, 3,260, 2,772 reported cases annually from *Salmonella, E. coli, Clostridium perfringens* respectively (Morbidity and Mortality Weekly Report, 1997). This enteric pathogen can be transmitted to humans through the consumption of poultry products (Labbe, 1991).

There are many things that can be done to reduce the threat of this foodborne illness. One approach is to educate the general public and another is to reduce the

number of pathogens entering the food chain. In animal agriculture, poultry and beef products are the predominant staples of the meat industry; with annual food consumption on a per capita basis of 68 and 63 lbs for poultry and beef respectively (Food Consumption, 2003). Prevention of food contamination in these agricultural arenas is very important to maintain a healthy population of people.

In the poultry industry, controlling the levels of *Clostridium perfringens* is also a very important issue. *Clostridium perfringens* is one of the etiologic agents of the disease necrotic enteritis (NE). The clinical signs of this disease include depression, decrease in appetite, diarrhea, and severe necrosis of the intestinal tract (Ficken and Wages, 1997).   The disease can be divided into two separate categories, clinical and subclinical NE. In a survey conducted in 2000, it was estimated that the subclinical form of the disease costs the poultry industry as much as 5 cents per bird.

In humans, NE is known as pig-bel syndrome and has many of the same clinical signs; however, the main concerns are associated with foodborne illness. *Clostridium perfringens* produces two types of toxins, an enterotoxin and an exotoxin. In humans, pig-bel syndrome is characterized by intense abdominal cramps, and diarrhea, and gas typically begining 8-22 h after consumption of foods containing large numbers of *Clostridium perfringens* that are capable of producing the toxin causing food poisoning. The illness is usually over within 24 h but less severe symptoms may persist in some individuals for 1 to 2 wk (Stevens, 1997). Therefore, necrotic enteritis is an important disease to control in the poultry industry. This is true not only from a production standpoint but also from a food safety aspect.

Understanding the disease progression of necrotic enteritis has been very difficult due to its complexity and several predisposing factors such as dietary components, immuno-suppression, mechanical irritation of the gut, and sudden gut microflora changes that appear to contribute to this syndrome (Ficken and Wages, 1997; Elwinger et al., 1992; Smith, 1965). Bacteria in the gastrointestinal tract derive most of their nutritional requirements for reproduction and growth from dietary components. These nutritional components are either not broken down by digestive fluids or absorbed slowly enough that bacteria populations can compete for them. Since many bacteria utilize different substrates for growth, it is important to understand that the dietary composition largely determines the microbial make-up of the gastrointestinal tract (Apajalahti and Bedford, 2000). Specific species of bacteria can be selected by administering certain feed ingredients that are specifically utilized by the bacteria and not by the host. Some of these ingredients include prebiotics, dietary fiber, and oligosaccharides. It has been shown that sudden changes in rations can alter the native microbial population and give rise to opportunistic bacteria such as *Clostridium perfringens* (Apajalahti and Bedford, 2000). Investigations evaluating the alimentary tract of the chicken, have shown that the onset of necrotic enteritis can be attributed to the diet fed to birds (Nairn and Banford, 1967; Smith, 1965). It has also been shown that high levels of fish meal and wheat in the diet exacerbate the outbreak of necrotic enteritis (Branton et al. 1987; Johnson and Pinedo, 1971; Truscott and Al-Sheikhly, 1977; Riddell and Kong, 1992). Increased disease prevalence could be associated with the high protein levels in the fish meal (menhaden) that cause a shift in the microbiota,

or in the case of the wheat diet could be associated with the high levels of non-starch polysaccharides such as hexose and pentose that are resistant to digestive enzymes. When working with a disease such as necrotic enteritis it is important to understand the effects of dietary components in maintaining the homeostatic microbial ecology of the gastrointestinal tract.

Necrotic enteritis is a disease that has been highly correlated with other infectious diseases such as coccidiosis. Coccidiosis causes immunosuppression and severe gastrointestinal damage, which may give rise to *Clostridium perfringens*. There have been a number of studies that show the correlation between *Eimeria* species and the development of NE (Arakawa and Ohe, 1975; Al-Sheikhly and Al-Saieg, 1979; Frame and Bickford, 1985; Shane et al., 1985). Clinical signs of coccidiosis in affected animals include diarrhea dehydration, weight loss and histopathological damage to the gastrointestinal lining (McDougald and Reid, 1997). Avian coccidial species are host and tissue specific within the gastrointestinal tract of chickens. These parasites have been shown to seriously alter bird performance, as seen through retarded growth and poor feed conversion (Yun et al., 1999). In the past, the commercial poultry industry has controlled coccidiosis through good management practices and by the inclusion of coccidiostats in the feed (Cox, 1997). However, the use of the chemotherapeutic agents has resulted in coccidia parasites becoming drug resistant. If these products are removed from the market because of public concerns of antimicrobial resistance, the disease could become an even larger problem, which could be further exacerbated by the opportunistic pathogen *Clostridium perfringens* and the disease condition of NE.

Clostridium perfringens *Toxins*

Clostridium perfringens (CP) produces a large variety of biologically active toxins that play a significant role in the pathogenicity of the organism. *Clostridium perfringens* produces both extracellular and intracellular toxins that contribute significantly to the disease condition of NE. There are five extracellular toxins including toxin types A through E (Hatheway, 1990). The α toxin is the most potent extracellular toxin produced by *Clostridium perfringens* and is responsible for the necrotic tissue formed in the disease condition. The α toxin contains phospholipase C and sphingomyelinase enzymes that are very active against epithelial cells, muscle cells, leukocytes, red blood cells and platelets. The effects of these enzymes have been shown to have close interactions with the cell membrane and hydrolysis of sphingomyelin and phosphatidylcholine, resulting in the lysis of the cell (Allen et al., 1999). The harmful effects of these enzymes can be overcome by the use of zinc-chelating agents such as ethylene diamine tetraacetic acid and o-phenanthroline which inactivate phospholipase C (Titball and Rubidge, 1990). The activity of these enzymes cause significant events to happen in the host, such as activation of the arachidonic acid pathway and activation of protein kinase C. The result of phospholipase C breaking down the phospholipids in the cell membrane is increased levels of diacylglycerol. High levels of this compound inside the cell will activate the arachidonic acid cascade producing the end products, leukotrienes and thromboxanes. These end products of the cascade cause inflammation, edema, platelet aggregation, muscle contraction, and increased vascular permeability (Samuelsson, 1983). Protein kinase C is also activated by increased concentrations of

diacylglycerol, resulting in the activation of intracellular phospholipase; which causes a positive feedback loop to occur inside the cell, thus exacerbateing the problem. The combination of these events causes catastrophic damage to the gastrointestinal tract and severely inhibits the immune response to the infection.

Clostridium perfringens also produces an enterotoxin during the sporulation event which is released at the completion of this process. Once the *Clostridium perfringens* enterotoxin (CPE) is released into the luminal contents, it binds to the epithelial cells, causing characteristic symptoms such as diarrhea and abdominal cramps. *Clostridium perfringens* enterotoxin has also been linked to sudden infant death syndrome, in which the toxin is absorbed through the gastrointestinal lining into the blood stream causing systemic effects, leading to death (Lindsay et al., 1993). Over the last 15 years, CPE has become a major factor in non-foodborne gastrointestinal diseases. Recent estimates show that CP influences 5-20% of all cases associated with antibiotic initiated diarrhea (Carman, 1997). The enterotoxin also has devastating effects on the mucosal lining of the intestine, inhibits glucose absorption, and causes large amounts of intestinal fluid and electrolyte loss, as well as extensive histopathological damage (Kokai-Kun and McClane, 1997).

The intestinal response to CPE begins when the enterotoxin binds to its receptor located on intestinal epithelial cells; this 50,000 k Da protein has been associated in all cells that are sensitive to the enterotoxin. When the CPE binds to the receptor, a 90,000 k Da protein known as the small complex, is formed in the plasma membrane of the affected cell (Wieckowski et al., 1994). Research has shown that the small complex

could be removed from affected cells by using protease treatments soon after the CPE was applied to the plasma membrane; however, as binding time increases the more resistant the small complex became to the proteases. This suggests that the small complex undergoes some sort of physical or conformational change in the plasma membrane. Once this change has occurred, a 70,000 k Da membrane protein fuses with the 90,000 k Da protein to form what is know as the large complex (160,000 k Da). Soon after the large complex is formed, the plasma membrane becomes permeable to a variety of chemical products such as anions, cations and amino acids. As membrane alterations increase, a cascade of events begins to occur and the cell begins to loose osmotic homeostasis, which will lead to the efflux of larger molecules and the lysis and death of the cell (McClane et al., 1988; McClane and Wnek, 1990; Kokai-Kun and McClane, 1997).

Sporulation

When bacteria are subjected to harsh natural environment they must adapt quickly or their fate will be doomed. *Clostridia* are very good at adapting to their environmental conditions, as demonstrated by their ubiquitous nature in the environment. If the conditions in which clostridia are placed are favorable, the bacteria will maintain normal cellular activity and reproductive functions. In response to nutrient depravation, the bacteria has several mechanisms which aid in the quest of nutrients to help alleviate the process of sporulation. The bacteria will synthesize flagella that help in the search for metabolizable carbon, nitrogen and phosphorous compounds. Also, intracellular and extracellular enzymes dramatically shift to the survival mode as well. Key enzymes of the TCA cycle and other carbon utilization enzymes are expressed, giving the bacteria a wider range of energy metabolism. The bacteria also increase their extracellular enzymes which include proteases, nucleases, amylases, phosphorilases and other hydrolytic enzymes that aid in energy acquiring techniques. If all of these fundamental changes do not result in adequate uptake of energy to support cellular function, then the cell will enter into a stage known as sporulation (Mitchell, 2001.; Labbe and Shih, 1997).

The molecular events involved with sporulation have been extensively characterized in the bacterium *Bacillus subtilis* and this organism serves as the model system for the process of sporulation (Errington, 1993; Harwood, 1989). Sporulation in the bacillus and clostridial species is initiated when GTP levels fall below a certain point. Sporulation consists of seven stages depending on the cytological changes which

occur during this process. In stage zero, there is no sporulation structure present and the cell appears to possess normal characteristics of a vegetative cell. Stage one is the first stage in which an identifiable change takes place; the forespore septum divides the cell asymmetrically into the forespore and the mother cell. The mother cell engulfs the forespore in the second stage of development, now the forespore has a double membrane and resides in the cytoplasm of the cell. Once this process has occurred the cell must complete the process of sporulation. Cortex formation begins in stage three of the sporulation process; the cortical membrane develops between the inner and outer forespore membrane. The cortical membrane is very important for survivability in nature; because it possesses refractile properties which reduce UV radiation and protects the spore from dehydration. In stage four, the inner spore coat protein deposition occurs on the surface of the outer forespore membrane. As this spore coat develops, the spore becomes increasingly resistant to environmental factors such as chemicals and heat. Maturation of the spore also takes place during the fifth stage of development. The release of the spore from the mother cell takes place in the sixth stage. The entire process of sporulation takes approximately seven to eight hours. Now that the bacteria has made a spore, it will be able to survive for many years in the environment until the appropriate conditions come along favoring germination (Errington, 1993; Harwood, 1989). Germination is the final stage of the sporulation process. Germination of the spore occurs very rapidly when compared to the sporulation process, and it involves three steps: 1) activation, 2) germination, and 3) outgrowth. The signals that initiate the activation stage of germination are poorly understood but when environmental

conditions are favorable for growth the spore actively starts the process of germination which ends with a viable vegetative cell. Several different conditions in the laboratory have been shown to stimulate this process and include: moderate heating, increasing nutrients such as amino acids, sugars, lactate, and nicotinamide (Labbe and Shih, 1997). Once the germination process begins, the cortical membrane loses is refractility and the core becomes a stainable cytoplasm containing ribosomes and a nuclear area, a process initiated by internal enzymes. The outgrowth period is characterized by the synthesis of RNA, protein, membranes, cell wall, and functional DNA. The outgrowth of the vegetative cell occurs in the sporangium, and is released through the exosporium. Once the vegetative cell is released into the environment, it begins normal cellular and reproductive functions (Harwood, 1989).

Alternative Technologies

In the United States, antibiotics are used in the agricultural arena on a daily basis prophylactically and therapeutically. The use of antibiotics improves the health and performance of the animals, as well as contributions to the economic welfare of the industries involved. Currently there are very few restrictions on antibiotic use in the United States. However, as the increase of antibiotic resistant bacteria become more prevalent in animal and human medicine, new and tougher regulations may be enforced. In 1999, almost all antibiotic growth promoters were pulled off the market in the European Union. Growth promoters have been shown to increase health status, uniformity, and production efficiency (Bedford, 2000). However, growth promoters have not been shown to have any benefits on performance in germ-free animals. These

antibiotics target gram-positive organisms which are associated with lower levels of performance and health. *Clostridium perfringens* is one of the bacteria that is specifically targeted by growth promoting antibiotics. If these products are removed from the market, producers are likely to see increased levels of diseases associated with *Clostridium perfringens*.

As pressure mounts to discontinue the use of antibiotics in the agriculture industry, it is important to develop new strategies to combat these costly enteric pathogens. Antibiotics are used extensively in the agricultural arena to combat bacterial infections and to improve feed efficiency. Unfortunately, these antibiotics are becoming less effective due to a dramatic increase in antibiotic-resistant bacteria. Recently, McDonald's Corporation announced that the company plans to phase-out the purchasing of animal products that have been exposed to growth promoting antibiotics, in an effort to reduce the potential effects of antibiotic resistance in many microbial populations that directly affect human medicine (McDonald's Press Release, 2003). McDonald's has asked suppliers, to certify that their meat products have not been treated with growth promoting antibiotics and to maintain records of antibiotic use that would be available for company audits and reviews.

Necrotic enteritis has been shown in the poultry industry to be controlled by growth promoting antibiotics such as bacitracin methlene disalicylate and antibiotics such as Narasin^{TM}. In a recent study, the combination of these antibiotics markedly reduced the clinical signs associated with NE (Brennan et al., 2003). Necrotic enteritis has been controlled for many years in the commercial poultry industry; however, if some

of these tools are removed from the market, this condition could be very costly to the industry. Consumer concerns are at the forefront of the decisions made by McDonald's; therefore, it is important for research to develop new alternative methodologies to control many of these bacterial populations.

One alternative method being investigated for pathogen reduction is a chlorate ion based product that exploits the respiratory nitrate reductase enzymatic pathway. The nitrate reductase enzymatic pathway can be utilized as part of a nitrate respiration process in some enteric and sulphate-reducing bacteria; the nitrate serves as a terminal electron acceptor during anaerobic metabolism (Richardson, 2001). *Clostridium perfringens* also possesses this pathway, but it differs slightly from assimilatory and respiratory reduction. The system utilized by CP is for energy production through the fermentation of nitrate (Fujinaga et al., 1999; Hasan and Hall, 1975). This pathway also co-metabolically reduces chlorate to a cytotoxic chlorite ion; bacteria containing nitrate reductase build up toxic levels of this chlorite ion which is lethal to the bacteria (Stewart, 1988). This chlorate ion has been shown to reduce enteric pathogens in chickens, sheep, beef cattle, and pigs (Anderson et al., 2001; 2002; Byrd et al., 2003; Edrington et al., 2003;). In the poultry industry, profit margins can vary significantly depending on disease prevalence. Because of such high cost associated with NE, research into areas involved with this disease are warranted. In the present investigation, an experimental chlorate product (ECP) was evaluated in vitro and in vivo to assess its effect on CP.

Another non-antibiotic method for the reduction of pathogens involves the use of a competitive exclusion (CE) culture to establish a healthy microflora in the

gastrointestinal tract of neonatal poultry. In the commercial poultry industry, young

chicks do not ingest intestinal microflora from adult chickens because broiler production

is based on an all-in, all-out system, which prevents the transfer of microorganisms from

one batch of birds to the next (Nurmi et al., 1996). The concept of CE cultures

protecting poultry from subsequent *Salmonella* challenge has been verified by several

different laboratories (Barnes et al., 1980; Corrier et al., 1995; Mead and Impey, 1986;

Nurmi and Rantala, 1973; Snoeyenbos et al., 1978; Weinack et al., 1979). Establishing a

normal gut microflora is a very important aspect in rearing healthy poultry. It is not

known what the effects these two non-antibiotic products (CE cultures and ECP) in

combination will be and if they might provide beneficial results that will aid in pathogen

reduction in the commercial poultry industry.

Utilization of Chlorate

Nitrate compounds are used in a number of different metabolic reactions that aid

in the lifecycle of prokaryotic and eukaryotic cells. These products are commonly used

in assimilation of essential cellular components or anaerobic respiration by many

facultative and obligate anaerobes. These bacteria can utilize both nitrates and sulfates

to aid in these metabolic processes. In both nitrate reduction (only nitrate reduction will

be discussed) and sulfate reduction, there are two types of pathways, assimilatory and

dissimilatory. The assimilatory pathway takes nitrates in the environment, moves them

into the cell and uses these products for biosynthesis of macromolecules. The

dissimilatory pathway uses the nitrates as a place to store electrons and generate energy

during the respiratory process. In nitrate assimilation, there are three steps involved in

the process that include: uptake of nitrate, reduction of nitrate to nitrite, which is catalyzed by nitrate reductase (NaR), and the six electron reduction of nitrite to ammonium by nitrite reductase ((NiR) Fujinaga et al., 1999). Nitrate respiration is another metabolic process that involves the reduction of nitrates; the nitrate compound in this process can be used as an electron acceptor under anaerobic conditions. The respiratory NaR couples substrate oxidation to nitrate reduction to generate a proton motive force. The electron transport system used in anaerobic respiration is somewhat similar to aerobic respiration, but the terminal electron transport protein donates its electrons to nitrate instead of oxygen. Respiratory NaR has been studied in many of the prokaryotes including, *Paracoccus denitrificans*, *Escherichia coli*, and *Rodobacter capsulatus* (Berks et al., 1995; Gennis and Stewart, 1996).

Nitrate reduction in strict anaerobes is not as well understood; however, some work has been performed on *Clostridium perfringens* and the organism has been shown to reduce nitrate to ammonium ions. Several studies have shown that nitrate reduction in *Clostridium perfringens* differs from traditional assimilatory and dissimilatory nitrate reduction reactions. This nitrate reducing system has not been linked to nitrate assimilation but to energy production, a process known as nitrate fermentation (Caskey and Tiedje, 1980; Hasan and Hall, 1975; Ishimoto et al., 1974; Takahashi et al., 1963). Several research groups have shown that *Clostridium perfringens* and *Clostridium thermoaceticum*, when using nitrate as an electron acceptor, increased their growth yields, compared to bacteria grown without nitrate (Hasan and Hall, 1975; Seifritz et al., 1993). Hasan and Hall showed that the growth increase was due to an increase in

metabolites which participate in phosphorylation reactions when an inorganic acceptor molecule was used. The researchers explained these greater growth yields a result of increased levels of adenosine triphosphate (ATP) produced per mole of glucose. The ATP was shown to be created through the utilization of nitrate as an electron receptor (Hasan and Hall, 1975). These results can be explained by: a net yield of 1 ATP from each of the three carbon glyceraldehyde-3-phosphate molecules produced during glycolysis, and one more ATP being produced from aceyl phosphate for each mole of acetate or butyrate formed during the growth cycle. This shows that for each mole of acetate appearing as a final product represents the synthesis of 2 ATP, for each butyrate 3 ATP, and for each lactate and ethanol 1 ATP (Hasan and Hall, 1975). In the glycolytic pathway when anaerobic conditions exist, the fermentation of products such as lactate and ethanol play a very integral role in restoring nicotinamide adenine dinucleotide (NAD) to a functional proton acceptor. In the production of lactate and ethanol, hydrogen protons are scavenged from the reduced form of NAD to create both of these products (Becker, 1983). Nitrate serves as a terminal electron acceptor when it is reduced to ammonia, reducing some of the production of lactate and ethanol from pyruvate. This is why the explanation of increased yields in growth rate in the presence of nitrate, can be accounted for by the increase in acyl phosphate in the experiments previously described.

To understand the utilization of chlorate as a bactericidal compound we must understand that this product exploits the respiratory nitrate reductase enzymatic pathway, which has been previously discussed. This pathway also co-metabolically reduces

chlorate to a cytotoxic toxic chlorite ion; bacteria containing nitrate reductase build up toxic levels of this chlorite ion which are lethal to the bacteria (Stewart, 1988). Seki-Chiba and Ishimoto (1977) have shown that *Clostridium perfringens* does have a nitrate reductase enzyme with a molecular weight of 80 k Da, thus the bacteria has the capability to reduce chlorate.

This cytotoxic chlorite ion has been shown to reduce enteric pathogens in several experimental settings. Byrd and coworkers (2003) showed that the ECP, when administered in the drinking water, significantly reduced *Salmonella* typhimurium (ST). The incidence of ST was reduced in the crop from 36.7 % in the controls to 2% in the treated groups. Similarly, reductions were seen in cecal colonization from a log_{10} value of 2.52 in the controls to .96 in the treated groups (Byrd et al., 2003). Edrington and *coworkers* (2003) showed that *Escherichia coli* (*E. coli*) O157:H7 could be significantly reduced in sheep by supplementing the ECP in the feed. The results from their study show that fecal shedding of *E. coli* O157:H7 was reduced from 3.89 log_{10} in the controls to 1.53 and 1.11 log_{10} in the, 2X and 3X chlorate treated animals, respectively (Edrington et al., 2003). Anderson and *coworkers* (2002) have also shown similar results, in that *E. coli* concentrations were significantly reduced in bovine ruminal and fecal contents by supplementing the experimental chlorate product in the feed. The ruminal concentrations where reduced by 0.3-0.7 log_{10} in the treated groups, but showed increase of 0.7-1.8 log_{10} in the untreated controls. Fecal *E. coli* concentrations in this experiment showed a significant 2.6 log_{10} reduction in the chlorate treated animals when compared to the controls (Anderson et al., 2002). Utilizing this enzymatic pathway to

reduce chlorate to cytotoxic chlorite has been shown to reduce enteric pathogens from a wide variety of host animals and pathogens. This product could serve as a potential non-antibiotic tool to aid in the reduction of *Clostridium perfringens* in chickens.

Conclusion

The United States commercial poultry industry raises billions of birds every year. It is important for researchers to develop new technologies to aid in the prevention of enteric diseases. One disease that is very likely to affect the industry over the next several years is the disease condition of NE. When consumers of the United States demand antibiotic free birds in the market place, the poultry industry will be forced to react with new innovative technologies, to replace the old technologies. It is the responsibility of the research community to be prepared to face the challenges of tomorrow today.

This dissertation evaluates the effects of an experimental chlorate product on the microbial population in a competitive exclusion culture as well as the effects on the disease condition NE. The research goals of this dissertation are to: 1) evaluate the effect of an experimental chlorate product on the independent bacteria that compose a competitive exclusion culture in vitro and in vivo, 2) develop a disease model that accurately describes the NE in young broilers, and 3) evaluate the experimental chlorate product on an active case of necrotic enteritis.

Clostridium perfringens is a pathogen that affects both human and animal reservoirs and its toxic effects are significant in both medicines. This organism causes serious disease conditions that cost both the human and agricultural industries an

enormous amount of money each year. Reducing this pathogen in the poultry industry will greatly reduce the threat of food borne illness related to this bacterium. The following chapters of this dissertation will provide data from this area of research, and will provide the industry with an alternative product for the reduction of this enteric pathogen.

CHAPTER III

**UTILIZATION OF THE NITRATE REDUCTASE PATHWAY FOR
DETERMINING SENSITIVITY OF A COMPETITIVE EXCLUSION CULTURE
IN VITRO AND *IN VIVO***

Introduction

Foodborne illness continues to be a serious problem in the United States. In the surveillance for foodborne disease outbreaks, *Salmonella, E. coli,* and *Clostridium perfringens* were reported to be the leading causes of foodborne disease in the United States with estimated 32,610, 3,260, and 2,772 reported cases respectively (Morbidity and Mortality Weekly Report, 1997). In the commercial poultry industry, *Salmonella* is a very serious problem, due to the fact that it has been identified in essentially all aspects of poultry production.   An investigation by Jones and coworkers (1991) was conducted to identify the incidence and location of *Salmonella* contamination in modern, commercial broiler operations. Data obtained showed that *Salmonella* could be found in essentially all areas of integrated production. *Salmonella* was most frequently isolated from feed mill samples (20%), processing plant (16 %), multiplier farm (13 %), hatchery (7 %), and broiler houses (4.5 %) were positive for salmonellae (Jones et al., 1991). The significance of the problem can not be truly realized until consideration is given to production statistics for poultry in the United States at present. Production statistics

representing total United States production reveal that greater than 7.9 billion broiler

chickens and 280 million turkeys were processed in 1998 alone, and estimates for 1999

production and beyond show a continuing trend of increasing production (Carey, 1998).

Since the United States population consumes more poultry than any other animal protein

source and poultry represent the largest *Salmonella* reservoir in animal agriculture, state

and federal regulatory agencies have mandated that United States poultry producers

comply with regulations set to reduce the overall incidence of *Salmonella* in poultry and

poultry products. *Salmonella* incidence data are now being collected in all United States

broiler processing plants. These assessments of incidence on processed carcasses are

being regulated by USDA-FSIS to ensure that producers do not exceed the mandated

maximum accepted level of contamination. To reduce the amount of *Salmonella* coming

into processing plants, which subsequently contaminates processed carcasses, poultry

producers have at their disposal several new intervention strategies.

These intervention strategies start with good management practices on the farm

to adequate handling of the product in the processing plant. One pre-harvest

intervention strategy that is currently being evaluated in our laboratory for pathogen

reduction is a chlorate ion based product that exploits the respiratory nitrate reductase

enzymatic pathway (NREP). The NREP is part of a nitrate respiration process in some

Enteric and sulphate-reducing bacteria; this pathway uses nitrate as a terminal electron

acceptor during anaerobic metabolism (Richardson, 2001). The NREP also co-

metabolically reduces chlorate to a cytotoxic chlorite ion; which will build up to toxic

levels, which is lethal to the bacteria (Stewart, 1988). This chlorate ion has been shown

to reduce enteric pathogens in chickens, sheep, beef cattle, and pigs (Anderson et al., 2001; 2002; Byrd et al., 2003; Edrington et al., 2003).

Another pre-harvest strategy utilized by the poultry industry for the reduction of pathogens involves a competitive exclusion (CE) culture. Competitive exclusion products are used to establish a healthy microflora in the gastrointestinal tract of neonatal poultry. The precise mechanisms by which CE products exclude pathogens are not fully understood. It has been postulated that CE cultures provide protection against pathogens through several mechanisms. One mechanism involves competition for mucosal intestinal attachment sites of the intestine (Nurmi et al., 1992). If the beneficial bacteria of the CE culture can fill all the intestinal attachment sites prior to exposure to a pathogen, then the pathogen will not be able to bind to the mucosa, and thus will pass through the animal. Another mechanism of excluding pathogens involves the competition for specific nutrients in the intestine of the animal. If a pathogen does not have the appropriate nutrients available for growth, it will not colonize the gastrointestinal tract of the host (Nurmi et al., 1992). Another postulated mechanism to prevent pathogen establishment is through production of antibacterial compounds which may be toxic to invading pathogens. The predominate antibacterial compounds appear to be the volatile fatty acids (VFA), acidic, propionic, and butyric (Nisbet et al., 1996). Under low intestinal pH conditions, VFA's are in the associated forms, which allow them to pass through the bacterial cell wall. Once inside the bacterial cell, VFA's dissociate, which results in an increase in protons in the cytosol of the cell. The cell will actively pump out the extra protons through the use of a ATP driven proton pump.

Resulting in a net loss of energy in the cell, ultimately leading to cell exhaustion or cell death.

Establishing a normal gut microflora is a very important aspect in rearing healthy poultry. Due to new regulations and consumer demands, research into areas involved with reducing these harmful pathogens is warranted. In the present investigation, an ECP was assessed *in vitro* and *in vivo* to evaluate its effect on the competitive exclusion culture.

Materials and Methods

Experimental Animals

For Experiment 2 Cobb X Ross broiler chicks were obtained from a local commercial hatchery on day-of-hatch. All chicks were placed in individual rearing pens at appropriate rearing temperature on clean pine shavings litter material. All chicks were provided water and a corn-soy based diet that met or exceeded National Research Council guidelines (1994) for *ad libitum* consumption.

Experiment 1

In this experiment, the CE culture Preempt[TM][1] was evaluated to determine the sensitivity of the component bacteria to an ECP and to measure the utilization of sodium nitrate. Component bacteria were cultured in triplicate with and without 5 mM

————————

[1] MS BioScience, Dundee, IL 60118

sodium nitrate[2] and sodium chlorate[3]. Additionally, *Pseudomonas* was cultured

aerobically for 48 h at 37ºC. A 1 mL sample of the culture was obtained at 0, 3, 6, 24,

and 48 h. The OD reading of the culture was recorded at each time point in a

spectrophotometer and recording the absorption at 625 nm. This reading was used to

determine the inhibition of growth of the bacteria. A previously described colormetric

assay was used to determine the utilization of nitrate (Cataldo et al. 1975).

Experiment 2

In this experiment, the objective was to evaluate both the CE culture and the ECP

in the reduction of a foodborne pathogen. The experiment contained four groups, a

negative control, CE culture, ECP and the combination of the two. On day 1, twenty-

five chicks from each group were challenged with 10^8-10^9 cfu novobiocin[3] (NO) and

nalidixic acid[3] (NA) resistant S.Typhimurium (Seeders) and placed in the pen with the

remaining unchallenged chicks (Contacts). One h later, a reconstituted competitive

exclusion (CE) product was administered by oral gavage (0.25 mL) to groups 2 and 4.

After an additional hour, chicks were provided either distilled water or water containing

the experimental chlorate product (ECP) [1X ECP is equivalent to a 15 mM chlorate ion

concentration] to groups 3 and 4 for 4 days. After this period, all chicks were provided

free access to water until termination of the experiment. On day 3, approximately 48h

post-CE treatment, ten chicks in each group were randomly selected and euthanized by

––––––––

[2] Mallinckrodt Baker Inc., Paris, KY 40361
[3] Sigma Aldrich Co., St. Louis, MO 63178

cervical dislocation. The concentration of propionic acid and total volatile fatty acids (VFA(acetic + propionic + butyric + isobutyric + valeric + isovaleric)) in the cecal contents were determined by gas liquid chromatography as reported previously (Corrier et al., 1993).

Recovery of Salmonella

On Day 10, all seeder and contact chicks were killed by cervical dislocation and evaluated for *Salmonella* colonization. An individual cecum was removed and 0.25 grams of cecal contents placed into a 6 mL snap cap polypropylene tube containing 2.25 mL of Butterfield's solution. Serial dilutions of each sample were performed using 0.5 mL of the sample, placed into 4.5 mL of Butterfield's solution for a final concentration of 10, 100, and 1000 cfu/mL. One hundred μL from each dilution tube was placed onto a brilliant green agar[4] (BGA) plate containing 25 μg/mL NO and 20 μg/mL NA and spread plated using a bacterial cell spreader. All of the plates were incubated for 24 hours at 41° C, and the number of *Salmonella* cfu's were determined and expressed as $\log_{10}$ *Salmonella* /g cecal contents. Cecal contents that were negative at a 100 fold dilution on BGA plates but were positive at a 10 fold dilution on BGA plating were assigned 1.50 $\log_{10}$ *Salmonella* /g cecal contents (Corrier et al., 1993; 1995)

Statistical Analysis

Differences in propionate levels in the cecal contents of treated chickens were evaluated using the General Linear Model procedure for one-way analysis of variance

---

[4] Becton, Dickinson and Company Sparks, MD 21152

(SAS Institute, 1996). Statistically different means (P≤ 0.05) were further separated using Duncan's Multiple Range Test (SAS Institute, 1996). The chi square test of independence was used to compare Salmonella Typhimurium incidence data following bacterial enrichment of cecal tonsils (Ott, 1993)

Results and Discussion

The results from Experiment one show how the ECP affected the CE culture *in vitro*. Of the 15 different facultative strains, 11 had slight NR utilization, 3 had moderate NR utilization; the remaining were NR negative (with slight and moderate utilization: ≥0.1 to ≤ 1.0 mM and ≥ 1.0 mM nitrate utilized within 6 h, respectively). Of the obligate anaerobes evaluated, 3 had slight utilization, and the remaining were NR negative (Figure 1). Of the total bacteria that are NR positive, fifty percent were chlorate sensitive, as indicated by marked inhibition of growth during the 48 h incubation period. *Enterococcus faecalis* had a slight NR utilization and had impaired growth when the ECP was present (Figure 2). *Serratia liquefaciens* had a moderate utilization of nitrate and had impaired growth when the ECP was present (Figure 3). *Enterococcus faecalis* and *Serratia liquefaciens* were selected as representatives of both slight and moderate utilization of the NR pathway which co-metabolically reduces the chlorate ion to cytotoxic chlorite. Although several of the bacterial populations are ECP sensitive, ECP does not eliminate all the bacterial constituents of this CE product.
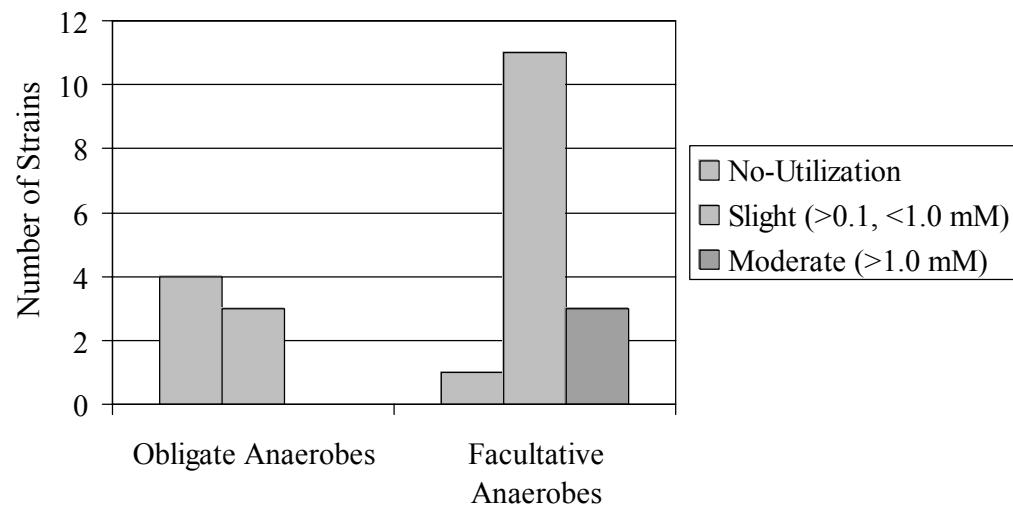
Figure 1. Nitrate (5 mM) utilization of obligate and facultative anaerobes in a commercial competitive exclusion culture.
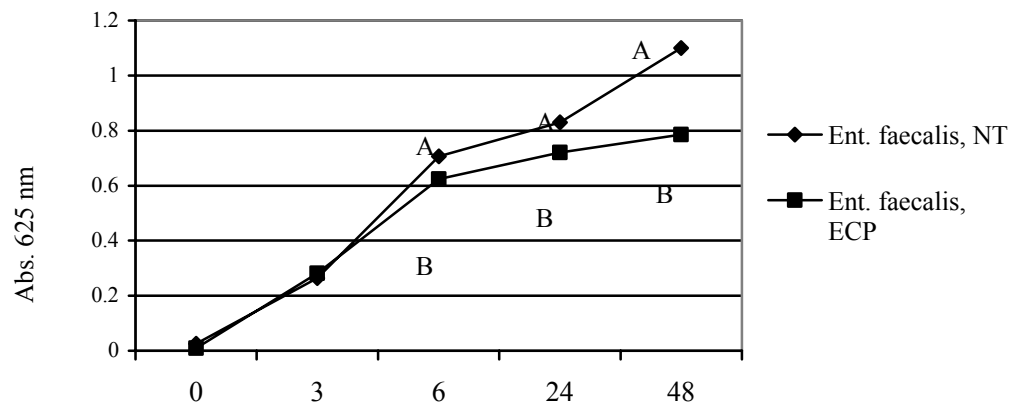
Figure 2. Utilization of the nitrate reductase pathway: a comparison of
Enterococcus faecalis **grown in the presence of nitrate (NT) or an experimental**
chlorate compound (ECP) for 0, 3, 6, 24, and 48 h. *ECP is a 15 mM chlorate ion
equivalent. [A,B] Mean values with no common superscripts differ significantly
$(P \leq 0.05)$.
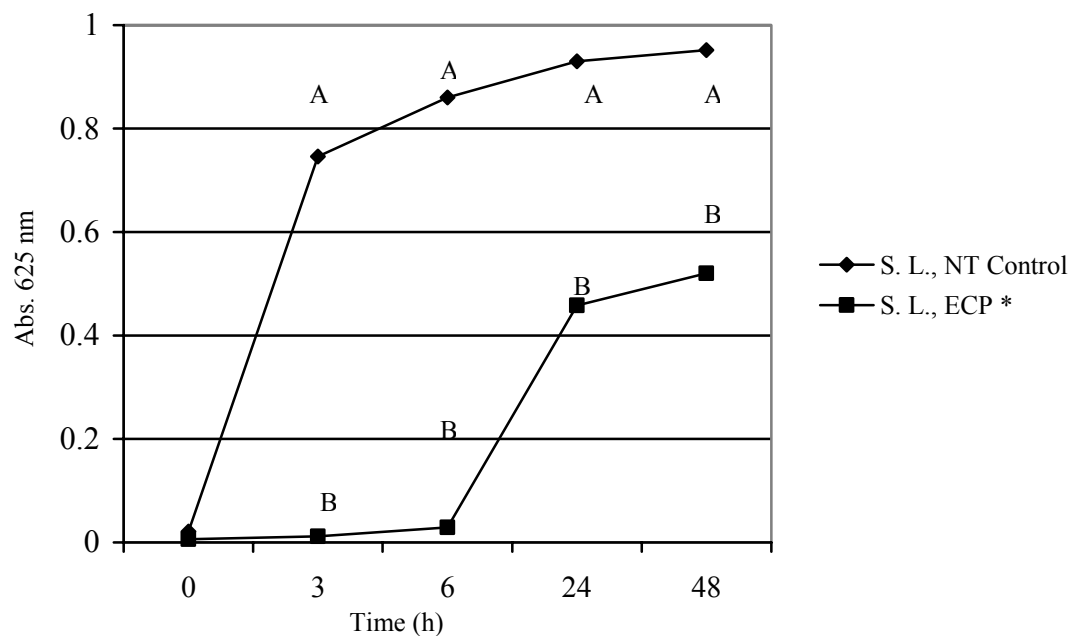
Figure 3. **Utilization of the nitrate reductase pathway: a comparison of *Serratia liquefaciens (S. L.)* grown in the presence of nitrate (NT) or an experimental chlorate compound (ECP) for 0, 3, 6, 24, and 48 h.***ECP is a 15 mM chlorate ion equivalent. [A,B] Mean values with no common superscripts differ significantly ($P \leq 0$.05).

Broiler chicks provided ECP in the drinking water of a CE product had significant reductions in the number of ST recovered and the overall incidence as compared to the controls (Table 1). *Salmonella*-challenged (Seeders) or non-challenged (Contacts) chicks had significantly lower numbers of cecal *Salmonella* cfu recovered and incidence of *Salmonella* recovered when compared to the controls. Experimental chlorate product and CE products had significantly lower numbers of *Salmonella* cfu recovered from the ceca and the incidence detected in the ceca as compared to the controls. Furthermore, the combination of ECP and CE was not significantly different from the ECP alone with the exception of non-challenged chicks provided ECP alone. ECP alone or in combination with CE provided to broiler chicks 3 days prior to sampling did not significantly affect the cecal volatile fatty acid concentrations compared to the controls or the CE treated (data not shown).

The data in the present study suggest that ECP provided in the drinking water during the first 4 days of life may reduce the incidence and the population of *Salmonella* in newly hatched chicks. Furthermore, data suggest that ECP does not adversely affect the microbial population naturally found in the gastrointestinal tract or artificially introduced in the CE culture.

These results agree with recent studies in our laboratory that demonstrated chlorate supplementation effectively decreased *E. coli* O157:H7 in cattle and pigs prior to harvest (Anderson et al., 2001; Callaway et al., 2002). Studies demonstrated that chlorate significantly reduced *E. coli* O157:H7 and Salmonella Typhimurium DT104 in gastrointestinal contents while not significantly altering normal total culturable

Table 1. **Effect of an experimental chlorate compound (ECP) provided in the drinking water and competitive exclusion culture (CE) during the first four days on *Salmonella* Typhimurium cecal colonization in broilers chicks (two trials)[1].**

Groups	*Salmonella* challenged (Seeders)		Non- *Salmonella* challenged (Contacts)	
	Log_{10} *Salmonella* per gram cecal contents	*Salmonella* positive chicks per total chicks (%)	Log_{10} *Salmonella* per gram cecal contents	*Salmonella* positive chicks per total chicks (%)
Control	5.37 ± 0.98^{A2}	$40/40 (100)^{A}$	3.94 ± 2.15^{A}	$33/39 (84.6)^{A}$
CE-gavage	4.21 ± 2.05^{B}	$35/40 (87.5)^{B}$	1.31 ± 2.08^{B}	$13/39 (33)^{B}$
ECP[1]	1.71 ± 2.31^{C}	$16/39 (41)^{C}$	0.42 ± 0.97^{C}	$7/40 (15)^{B}$
CE + ECP	1.76 ± 2.37^{C}	$15/40 (37.5)^{C}$	0.07 ± 0.45^{C}	$1/40 (2.5)^{C}$

[1]ECP is equivalent to a 15 mM chlorate ion concentration
[2]Mean $\pm$ standard deviation
[a-c]Mean values within the same column with no common superscripts differ significantly (P $\leq$ 0 .05).

anaerobic bacteria counts suggesting that chlorate supplementation may be a viable strategy to reduce foodborne pathogens that posses nitrate reductase enzyme. Previously, Davies and coworkers (1996) suggested that preharvest *Salmonella* control may have the greatest effect when applied in the final period before harvesting. Because poultry have access to water during the initial placement or during the feed withdrawal period and ECP can be administered in the drinking water, ECP could have an impact on the pathogen load during grow-out or that will be transported to the processing plant. The combined effects of the ECP with other products such as CE, could provide a practical approach for reducing foodborne pathogens both at initial placement and entry into the processing plant. Government regulations (Pathogen Reduction Act) have caused a need for cost efficient approaches to reducing food borne pathogens without dramatically altering present management techniques. The results of the present study suggest a possible method to reduce food borne pathogens that can be incorporated into existing commercial management procedures.

CHAPTER IV

DEVELOPMENT OF AN EXPERIMENTAL DISEASE MODEL FOR

NECROTIC ENTERITIS UTILIZING IMMUNOSUPPRESSANTS AND

DIETARY MECHANISMS

Introduction

When the topic of food safety arises, many people think of foodborne disease and the microorganisms that are associated with this common illness. There are a variety of organisms that cause disease in humans; including pathogens such as *Salmonella, E. coli, Clostridium perfringens* (CP), *Campylobacter, Shigella and Staphylococcus aureus*. In the surveillance for foodborne disease outbreaks, *Clostridium perfringens* was reported to be the third leading cause of foodborne disease behind *Salmonella*, and *E. coli* in the United States with an estimated 32,610, 3,260, 2,772 reported cases annually from *Salmonella, E. coli, Clostridium perfringens* respectively (Morbidity and Mortality Weekly Report, 1997). This enteric pathogen has been shown to be transmitted to humans through the consumption of poultry products (Labbe, 1991). *Clostridium perfringens* is a spore-forming, gram positive rod-shaped bacterium, which grows in anaerobic conditions and is ubiquitous in nature. Some of the primary host reservoirs of these bacteria include humans, cats, cows, pigs, sheep, and chickens (Maier et al., 2000).

In the poultry industry, *Clostridium perfringens* is one of the etiologic agents of the disease necrotic enteritis (NE). The disease can be divided into two separate

categories, clinical and subclinical NE. Clinical signs of NE include depression,

decreased appetite, diarrhea, and severe necrosis of the intestinal tract (Ficken and

Wages, 1997). In a survey conducted in 2000, it was estimated that the subclinical form

of the disease costs the producer as much as 5 cents per bird, due to decreased

performance. Combined with the 1999 broiler meat production estimate, cost of this

disease including clinical and subclinical infections, was close to $2 billion dollars

worldwide (World Growth, 2000; Van der Sluis, 2000).

Understanding the disease progression of NE has been very difficult due to its

complexity and several predisposing factors such as dietary components, immuno-

suppression, mechanical irritation of the gut, and sudden gut microflora changes that

appear to contribute to this syndrome (Ficken and Wages, 1997; Elwinger et al., 1992;

Smith, 1965). Certain dietary components in the gastrointestinal tract give rise to

specific bacterial populations by supplying them with their nutritional requirements.

These nutritional components are either not broken down by digestive fluids or absorbed

slowly enough so that bacterial populations are able to compete for them. Since many

bacteria species utilize different substrates for growth, it is important to understand that

dietary constituents play a role in determining the microbial composition within the

gastrointestinal tract (Apajalahti and Bedford, 2000). It is not clear which bacterial

populations may be enriched during the feeding of some commonly used feed additives

that have been associated with NE. It has been shown that sudden changes in rations can

alter the native microbial population and give rise to opportunistic bacteria such as

Clostridium perfringens (Apajalahti and Bedford, 2000). Investigations of the

alimentary tract of the chickens have shown that the onset of necrotic enteritis can be in part attributed to the diet fed to chicks (Narin and Bamford, 1967; Smith, 1965). High concentrations of fish meal or wheat in the diet exacerbate the outbreak of necrotic enteritis (Branton et al. 1987; Johnson and Pinedo, 1971; Ridell and Kong, 1992; Truscott and Al-Sheikhly, 1977). Increased disease prevalence could be associated with the high protein levels in the fish meal causing a shift in the microbiota, or in the case of the wheat diet, could be a result of the high levels of non-starch polysaccharides such as hexose and pentose that are resistant to digestive enzymes. When working with a disease such as necrotic enteritis, it is important to understand the effects of dietary components in maintaining the homeostatic microbial ecology of the gastrointestinal tract.

Necrotic enteritis is a disease that has been highly correlated with other infectious diseases such as coccidiosis. Coccidiosis causes immunosuppression and severe gastrointestinal damage, which may give rise to *Clostridium perfringens*. There have been a number of studies that show a correlation between Eimeria species and the development of NE (Al-Sheikhly and Al-Saieg, 1979; Arakawa and Ohe, 1975; Frame and Bickford, 1985; Shane et al., 1985). Avian coccidial species are host and tissue specific within the gastrointestinal tract of chickens. These parasites have been shown to seriously alter bird performance, as seen through retarded growth and poor feed conversion (Yun et al., 1999). The commercial poultry industry has controlled coccidiosis through good management practices and by the inclusion of coccidiostats in the feed (Cox, 1997). However, the use of the chemotherapeutic agents has resulted in

coccidia parasites becoming drug resistant (Stephan et al., 1997). If increasing public concerns of antimicrobial resistance causes the removal of certain antibiotics, then the disease could become an even larger problem. Coccidiosis could be further exacerbated by the opportunistic pathogen *Clostridium perfringens* and the disease condition of NE. The purpose of the present investigation is to evaluate the development of a disease model for NR following the administration of a wheat diet and the individual combined effects of a commercial coccidia vaccine[5] (CCV), and commercial bursal disease vaccine[5] (CBDV).

Materials and Methods

Experimental Animals

Ross X Ross straight run broiler chicks were obtained from a local commercial hatchery on day-of-hatch and were placed in rearing pens on clean pine shaving litter. Chicks were provided with water and a 55 % wheat/corn based broiler starter diet for *ad libitum* consumption. The diet met or exceeded National Research Council guidelines for broiler chicks (NRC, 1994).

Experimental Design

The objective was to create an experimental model of NE. Day-of-hatch straight-run broilers that were randomly assigned to one of the following groups containing 25

_____

[5] Schering Plough Animal Health, Millsboro, DEL 19966

birds/treatment: control, CCV, CBDV, or CCV + CBDV, and challenged with 10^7 cfu

CP twice daily for three days. Each treatment group had an appropriate non-

challenged control. The commercial products (CCV and CBDV) were used as

predisposing factors to aid in the development of this disease model. Chicks in the

CBDV treatment groups were administered the vaccine at a level 10 X the manufacturers

recommended dose via ocular administration in order to immunocompromize the chicks.

The CCV was administered at a level 24 X the manufacturers recommended dose via

oral gavage.

Isolation and Administration

Four field isolates of *Clostridium perfringens* (type A) from different

geographical locations (one isolate from Texas and Virginia, and two isolates from

Georgia) were cultured separately and provided to the appropriate treatment groups. The

gastrointestinal contents from NE identified chicks were shipped overnight to our

laboratory where CP isolation was performed. The gut was sealed with a zip tie just

distal to the gizzard and just cranial to the illeocecal junction in the lower gut. The

sample was placed in a plastic bag and put on ice for shipment. Upon arrival at our

laboratory, the GI tract was placed in an anaerobic chamber and approximately 1 g of the

gut contents was placed into 10 mL of fluid thioglycollate medium[6] and incubated for 24

hrs at 37°C. A 10μL loop was used to streak the bacteria onto Brucella blood agar

plates[7] and incubated (24 hrs at 37°C). A single colony was removed from the plate and

----

[6] Becton, Dickinson and Company Sparks, MD 21152
[7] Anaerobe Systems, Morgan Hill, CA 95037

restreaked on Brucella blood agar plates. The bacteria was grown in pure culture and frozen with 20% glycerol at -80°C. For challenge, the isolates were grown in thioglycollate medium for 12 h and the chicks were challenged via oral gavage with 3 mL of 10^7 cfu/mL. Birds where administered CP at two and a half weeks of age twice daily for 3 days.

Bacterial Culture

To quantitatively measure populations of *CP* and *E. coli*, a section of the small intestine about six to eight inches in length, just cranial to Meckel's diverticulum was removed. The sample was placed in 10 mL of anaerobic thioglycollate, stomached for 30 s and 0.5 mL of gut contents were removed and placed into 4.5 mL of neutral phosphate buffered saline. Ten fold serial dilutions were performed and plated on Mac-Conkey[8] agar and incubated (24 hrs at 37°C). Colonies exhibiting typical *E. coli* morphology were counted and recorded. Another 0.5 mL was removed from the stomached material and placed into an anaerobic vial containing 4.5 mL of anaerobic thioglycollate and placed into an anaerobic chamber. The stomached material was incubated for 24 h at 37°C and streaked on blood agar. The following day, the plates were examined for the presence of CP.

Necrotic Enteritis Lesion Scores

To evaluate gross lesions associated with NE, the jejunum and ileum of the small intestine were examined. Lesion scores were recorded using the following criteria:

----

[8] Becton, Dickinson and Company Sparks, MD 21152

0 = No gross lesions, normal intestinal appearance.

1 = Thin-walled or friable, gray appearance

2 = Thin-walled, focal necrosis, gray appearance, small amounts of gas production.

3 = Thin walled, sizable patches of necrosis, gas filled intestine, small flecks of blood.

4 = Severe extensive necrosis, marked hemorrhage, large amounts of gas in intestine.

Statistical Analysis

Isolation frequencies (+/-) of the recovery of *Clostridium perfringens* from the intestinal cultures were compared using the Chi-Square Test of Independence. Lesion scores, mortality and Log $_{10}$ values of *E. coli* were subjected to analysis of variance using the General Linear Model procedure of SAS. Treatment means (P $\leq$ 0.05) were separated using Duncan's Multiple Range Test (SAS Institute, 1996)

Results and Discussion

In the present investigation, the development of NE was evaluated with several predisposing factors. All chicks administered CP showed an increase (P $\leq$ 0.05) in intestinal lesion scores when compared with the controls (Figure 4). The CBDV and the CCV + CBDV treatment groups showed a significant increase in mean lesion scores when compared to the CP treated chicks. These increased levels of disease in part can be attributed to the immunosuppression caused by the CCV and the CBDV. The CCV contains *Eimeria acervulina, E. mivati, E. maxima* which specifically targets areas of the

digestive tract that may allow CP to invade. In the present investigation, intestinal lesions were seen (data not shown), and the lesions were consistent with typical coccidia infections. These protozoa have devastating effects on the epithelial lining of the gastrointestinal tract, which give rise to opportunistic pathogens, such as CP. It has been well documented that coccidia infections increase the risk of NE (Al-Sheikhly and Al-Saieg, 1979; Arakawa and Ohe, 1975; Frame and Bickford, 1985; Shane et al., 1985). Another potential problem in the commercial poultry industry is immunosuppression. In this disease model, CBDV was utilized because of its immunosuppressive properties. This virus affects the lymphoid cells and specifically targets the bursa of Fabricius, which is responsible for the development of humoral immunity in chicks. A major factor involved with this disease is the prolonged immunosuppression that is associated with infected chicks (Lukert and Saif, 1997). Chicks infected with this disease often have secondary infections such as CP and *E. coli* that exacerbate the disease problem.
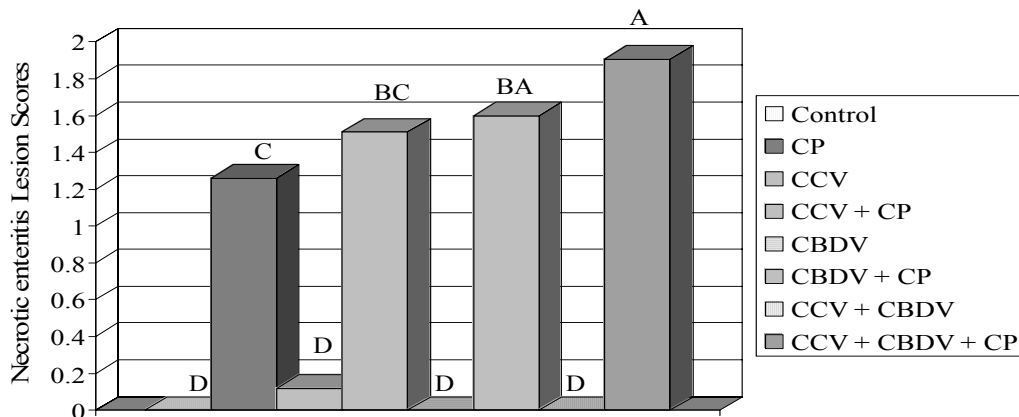
Figure 4. **Evaluation of lesion scores in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination of both (CCV + CBDV) in an experimental disease model of necrotic enteritis (two replicates).** [1](n = 50/replicate). [A-D]Values within treatment groups with different superscripts are significantly different (P≤ 0.05).

Our laboratory evaluated the incidence of CP and populations of intrinsic *E. coli* and the results are as follows. All of the CP treated chicks had a increase (P≤ 0.001) in incidence of CP (Figure 5) are as follows: The CP treated chicks had a 79 % recovery rate, chicks that received CCV had a 76 % recovery rate, the CBDV had a recovery rate of 100 % and the combination of the two had an 85 % recovery rate. Chicks that did not receive the CP challenge did have some incidence of CP; however, this was not significantly different compared to the control group. The concentration of CP in non-challenged chicks can be attributed to intrinsic CP bacteria in the gut or to cross contamination between the treatment groups. Interestingly, chicks in the control groups did not elicit any of the clinical signs associated with the disease when the intestinal tract was evaluated. When evaluating populations of intrinsic *E. coli,* increases (P≤ 0.05) were seen in all treatment groups that received CP (Figure 6). The increase in *E. coli* in the control CCV + CBDV group can probably be attributed to the immunosuppression caused by both of these products. This data is consistent with other studies performed by Apajalahti and Bedford (2000). Their data showed that chicks with NE that were infected with *Eimeria,* had a gut composition that consisted of *E.coli* (52%), CP (14%), *Enterococcus sp*. (29%) and *Proteus vulgaris* (5 %). These increased levels of *E.coli* are consistent with results of the present investigation and other work preformed with NE in our laboratory.
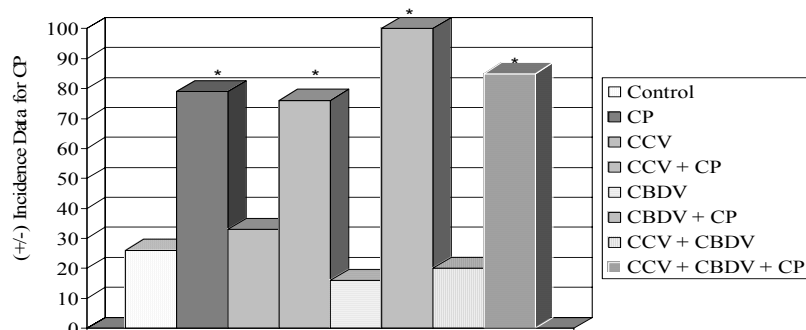
Figure 5. Evaluation of *Clostridium perfringens* (CP) isolation frequencies in day-of-hatch broilers fed a wheat diet and challenged with CP in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination of both (CCV + CBDV) in an experimental disease model of necrotic enteritis (two replicates). [1](n = 50/replicate).
Values within treatment groups with different superscripts (*) are different (P≤ 0.001)

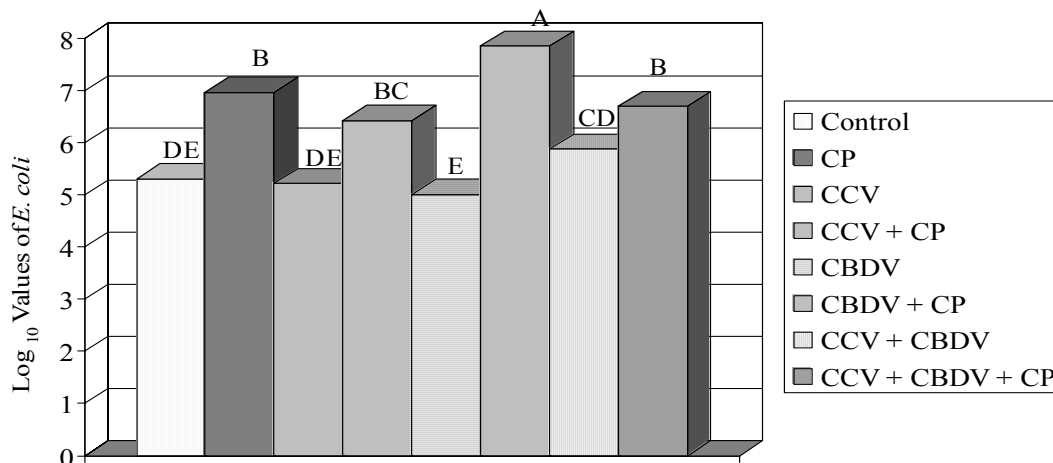**Figure 6**. **Evaluation of *Escherichia coli* in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination of both (CCV + CBDV) in an experimental disease model of necrotic enteritis (two replicates).**[1](n = 50/replicate). [A-E]Values within treatment groups with different superscripts are significantly different (P≤ 0.05).

In this disease model, mortality increased (P≤ 0.025, P≤ 0.001) in all CP challenge treatment groups (Figure 7). The chicks challenged with CP alone had a mortality rate of 26 %, compared to 16, 35, 28, and 2% for the CCV, CBDV, CCV + CBDV and control treatment groups, respectively. In the commercial poultry industry, there are many factors that can lead to immunosuppression or a disease infection. To minimize this condition, the poultry industry protects broiler flocks through the use of good management practices and therapeutic use of several different antibiotics. Coccidiosis has been controlled for many years through the use of ionophores and growth promoting antibiotics targeting Gram-positive bacteria. Although these products have been very effective for controlling these conditions, some of the antibiotics and coccidiostats are loosing their effectiveness because of the acquisition of resistance among targeted organisms. Consumers have also caused increased pressure on the agricultural community to remove many of these products from commercial operations. If the United States follows the same trend as the UK, many of the commercial antibiotics will be removed from the agricultural arena. Removing these products could cause the poultry industry to see higher levels of bacterial, viral and protozoal infections. Increases in disease conditions could significantly increase the risk of NE in commercial poultry operations. The disease model developed in this investigation utilizes several of the predisposing factors that commercial poultry operations face on daily basis. This research will provide the industry with a realistic disease model that will aid in providing a better understanding of this disease condition.
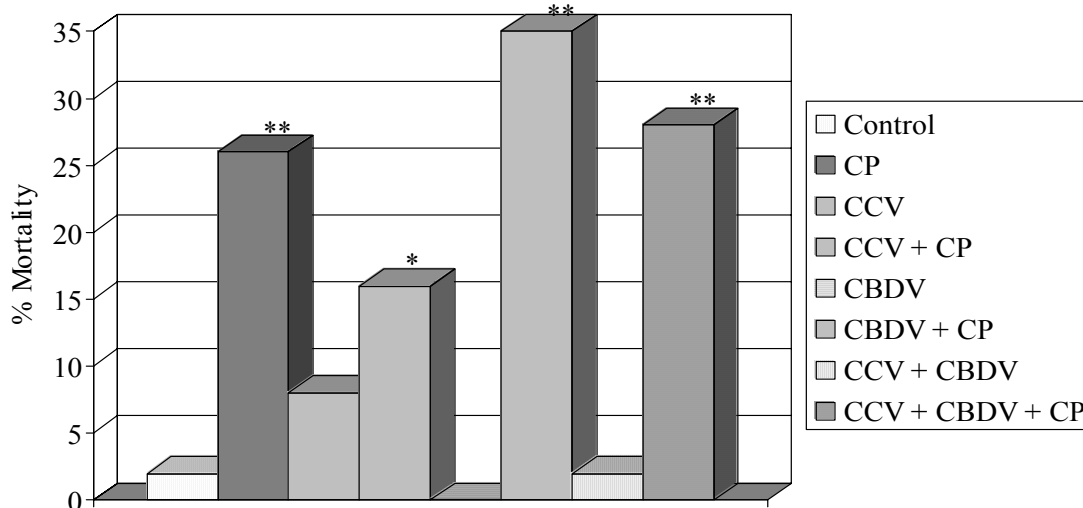
Figure 7. Evaluation of mortality in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination of both (CCV + CBDV) in an experimental disease model of necrotic enteritis (two replicates).[1] (n = 50/replicate). Values within treatment groups with different superscripts are different * (P≤ 0.025), ** (P≤0 .001).

CHAPTER V

EVALUATION OF AN EXPERIMENTAL CHLORATE PRODUCT ON CLOSTRIDIUM PERFRINGENS *IN VITRO* AND *IN VIVO*

Introduction

Necrotic enteritis (NE) is an acute disease with worldwide distribution that produces marked destruction of the gastrointestinal lining of the digestive tract in commercial poultry (Ficken and Wages, 1997). The etiologic agent of the disease is *Clostridium perfringens* (CP), a spore-forming, gram positive rod-shaped bacterium that can be found in many areas of our environment ((soil, dust, feces, feed, poultry litter, and in many domestic animals) Maier et al., 2000). This bacterium and the toxins it produces are the primary cause of the disease but many predisposing factors such as immunosuppression, mechanical irritation of the gut, dietary components, and sudden gut microflora changes contribute to this devastating disease (Ficken and Wages, 1997; Elwinger et al.1992; Smith 1965,). The disease can be divided into two separate categories: clinical and subclinical necrotic enteritis. In a survey conducted in 2000, it was estimated that the subclinical form of the disease cost the poultry industry producers as much as 5 cents per bird. Combined with the 1999 broiler meat production, estimated disease cost for both clinical and subclinical infections was close to $2 billion dollars worldwide (Anonymous, 2000; Van der Sluis, 2000).

As pressure by the general populace and other special interest groups mount to decrease the use of antibiotics in the agricultural industry, it is important to develop new strategies to combat these costly enteric pathogens. Recently McDonald's Corporation announced that the company plans to phase-out the use of animal growth promoting antibiotics, in an effort to reduce the effects of antibiotic resistance in many microbial populations that directly effect human medicine (McDonald's Press Release, 2003). The disease NE in the poultry industry is controlled by antibiotics such as bacitracin methlene disalicylate and the ionophore narasin. In a recent study, the combination of these antibiotics markedly reduced the clinical signs associated with the disease condition (Brennan et al., 2003). Necrotic enteritis has been controlled for many years in the commercial poultry industry; however, if some of these tools are removed from the market, this condition could be very costly to the industry. Therefore it is important for research to develop new alternative methodologies to control this disease.

One alternative method being investigated is an experimental chlorate product (ECP), which utilizes the nitrate reductase enzymatic pathway. Nitrate plays a key role in the nitrogen cycle in which both prokaryotic and eukaryotic organisms are involved. The nitrate reductase enzymatic pathway can be utilized as part of a nitrate respiration process in some enteric and sulphate-reducing bacteria, where nitrate serves as a terminal electron acceptor during anaerobic metabolism (Richardson, 2001). *Clostridium perfringens* also possesses this pathway, but it differs slightly from assimilatory and respiratory reduction, as the system utilized by CP is for energy production through the fermentation of nitrate (Fujinaga et al., 1999, Hasan and Hall,

1975). Previous reports have shown that some facultative bacteria that utilize the nitrate

reductase enzymatic pathway cometabolically reduce chlorate to a cytotoxic chlorite ion.

Bacteria containing nitrate reductase build up toxic levels of chlorite, which is lethal to

the bacteria (Stewart, 1988). The ECP has been shown to reduce enteric pathogens in

chickens, sheep, beef cattle, and pigs (Anderson et al., 2001; 2002;

Byrd et al., 2003; Edrington et al., 2003).   In the poultry industry, profit margins can

vary significantly depending on antibiotic use and disease. The objectives of the present

investigation were to assess the effects of ECP in vitro and in vivo to evaluate its effects

on *CP*.

Materials and Methods

Experimental Animals

In Experiment 2Ross X Ross broiler chicks were obtained from a local

commercial hatchery on day-of-hatch and placed in rearing pens on clean pine shaving

litter. Broilers were fed a commercial 55% wheat/corn based broiler starter diet from

day 0-8 and 14-termination. On day 8 through the 13th day, birds were changed to a

high protein starter diet containing 30 % menhaden fishmeal as fishmeal diets have been

reported to be a predisposing factor in the disease condition of NE (Brennan, 2001).

In Experiment 3, broilers with an active case of NE were obtained from a local

poultry producer at 3 weeks of age and placed in individual rearing pens at appropriate

rearing temperature on clean pine shavings. All birds were provided water and a

wheat/corn/soy ((WD) 55 %) or a corn/soy (CS) based diet that met or exceeded

National Research Council guidelines for ad libitum feeding (NRC, 1994).

Experiment 1

In the first experiment, our laboratory evaluated the efficacy of ECP in reducing

Clostridium perfringens in vitro. The gastrointestinal tract from three single comb

White Leghorn hens were removed and placed in an anaerobic chamber and the gut

contents were pooled into a 500 mL beaker.   One hundred g of pooled contents were

diluted at a 1:1 ratio with thioglycollate medium. In the pooled solution of gut contents,

1 mL of both sodium nitrate (1000 mM) and sodium lactate[9] (4000 mM) was added to

the solution to make 5 mM and 20 mM final concentrations, respectively. The initial

stock cultures of CP in the investigation were incubated at 37°C, and transferred into

new thioglycollate media every 12 h for a total of three passes. *Clostridium perfringens*

was added to the gut contents so that the final concentration of bacteria was 10^6 cfu CP

per g of gut contents. The contents were divided into 10 mL aliquots, and placed into

glass test tubes containing distilled water or the appropriate concentration of sodium

chlorate (5 mM or 10 mM) and each experimental group were run in triplicate. A 1 mL

aliquot from each of the experimental tubes was removed at 0, 1, 2, 3 h to enumerate CP.

The samples were serially diluted, placed on anaerobic blood agar plates and incubated

at 37° C for 24 h. Bacteria were counted and recorded for statistical analysis.

————————

[9] Sigma Aldrich Co., St. Louis, MO 63178

Experiment 2

In Experiment 2, the objective was to qualitatively and quantitatively evaluate the effects of the ECP on an active case of NE in broiler chickens. Mortality, morbidity, and intestinal lesion scoring and plate cultures of *Clostridium perfringens* and *Escherichia coli* were the parameters used to evaluate the efficacy of the product. The chicks received a challenge dose of commercial coccidia vaccine (CCV) to serve as a coccidial infection. Coccidial infections have been highly correlated with the disease condition of NE (Al-Sheikhly and Al-Saieg, 1979; Arakawa and Ohe, 1974; Frame and Bickford, 1985 Shane et al., 1985). The CCV was administered at a level 24 X above the manufacturers recommended dose via oral gavage; which was chosen because of the clinical signs associated with the coccidial infection. In this investigation, the birds were exposed to CP through a natural challenge. The ECP (1X ECP is equivalent to a 15 mM chlorate ion concentration) was added in the drinking water to the experimental groups, when the birds started eliciting clinical and subclinical signs of the disease.

Experiment 3

In the third experiment, birds with an active case of NE were purchased, and transported to the rearing facilities. Treatments consisted of a control group that received tap water and regular broiler feed, a group that received ECP (1X ECP is equivalent to a 15 mM chlorate ion concentration) in the drinking water (DW) a group that received the ECP at a equivalent concentration in the feed (FD), and a group that received the product at the same concentrations in both the feed and drinking water.

Bacterial Culture

To quantitatively measure populations of *CP* and *E. coli*, a section of the small intestine about six to eight inches in length, just cranial to Meckel's diverticulum was removed. The section was placed in 10 mL of anaerobic thioglycollate, stomached (30 s) and 0.5 mL of gut contents removed and placed into 4.5 mL of neutral phosphate buffered saline. Ten fold serial dilutions were performed and plated on Mac-Conkey's agar and incubated for 24 h at 37°C. Colonies exhibiting typical *E. coli* morphology were counted and recorded. An additional 0.5 mL was removed from the stomached material and placed into an anaerobic vial containing 4.5 mL of anaerobic thioglycollate and placed into an anaerobic chamber where it was incubated for 24 h at 37°C before streaking on blood agar. Plates were evaluated on the following day for the presence of CP.

Necrotic Enteritis Lesion Scores

To evaluate gross lesions associated with NE, the jejunum and ileum of the small intestine were examined. Lesion scores were recorded using the following criteria:

0 = No gross lesions, normal intestinal appearance.

1 = Thin-walled or friable, gray appearance.

2 = Thin-walled, focal necrosis, gray appearance, small amounts of gas production.

3 = Thin walled, sizable patches of necrosis, gas filled intestine, small flecks of blood.

4 = Severe extensive necrosis, marked hemorrhage, large amounts of gas in intestine.

Statistical Analysis

Isolation frequencies (+/-) of the recovery of CP from intestinal cultures were compared using the Chi-Square Test of Independence. Lesion scores, mortality and log $_{10}$ values of *E. coli* from the in vivo study were subjected to analysis of variance using the General Linear Model procedure of SAS. Treatment means ($P \leq 0.05$) were separated using Duncan's Multiple Range Test (SAS Institute, 1996).

Results and Discussion

The results from the first experiment demonstrated that CP populations could be decreased ($P \leq 0.05$) in a mixed gut culture with ECP (Figure 8). The $\log_{10}$ value of CP was not significantly reduced at the 0 or 1hour time points. However, a reduction in CP numbers ($P \leq 0.05$) was observed at 2 and 3 h. $\log_{10}$ values at the 2 h time point showed that both ECP concentrations (5 mM and 10 mM) decreased CP from 5.46 in the controls to 5.13 and 4.93 in the 5 and 10 mM treatment groups, respectively. Similar results seen with both concentrations of the ECP at the 3 h time point, in which CP $\log_{10}$ values decreased from 5.51 in the controls to 3.38 in the 5 mM and another significant reduction of 3.29 in the 10 mM and treatment group. These results suggest that CP does possess a respiratory nitrate reductase enzyme, which appears to co-metabolically reduce chlorate to a cytotoxic toxic chlorite ion (Stewart, 1988). Respiratory nitrate reduction in strict anaerobes is not as well understood as in the facultative bacteria; however, some work has been performed on CP and the organism has been shown to reduce nitrate to
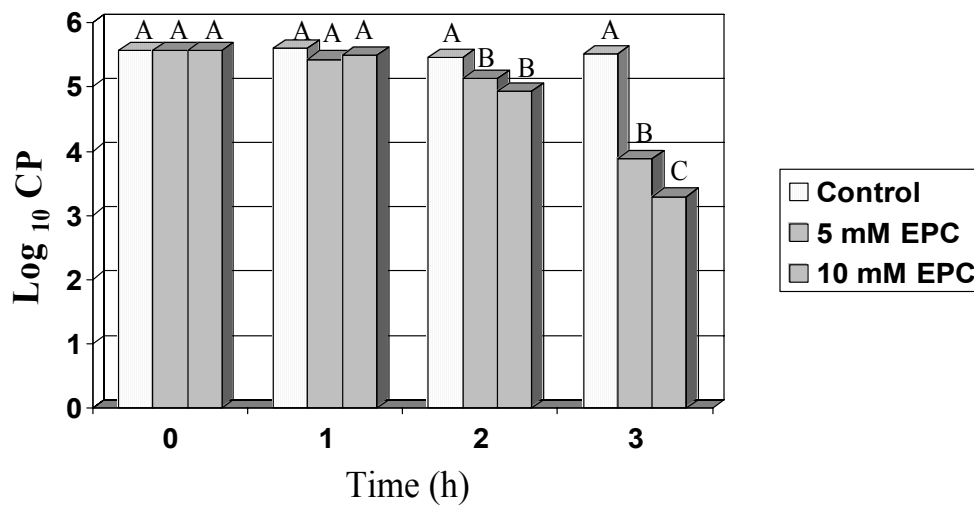
Figure 8. Effects of an experimental chlorate product (ECP) (5 mM or 10 mM) was evaluated at 0, 1, 2, 3 h to enumerate *Clostridium perfringens* (CP) in a mixed gut culture in vitro. [A-C]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

ammonia. These studies have shown that nitrate reduction in CP differs from traditional assimilatory and dissimilatory nitrate reduction reactions (Caskey and Tiedje, 1980; Hasan and Hall, 1975; Ishimoto et al., 1974; Takahashi et al., 1963). This nitrate reducing system has not been linked to nitrate assimilation but to energy production, a process known as nitrate fermentation. The results from the in vitro experiment show that the ECP does have the potential to reduce CP in a mixed gut culture and that an appropriate enzymatic pathway does exist in this bacterium to reduce chlorate to cytotoxic chlorite.

In Experiment 2, all chicks administered ECP showed a significant reduction ($P \leq$ 0.05) in intestinal lesion scores when compared with the controls (Table 2). Controls had an intestinal lesion score of 2.28 compared to the ECP treatment groups with lesion scores of 0.57 to 1.00. Furthermore, the incidence of CP and populations of intrinsic *E. coli* were significantly lower in all of the treatment groups compared with the controls. All ECP treated chicks had a reduction ($P \leq$ 0.025) in the incidence of CP. A recovery rate of CP was 33 % or lower, when compared to the 100 % recovery rate of the controls. When evaluating the populations of intrinsic *E. coli,* significant reductions ($P \leq$ 0.05) were seen in all treatment groups that received ECP, when compared with the controls. *E.coli* in the controls had a $\log_{10}$ value of 8.5 which was reduced to 4.2 and 2.4 in ECP treatment groups respectively. Apajalahti and Bedford (2000) showed that chicks with NE that were infected with *Eimeria* had a gut composition of bacteria that consisted of *E.coli* (52%), CP (14%), *Enterococcus sp*. (29%) and *Proteus vulgaris* (5 %). This chlorate ion has been shown to reduce enteric pathogens such as *Clostridium*

Table 2.Effect of an experimental chlorate product (ECP) provided in the drinking water for five days during a natural challenge of *Clostridium perfringens* in broilers fed a wheat/corn diet (WD) or a corn/soy diet (CS), and administered a commercial coccidia vaccine (CCV).

	NE Lesion Scores	Incidence of CP in the GI tract (+/-)	Mortality (+/-)	Intestinal *E.coli* ($\log_{10}$ *E.coli*/g GI contents)
Control	2.28[A]	100%	84%	8.5[A]
ECP + CCV	1.00[B]	26%[*]	4%[**]	4.2[BC]
ECP	0.84[CB]	26%[*]	24%[**]	2.4[D]
ECP + CS	0.57[CD]	33%[*]	16%[**]	2.8[CD]

[1]ECP is equivalent to a 15 mM chlorate ion concentration
[a-c]Mean values within the same column with no common superscripts differ significantly (P ≤ 0.05).
* A difference (*P≤ 0.001) was found between the number of positive and negative samples containing *Clostridium perfringens,* a difference (**P≤ 0.025) was also seen in mortality.

perfringens, S*almonella* and *E. coli* in chickens, sheep, beef cattle, and pigs (Byrd et al.,

2003; Edrington et al., 2003; Anderson et al., 2001; 2002). The results of this study

show that the ECP significantly reduced two of the major bacterial populations

associated with NE. Reducing these populations of bacteria from the disease condition

may significantly alter the severity of the disease. The control chicks experienced 84 %

mortality compared to the ECP treatment groups that had mortality ranging from 4 to 24

%. Although the mortality rate in the control chicks is not comparable to industry levels

of mortality associated with NE, these data demonstrate that the ECP did significantly

alter the severity of the disease conditions associated with NE.

In Experiment 3, chicks administered ECP in only the feed had significantly

lower (P≤ 0.05) intestinal lesion scores when compared with the controls (Table 3).

Controls had an average intestinal lesion score of 2.28, compared to the ECP/DW,

ECP/FD, and ECP/DW/FD with lesion scores of 1.75, 1.25, and 1.80 respectively.

Intestinal lesion scores show that the ECP was not as beneficial as in Experiment 2. One

possible explanation was that these birds had already developed an active case of NE

and the lesion scores were already present when the birds arrived at our facility. These

results suggest that feed administration may provide a better delivery of the product to

these birds, which is further supported by the incidence data. The incidence of CP in the

chicks that were administered ECP in only the feed showed a significant reduction

as compared to the controls (70 vs 95 %). No significant differences were observed in

mortality in any of the treatment groups. Broilers fed ECP had a significant reduction in

intrinsic *E. coli* when compared to the controls. In Experiments 2 and 3, *E. coli* levels

Table 3. Effect of an experimental chlorate compound (ECP) provided in the

drinking water (DW), or in the feed on a preexisting case of necrotic enteritis (NE).

	NE Lesion Scores	Incidence of CP in the GI tract (+/-)	Mortality (+/-)	Intestinal *E.coli* ($\log_{10}$ *E.coli*/g GI contents)
Control	2.21[A]	95%	15%	3.25[A]
ECP[1] / DW	1.75[AB]	95%	10%	1.03[B]
ECP / FEED	1.25[C]	70%[*]	15%	1.27[B]
ECP / DW / FEED	1.80[AB]	80%	20%	.80[B]

[1]ECP is equivalent to a 15 mM chlorate ion concentration.

[a-c]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

*A difference ($P \leq 0.05$) was found between the number of positive and negative samples containing *Clostridium perfringens*.

here significantly reduced in chicks that received the ECP. Although the interactions between *E. coli* and CP are not completely understood, there appears to be a relationship between these bacteria and NE. Future work may explore how fast the diseased chicks would recover with the ECP, when compared to the control chicks.

Data presented in this paper suggest that the ECP can reduce many of the associated factors involved with NE and should be administered in the very early stages of disease development. The results of the present study also suggest that ECP can significantly reduce CP and *E. coli* in the gastrointestinal contents, further demonstrating that ECP may be a viable non-antibiotic strategy to reduce the signs associated with NE in the commercial poultry industry.

CHAPTER VI

CONCLUSIONS

Poultry is the most commonly consumed meat source in the United States and has been associated as a vehicle for food borne pathogens such as *Salmonella*, *E. coli*, and *Clostridium perfringens*. The research presented in this dissertation utilized new technologies in an effort to reduce these food borne pathogens. One approach was through the use of an experimental chlorate product (ECP), that utilizes the nitrate reductase enzyme (NR). This enzyme reduces nitrate to nitrite and also has been shown to co-metabolize chlorate to cytotoxic chlorite. Data presented in Chapter 3 evaluated the susceptibility of a competitive exclusion culture (CE) to the ECP. A commercially available CE product was evaluated for its nitrate reductase activity, to correlate the constitutive bacteria's chlorate sensitivity. The 29 constituent bacteria of the CE culture, encompassing 10 different genera, of which 15 strains are facultative and 14 are obligate anaerobes. Of the 15 different facultative strains, 11 had slight utilization of NR, 3 had moderate utilization of NR; the remaining were NR negative (with slight and moderate utilization: >0.1 to < 1.0 mM and > 1.0 mM nitrate utilized within 6 h, respectively). Of the obligate anaerobes evaluated, 3 had slight utilization of NR, the remaining were NR negative. Of the total NR positives, fifty percent were chlorate sensitive, as evidenced by marked inhibitions in growth over the 24 h incubation period. In vivo studies utilizing both products (CE and ECP) in a horizontal transmission challenge model (seeders +

contacts) showed significant reductions from 5.37 to 1.76 $\log_{10}$ cfu/g and 3.94 to .07 $\log_{10}$ cfu/g, respectively. Although some of the bacteria are affected by chlorate product as seen in the in vitro data, the CE culture maintained its integrity and significantly reduced *Salmonella* in commercial chicks. These data demonstrate that the ECP does not destroy the ecology of the gastrointestinal tract, and that many populations in the CE were not affected by the ECP product.

Altering the commensal microflora of the gastrointestinal tract with any antibacterial compound can have dynamic effects on the ecology of the ecosystem. Changing the microbial ecology can sometimes destroy many of the beneficial commensal bacteria that protect the host from the damaging effects of opportunistic pathogens. One such pathogen in the commercial poultry industry is *Clostridium perfringens* (CP). Understanding the disease progression of NE has been very difficult due to its complexity and several predisposing factors that appear to contribute to this syndrome. In Chapter 4, our laboratory shows the development of a disease model for NE, utilizing several of the predisposing factors involved in the diseases. Day-of-hatch broilers were fed a wheat/corn diet and randomly assigned to the following groups: control, commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or the combination of the two, and challenged with 10^7 cfu CP. Broilers in each treatment group had a significant increase ($P \leq 0.05$) in lesion scores when compared with controls with mean lesion scores of 1.05 and 2.05 in the CP and CBDV + CP treatment groups, respectively. Incidence data of CP was also significantly increased ($P \leq 0.05$) in all treatment groups when compared to controls with percentages of 73%

and 100% in the CCV + CP and CBDV + CP treatment groups, respectively. When compared with controls % mortality was also significantly increased (P≤ 0.05) to 23 % in the CP group and to 43 % and CBDV + CP treatment groups. These results demonstrate that a reproducible model for NE can be established using these methodologies. Having a disease model will allow many researchers the opportunity to better understand the complexity and the progression of NE.

Many of the predisposing factors of NE have been controlled through the use of vaccines and subtherapeutic agents. *Clostridium perfringens* is one of the gram-positive organisms that has been controlled in the commercial poultry industry by growth promoting antibiotics. Another predisposing factor, coccidia has been effectively controlled through the use of ionophores for many years. Increased pressure to remove these antibiotic products because of resistance and consumer preference could greatly increase the risk of NE.

As pressure mounts for discontinuing the use of antibiotics in the agriculture industry, it is important to develop new strategies to combat these costly enteric pathogens. One such product being evaluated for pathogen reduction in the poultry industry is an ECP.   Unlike most anaerobes, CP possesses a respiratory nitrate reductase pathway, thus this anaerobe may be susceptible to an experimental chlorate product (ECP). In chapter 5, one in vitro and two in vivo studies were performed to determine the effects ECP on CP. The in vitro experiment, consisted of a control, ECP with a 5 mM chlorate ion equivalent, and ECP with a 10 mM chlorate ion equivalent treatment groups. The effects of ECP were evaluated in vitro over several different time

intervals 0-3 h. By 3 h there was a significant reduction ($P \le 0.05$) in the 5 mM ECP (3.88 Log $_{10}$), and 10 mM ECP (3.29 Log $_{10}$) when compared to the control (5.51 Log $_{10}$). In the first in vivo experiment, evaluations of the ECP administered in the drinking showed reductions in many of the associated factors involved with NE. Controls had an intestinal lesion score of 2.28 compared to the ECP treatment groups with lesion scores of 0.57 to 1.00. The incidence of CP and populations of intrinsic *E. coli* were significantly lower in all of the treatment groups, when compared with the controls. In the second in vivo experiment, birds administered ECP in the feed showed significant reductions in lesion scores, incidence of CP and had reduced *E. coli* log$_{10}$ values. The data show that ECP reduces many of the clinical signs of NE, and potentially could provide the poultry industry with a new management tool for controlling NE.

The data presented in this dissertation show how an ECP can be used as a tool for the reduction of food borne pathogens such as *Salmonella*, *E. coli*, and *Clostridium perfringens*. The product not only has the potential to reduce these food borne pathogens but also shows promising results in the reduction of clinical signs associated with NE. Reducing the effects of this disease will help the poultry industry produce a better product for the consumer. This research will have a positive impact on the development of new technologies and the combination of these technologies to will further reduce the potential of diseased flocks and contaminated food products.

REFERENCES

Allen, S. D., C. L. Emery, J. A. Siders. 1999. Clostridium. Pages 654-671 in Manual of clinical microbiology. P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken, ed. ASM Press. Washington, DC.

Al-Sheikhly, F., A. Al-Saieg. 1979. Role of coccidia in the occurrence of necrotic enteritis if chickens. Avian Dis. 24:324-333.

Anderson, R. C., T. R. Callaway, T. J. Anderson, L. F. Kubena, and D. J. Nisbet. 2002. Bactericidal effect of sodium chlorate on *Escherichia coli* concentrations in bovine ruminal and fecal contents *in vivo*. Microbial Ecology Health and Dis. 14:24-29.

Anderson, R. C., T. R. Callaway, S. A. Buckley, T. J. Anderson, K. J. Genovese C. L. Sheffield, and D. J. Nisbet. 2001. Effect of oral sodium chlorate administration on *Escherichia coli* O157:H7 in the gut of experimentally infected pigs. Inter. J. Food Microbiol. 71:125-130.

Apajalahti, J., M. Bedford. 2000. Impact of dietary and environmental factors on microbial communities of the avian GI tract. Online 4/8/2002. http://www.poultry-health.com/fora/inthelth/apabed01.htm.

Arakawa, A., and O. Ohe. 1975. Reduction of *Clostridium perfringens* by feed additive antibiotics in the ceca of chickens infected with *Eimeria tenella*. Poult. Sci.. 54:1000-1007.

Barnes, E. M., C. S. Impey, and D. M. Cooper, 1980. Manipulation of the crop and intestinal flora of the newly hatched chick. Am. J. Clin. Nutr. 33:2426-2433.

Becker, W. M. 1983. Anaerobic production of ATP: The TCA cycle. Pages 283-321in Biochemistry. G. Zubay ed. Addison-Wesley Publishing Company, Reading , MA.

Bedford, M. 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimize subsequent problems. World's Poult. Sci. J. 56:347-365.

Berks, B. C., S. J. Ferguson, J. W. Moir, and D. J. Richardson. 1995. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim Biophys Acta 1232:97-173.

Brennan, J., G. Moore, S. E. Poe, A. Zimmermann, G. Vessie, D. A. Barnum, and J. Wilson. 2001. Efficacy of in-feed tylosin phosphate for the treatment of necrotic enteritis in broiler chickens. Poult. Sci. 80:1451-1454.

Brennan J., J. Skinner, D. A. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poult. Sci. 82:360-3, 2003

Branton, S. L., F. N. Reece, and W. M. Hagler, Jr. 1987. Influence of a wheat diet on mortality of broiler chickens associated with necrotic enteritis. Poult. Sci. 66: 1326–1330.

Byrd, J. A., R. C. Anderson, T. R. Callaway, R. W. Moore, K. D. Knape, L. F. Kubena, R. L. Ziprin, and D. J. Nisbet. 2003. Effect of experimental chlorate product administration in the drinking water on *Salmonella typhimurium* contamination of broilers. Poult. Sci. 82:1403-1406.

Callaway, T.R., R.C. Anderson, K. J. Genovese, T. L. Poole, T. J. Anderson, J. A. Byrd, L. F. Kubena, and D. J. Nisbet. 2003. Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle. J Animal Sci. In Press

Carey, J. B., 1998. Poultry Statistics. Marketing Poultry, Texas Agricultural Extension Service. Online 6/18/2002. http://gallus.tamu.edu/

Carman, R.J. 1997. *Clostridium perfringens* in spontaneous and antibiotic-associated diarrhea of man and other animals. Rev. Med. Microbiol. 8 (Suppl. 1): S43–S45.

Caskey, W. H., and J. M. Tiedje. 1980. The reduction of nitrate to ammonium by *Clostridium* sp. isolated from soil. J. Gen. Microbiol. 119:217-223.

Cataldo, D. A., M. Haroon, L. E. Schrader, and V. L. Youngs, 1975. Rapid colormetric determination of nitrate and nitrite in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6:71-80

Corrier, D. E., J. A. Byrd, B. M. Hargis, M. E. Hume, R. H. Baliey, L. H. Stanker. 1999. Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawl. Poult. Sci. 78:45-49.

Corrier, D. E., J. A. Byrd, B. M. Hargis, M. E. Hume, R. H. Bailey, and L. H. Stanker. 1999. Survival of *Salmonella* in the crop contents of market-age broilers during feed withdrawal. Avian Dis. 43:453-460.

Corrier, D. E., and D. J. Nisbet, 1999. Competitive exclusion in the control of *Salmonella enterica* serovar enteritidis infection in laying poultry. Pages 391-396 in *Salmonella enterica enteritidis* in humans and animals: epidemiology, pathogenesis, and control. A. M. Saeed, R. K. Gast, M. E. Potter, and P. G. Wall, ed. Iowa State University Press, Ames, IA.

Corrier, D. E., D. J. Nisbet, J. A. Byrd, B. M. Hargis, N. K. Keith, M. Peterson, J. R. DeLoach. 1998. Dosage titration of a characterized competitive exclusion culture to inhibit *Salmonella* colonization in broiler chickens during grow out. J. Food Prot. 61:796-801.

Corrier, D. E., D. J. Nisbet, A. G. Hollister, C. M. Scanlan, B. M. Hargis, and J. R. DeLoach. 1993. Development of defined cultures of indigenous cecal bacteria to control salmonellosis in broiler chicks. Poult. Sci. 72:1164-1168.

Corrier, D. E., D. J. Nisbet, C. M. Scanlan, A. G. Hollister, and J. R. DeLoach. 1995. Control of *Salmonella typhimurium* colonization in broiler chicks with a continuous-flow characterized mixed culture of cecal bacteria. Poult. Sci. 74:916-924.

Cox, F. E. G. 1997. Control of coccidiosis: lesions from other sporozoa. Inter. J. for Parasitology. 28:165-179.

Cox, N. A., J. S. Bailey, L. C. Blankenship. 1991. Alternative administration of competitive exclusion treatment. Pages 105-118.in Colonization control of human bacterial enteropathogens in poultry. L.C. Blankenship, ed. Academic Press Inc, San Diego, CA.

Davies R. H., C. Wray. 1996. Determination of an effective sampling regime to detect *Salmonella enteritidis* in the environment of poultry units. Vet. Micro. 50:117-127.

DuPont, Quailcon. 2002. The Riboprinter Microbial Characterization System. Online 3/12/2002. http://www.quailcon.com/rp.htmL

Edrington, T. S., T. R. Callaway, R. C. Anderson, K. J. Genovese, Y. S. Jung, J. L McReynolds, K. M. Bischoff, and D. J. Nisbet. 2003. Reduction of *E.coli* 0157:H7 populations in sheep by supplementation of an experimental sodium chlorate product. Small Ruminant Res. 49:173-181.

Elwinger, K., C. Schneitz, F. Berndtson, O. Fossum, B. Teglof, and B.Bagstrom. 1992. Factors affecting the incidence of *necrotic enteritis*, cecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta. Vet. Scand. 33:369-378.

Errington, J. 1993. Bacillus subtilis sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. 57:1-33.

Ficken, M. D. and D. P. Wages. 1997. Necrotic enteritis . Pages 261-264 in Diseases of poultry. B. W. Calnek, ed. Iowa State University Press, Ames, IA

Food Consumption (per capita) Data System. 2003. Online 8/18/2003.
http://www.ers.usda.gov/data/foodconsumption/DataSystem.asp?ERSTab=3

Frame, D. D., and A. A. Bickford. 1985. An outbreak of coccidiosis and necrotic entertis in 16-week-old cage-reared layer replacement pullets. Avian Dis. 30: 601-602.

Franks, A. H., J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, and G. W. Welling. 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 64:3336-3345.

Fujinaga, K., Y. Taniguchi, Y. Sun, S. Katayama, J. Minami, O. Matsushita and A. Okabe. 1999. Analysis of genes involved in nitrate reduction in *Clostridium perfringens*. Microbiol. 145:3377-3387.

Gennis, R. B., and V. Stewart. 1996. Respiration. In *Escherichia coli* nitrate reductase of *Clostridium perfringens*. II. Purification and some properties of ferredoxin. J. Biochem 85:833-838.

Guandalini S. 1997. Enteric nervous system: intestinal absorption and secretion. J. Pediatr Gastroenterol Nutr. 25:S5–6.

Harwood, C. R. 1989. Biotechnology handbooks. II *Bacillus*. Plenum Press, New York.

Hasan, S. M. and J. B. Hall. 1975. The physiological function of nitrate reduction in *Clostridium perfringens*. J. Gen. Microbiol. 87:120-128.

Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3:66-98.

Hentges, D. J. 1993. The anaerobic microflora of the human body. Clin. Infect. Dis. Suppl. 4 16: S175-80.

Hirn, J., E. Nurmi, T. Johansson, L. Nuotio. 1992. Long-term experience with competitive exclusion and *Salmonella*s in Finland. J. Food Microbiol. 15:281-285.

Hugenholtz, P., B. M. Goebel, N. R. Pace. 1998. Imact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. Sept: 4765-4774.

Hume, M. E., D. E. Corrier, D. J. Nisbet, and J. R. DeLoach. 1998. Early *Salmonella* challenge time and reduction in chick cecal colonization following treatment with a characterized competitive exclusion culture. J. Food Prot. 61:673-676.

Ishimoto, M., M. Umeyama, and S. Chiba. 1974. Alteration of fermentation products from butyrate to acetate by nitratereduction in *Clostridium perfringens*. Z Allg Mikrobiol 14:115-121.

Johnson, D. C., and C. Pinedo. 1971. Gizzard erosion and ulceration in Peru broilers. Avian Dis. 15:835–837.

Jones, F., R. C. Axtell, F. R. Tarver, D. V. Rives, S. E. Scheideler, M. J. Wineland. 1991. Enviromental factors contributing to *Salmonella* colonization of chickens. Pages 3-21 in Colonization control of human bacterial enteropathogens in poultry. L. C. Blankenship, ed. Academic Press Inc, San Diego, CA.

Kokai-Kun, J. F., B. A. McClane. 1997. The clostridium perfringens enterotoxin. Pages 325-357 in The clostridia. J. I. Rood, B. A. McClane, J. G. Songer, R. W. Titball, ed. Academic Press, San Diego, CA

Labbe, R. G. 1991. *Clostridium perfringens*. J. Assoc. Off. Anal. Chem. 74:711-714.

Labbe, R. G., N-J. R. Shih. 1997. Physiology of sporulation of clostridia. Pages 21-32 in The clostridia. J. I. Rood, B. A. McClane, J. G. Songer, R. W. Titball, ed. Academic Press, San Diego, CA

Lindsay, J., A. Mach, M. Wilkerson, L. Wallace, F. Keller, and M. Wojciechowski. 1993. *Clostridium perfringens* type A cytotoxic-enterotoxin(s) as triggers for death in sudden infant death syndrome: development of a toxico-infection hypothesis. Curr. Microbiol. 27:51-59

Lukert, P. D., Y. M. Saif. 1997. Infectious bursal disease. Pages 721-738 in Diseases of poultry. B. W. Calnek, ed. Iowa State University Press, Ames, IA

Maier, R. M., I. L.Pepper., and C. P. Gerba. 2000. Environmental microbiology, Academic Press, San Diego, CA.

McClane, B.A. and A. P. Wnek. 1990. Studies of *Clostridium perfringens* enterotoxin action at different temperatures demonstrate a correlation between complex formation and cytotoxicity. Infect. Immun. 58:3109–3115

McClane, B.A., A. P. Wnek, K. I. Hulkower and P. C. Hanna. 1988. Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin. J. Biol. Chem. 263:2423–2435.

McDonald's Press Release. 2003. McDonald's calls for phase-out of growth promoting antibiotics in meat supply, establishes global policy on antibiotic use. Online 6/19/03 (http://www.mcdonalds.com/corporate/press/)

McDougald, L. R., W. M. Reid. 1997. Coccidiosis. Pages 865-890 in Diseases of poultry. B. W. Calnek, ed. Iowa State University Press, Ames, IA

Mead, G. C. 1989. Microbes of the avian cecum: types present and substrates utilized. J. Exp. Zool. Suppl. 3:48-54.

Mead, G. C., and C. S. Impey. 1986. Current progress in reducing *Salmonella* colonization of poultry by competitive exclusion. J. Appl. Bacteriol.61(Suppl.): S67-S75.

Milner, K. C., M. F. Schaffer. 1952. Bacteriologic studies of experimental *Salmonella* infections in chicks. J. Infect. Dis. 90:81-96.

Mitchell, W. J. 2001. Biology and physiology. Pages 49-104 in Clostridia. H. Bahl, P. Durre, ed. Wiley-VCH, New York.

Morbidity and Mortality Weekly Report. 1997. Surveillance for Foodborne Disease Outbreaks Online 7/19/2003. http://www.cdc.gov/mmwr/preview/mmwrhtmL/ss4901a1.htm

Naido, A. S., W. R. Bidlack, R. A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit. Rev. in Food Sci. and Nutr. 38: 13-126.

Nairn, M. E., and V. W. Banford. 1967. Necrotic enteritis of broiler chickens in western Australia. Aust. Vet. J. 43:49–54.

National Research Council. 1994. Nutrient requirements of poultry. 8th Rev. Ed. National Academy Press, Washington, DC.

Nisbet, D. J., D. E. Corrier, S. C. Ricke, M. E. Hume, J. A. Byrd II, and J. R. DeLoach. 1996. Cecal propionic acid as a biological indicator of the early establishment of a microbial ecosystem inhibitory to *Salmonella* in chicks. Anaerobe 2:345-350.

Nurmi, E., M. Hakkinen, L. Nuotio. 1996. Competitive exclusion. 31st National Meeting on Poultry Health and Processing. Ocean City, Maryland. Pages 72-77.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infections in broiler production. Nature 241:210-211.

Nurmi, E., L. Nuotio, and C. Schneitz. 1992. The competitive exclusion concept development and future. J. Food Microbiol. 15:237-240.

Ott, R. L. 1993. An introduction to statistical methods and data analysis. Duxbury Press, Belmont, CA.

Pillai, S.D. 2002. POSC 619 Class Notes and Readings. Texas A&M Department of Poultry Science, College Staion, TX.

Raskin L., W. C. Capman, R. Sharp, and D. A. Stahl. 1997. Molecular ecology of gastrointestinal ecosystems. Pages 243-298 in Gastrointestinal microbiology, Vol 2. R. I. Mackie, B. A. White, and R. E. Isaacson, ed. Chapman and Hall, New York

Richardson, D. J. 2001. Introduction: nitrate reduction and the nitrogen cycle. Cellular and Molecular Life Sci. 58:163-164.

Riddell, C., and X.-M. Kong. 1992. The influence of diet on *necrotic enteritis* in broiler chickens. Avian Dis. 36:499–503.

Rolfe R. D. 1984. Interactions among microorganisms of the indigenous intestinal flora and their influence on the host. Rev Infect Dis. 6: S73–9.

Samuelsson, B. 1983. Leukotriens: mediators of immediate hypersensitivity reactions and inflammation. Science. 220:568-575.

SAS Institute. 1996. SAS/STAT Software: Changes and enhancements through release 6.11. Version 8. SAS Institute, Inc., Cary, NC.

Savage D. C. 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. 31:107-133.

Schmidt, T.M. 1994. Fingerprinting bacterial genomes using ribosomal RNA genes and operons. Methods in Molecular and Cellular Biology. 5:3-12.

Seifritz, C., S. L. Daniel, A. Gobner, and H. L. Drake. 1993. Nitrate as a preferred electron sink for the acetogen *Clostridium thermoaceticum*. J. Bacteriol. 145:8008-8013.

Seki-Chiba, S. and M. Ishimoto. 1977. Studies on nitrate reductase of *Clostridium perfringens*. I. Purification, some properties, and effect of tungstate on its formation. J. Biochem. 82:1663-1671.

Shane, S. M., J. E. Gyimah, K. S. Harrington, and T. G. Snider. 1985. Etiology and pathogenesis of necrotic enteritis. Vet. Res. Comm. 9:269-287.

Silverman, H., J. Romano, G. Elmer. 1999. The vitamin book. Bantam Books, New York.

Smith, H. W. 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteria. 90:495-513.

Snoeyenbos, G. H., O. M. Weinack, and C. F. Smyser. 1978. Protecting chicks and poults from *salmonella* by oral administration of normal gut microflora. Avian Dis. 22:273-278.

Stavric, S., T. M. Gleeson, B. Blanchfield. 1991. Efficacy of undefined and defined bacterial treatment in competitive exclusion of *Salmonella* from chicks. Pages 323-330 in Colonization control of human bacterial enteropathogens in poultry. L.C. Blankenship, ed. Academic Press Inc, San Diego, CA.

Stephan, B., M. Rommel, A. Daugschies, and A. Haberkorn. 1997. Studies of resistance to anticoccidials in *Eimeria* field and pure *Eimeria* strains. Vet. Parasitology 69:19-29.

Stevens, D. L. 1997. Necrotizing clostridial soft tissue infections. Pages 141-151 in The clostridia. J. I. Rood, B. A. McClane, J. G. Songer, R. W. Titball, ed. Academic Press, San Diego, CA

Stewart, V. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. 52:190-232.

Takahashi, H., S. Taniguchi, and F. Egami. 1963. Inorganic nitrogen compounds: distribution and metabolism. Pages 91-202 in Comparative biochemistry. M. Florkin and H. S. Mason, ed. Academic Press, New York.

Titball, R. W. 1993. Bacterial phospholipase C. Microbiol. Rev. 57:347-366.

Titball, R. W., T. Rubidge. 1990. The role of histodine residues in the alpha toxin of *Clostridium perfringens*. FEMS Micrbiol Lett. 68:261-266.

Truscott, R. B., and F. Al-Sheikhly. 1977. Reproduction and treatment of necrotic enteritis in broilers. Am. J. Vet. Res. 38:857–861.

Van der Sluis, W. 2000. Clostridial enteritis is an often underestimated problem. World Poult. 16, No. 7:42-43.

Weinack, O. M., G. H. Snoeyenbos, C. F. Smyser. 1979. A supplemental test system to measure competitive exclusion of salmonellae by native microflora in the chicken gut. Avian Dis. 24:1019-1030.

Wieckowski, E. U., A. P. Wnek, B. A. McClane. 1994. Evidence that an ~50 k Da mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specially bound *Clostridium perfringens* enterotoxin. J. Biol. Chem. 269:10838-10848.

World growth continues. Poultry International 39, No. 1:8-14.

Yun, H., H. S. Lillehoj, J. Zhu, and W. Min. 1999. Kinetic differences in intestinal and systemic interferon and antigen-specific antibodies in chickens experimentally infected with Eimeria maxima. Avian Dis. 44:305-312.

VITA

JACKSON LEE MCREYNOLDS

BORN:

March 10 1975

Nacogdoches, Texas

EDUCATION:

B.S. Poultry Science, Texas A&M University, 1997

M.S. Poultry Science, Texas A&M University, 2000

MARITAL STATUS:

Married March 17, 2001 to Janet Faye Hancock

PERMANENT ADDRESS:

11810 Forsthoff

Bryan, Texas 77808

HOBBIES:

Duck Hunting,

Skeet Shooting