EVALUATION OF AGRICULTURAL DISINFECTANTS AND NECROTIC ENTERITIS PREVENTATIVES IN BROILER CHICKENS

A Thesis

by

KENDRE DUARON STRINGFELLOW

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

MASTER OF SCIENCE

December 2008

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Morgan Farnell
Committee Members,	David Caldwell
	Alejandro Castillo
	Jason Lee
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ABSTRACT

Evaluation of Agricultural Disinfectants and Necrotic Enteritis Preventatives in Broiler Chickens. (December 2008)

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Chair of Advisory Committee: Dr. Morgan Brian Farnell

The objective of this study was to determine the effect of time, temperature and organic matter on disinfectant efficacy. *Staphylococcus aureus* (SA) and *Salmonella* Typhimurium (ST) were used as organisms to represent Gram positive and Gram negative bacteria, respectively, commonly found in poultry housing. Three independent experiments evaluated the effect of temperature, time, and organic matter on the efficacy of working concentrations of disinfectants against representative organisms found in commercial poultry housing. Quaternary ammonium, chlorhexidine, phenolic and binary ammonium based solutions represented disinfectants commonly used within the poultry industry. Results from these experiments indicated that long term storage of disinfectants will reduce their efficacy against SA. However, a reduction ($p \leq 0.05$) in efficacy was observed with the phenolic compound against ST at elevated temperatures. Following the inclusion of organic matter (OM), reduced ($p \leq 0.05$) efficacy of all disinfectants was observed in a dose dependent manner against both organisms, with the exception of the phenolic compound against SA. Fresh disinfectant performed better (*p*

$\leq$ 0.05) in the presence of OM than 30 wk old disinfectant. These results emphasize the need to use fresh disinfectants and that OM should be removed prior to disinfection.

We also evaluated the effect of bismuth citrate, lactose and citric acids on the development of necrotic enteritis in broilers. *Clostridium perfringens'* associated necrotic enteritis in poultry causes significant loss and increased morbidity in the industry. Due to the reduced usage of antibiotic growth promoters, the incidence of necrotic enteritis has increased. These experiments evaluated different levels of bismuth citrate and bismuth citrate with lactose or citric acid added, on lesion development, bacterial intestinal colonization of *C. perfringens* and pH levels in the gut of broilers orally challenged with *C.perfringens*. Results from this investigation indicate that bismuth citrate at 100 ppm and 200 ppm caused a reduction ($p \leq 0.05$) in *C. perfringens* colonization and intestinal lesion development. The addition of dietary lactose to bismuth citrate enhanced the effect of bismuth citrate on intestinal lesion development. These data suggest that bismuth citrate alone or in combination with dietary lactose will reduce intestinal lesion development in broilers with necrotic enteritis.

DEDICATION

First and foremost I would like to thank the good Lord for giving me the opportunity to study at Texas A&M and for the continued support, love, and strength he has given me.

This thesis is dedicated to my caring and devoted mother, Mrs. Debra Stringfellow, for all your love, support and encouragement. Despite all the obstacles in your way, you managed to transform me from a young immature child into a responsible adult. I will always be proud to call you “Mom”.

Also to my father, Mr. Larry Stringfellow, I appreciate your inspiration and motivation for me to succeed. You, along with my mother, managed to raise your children with the strength and determination to achieve excellence in all aspects of our lives. I will always be proud to call you “Dad”.

To my brothers, Kendric and Kwame Stringfellow and my sister, Kiaa Stringfellow, I appreciate your continued moral support and sound advice. If it were not for your continuous love and motivation, I would not be where I am today.

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CHAPTER I

INTRODUCTION

The presence of avian and zoonotic pathogens is costly and difficult to control (Fussell, 1998). These pathogens cost poultry producers millions of dollars in lost revenue. The subclinical form of *Clostridium perfringens*' associated necrotic enteritis (NE) causes a reduction in performance and overall health of poultry (Kaldhusdal and Lovland, 2000). Recent estimations suggest that the subclinical form of NE cost the industry as much as 5 cents per bird or approximately $450 million/yr in the United States (Van der Sluis, 2000). *Escherichia coli* is one of the main etiologic agents that cause inflammatory processes in chicks which often results in a downgraded or rendered carcass (Barnes et al., 2003).

One of the key preventative measures for reducing the incidence of these infectious agents includes an effective biosecurity program. Biosecurity encompasses all measures that can or should be taken to keep disease (viruses, bacteria, fungi, protozoa, parasites), from a farm and to prevent the transmission of disease within an infected farm to a neighboring farm (Poss, 1998). Major components of a biosecurity program include isolation of infected birds, traffic control, rodent and insect control, and effective cleaning and disinfection.

This thesis follows the style of Poultry Science.

A disinfectant regimen is an important and inexpensive component of a biosecurity program. Disinfectants kill microorganisms by membrane disruption, macromolecule dysfunction, and metabolic inhibition (Maillard, 2002). Selection of a disinfectant depends on the chemical category and formulation, correct dilution or dosage, contact time, application surface, presence of organic matter, type and quantity of microorganisms, temperature, pH, and water hardness (Doerning, 1998). The widespread use of disinfectants has prompted some speculation on the effectiveness of these chemicals. Past research has shown variations in the efficacy of commercial disinfectants (Walker et al., 2002). In the field, disinfectants are often diluted and stored in the back of pickup trucks for long periods of time. Storage in this manner allows disinfectants to be exposed to extreme environmental conditions prior to their actual application. Disinfecting agents have a limited lifespan after their initial dilution, and organic matter (fecal matter, fat, blood, protein), heat, sunlight, time, adulterants (antifreeze, windshield wiper fluid) can reduce efficacy significantly (Davison et al., 1999; Ruano et al., 2001; Dvorak, 2005). In addition to the effective use of disinfecting agents, alternative feed additives, such as bismuth compounds may be used to reduce or prevent disease in poultry production.

For over 300 years, bismuth compounds have been used to treat gastric disorders in humans (Marshall, 1991). Studies evaluating the antimicrobial effect of bismuth subsalicylate demonstrated that it (Manhart, 1990; Scarpignato and Pelosini, 1999) is effective in the treatment of traveler's diarrhea, and treatment of gastritis and ulcer disease in humans caused by *Helicobacter pylori* (Gorbach, 1990). There is limited

research evaluating the administration of bismuth compounds in poultry. The similarities of *Helicobacter pylori* to *Campylobacter* species, prompted Farnell and coworkers (2006) to evaluate the effect of bismuth compound treatments on *Campylobacter* colonization in broilers. They found that bismuth citrate and colloidal subcitrate reduced ($p \leq 0.05$) cecal colonization by *Campylobacter* in broilers.

Additionally, bismuth salts are involved in gut protection (Larsen et al., 2003). Following treatment of bismuth subcitrate of a duodenal ulcer, Moshal and colleagues (1979) microscopically observed an increase in mucus secretory granules in duodenal epithelial cells, suggesting that there is an enhancement of mucus secretion following administration. The surface of the chicken gastrointestinal (GI) tract is covered by a layer of mucus which is essential in the protection of the GI tract against gut colonization of potential pathogens. Problems occur when there are changes in the intestinal mucosa, which possibly lead to an increase in the incidence of necrotic enteritis. The administration of bismuth compounds may be used to prevent damage to intestinal mucosa subsequently reducing necrotic enteritis in poultry.

Lactose is a naturally occurring disaccharide found in mammalian milk. An investigation in day of hatch broilers found that dietary lactose and probiotics decreased cecal pH and increased concentrations of bacteriostatic volatile fatty acids (Corrier et al., 1990a). This decrease in pH can be conducive to the activity of bismuth compounds. CBS forms a precipitate (an active metabolite) at pH levels less than 5, facilitating the infiltration of bismuth into human gastrointestinal microvilli (Wagstaff et al., 1988). A study conducted by Tasman-Jones and colleagues (1987) found that as pH increases,

adherence of CBS to gut epithelium decreases. The addition of lactose in diets may also promote gut integrity. Past research has shown that the addition of lactose to the diet or drinking water results in a reduction of *Salmonella* in the lower intestine, promotes growth, and reduces chick mortality (Corrier et al., 1990b; Hinton et al., 1990). McReynolds and coworkers (2007) recently published a paper examining the effects of dietary lactose on the disease condition of *Clostridium perfringens'* associated necrotic enteritis in broilers. They found that birds fed lactose had significantly lower lesion scores, suggesting that addition of lactose may have protective. Similar to lactose, organic acids have potential antimicrobial effects against pathogenic organisms (Barnhart et al., 1999).

Organic acids can change the pH of the gut, creating an inhospitable environment for some microorganisms (Ricke, 2003). Citric acid is a weak organic acid and it has been speculated to cause a decrease in the pH of intestinal contents by contributing hydrogen ions to the intestinal environment in chickens (Brown and Southern, 1985). Similar to lactose, citric acid may also have anti- *Salmonella* properties.

In an evaluation of potential disinfectants for preslaughter broiler crop decontamination, researchers found that citric acid at or greater than 10% caused a reduction in *Salmonella* in a simulated crop environment, suggesting that citric acid may have antimicrobial effects (Barnhart et al., 1999).

Protection against the harmful effects of poultry pathogens has relied on vaccines and antibiotics (Lowry et al., 1997). Due to the reduced usage of in-feed antibiotic growth promoters, alternative and inexpensive methods should be researched (Van Immerseel, 2004). The addition of bismuth compounds may have the potential to promote better animal health by reducing the colonization of pathogens. In addition, the appropriate use of a disinfectant has the potential to save the industry a substantial amount of money. This research investigated the effects of long term storage, temperature, and organic matter on disinfectants commonly used by a commercial poultry integrator. This work also investigated the effects of bismuth citrate, lactose or citric acid in broilers challenged with *Clostridium perfringens* by evaluating colony forming units and intestinal lesion development.

CHAPTER II

REVIEW OF LITERATURE

Biosecurity

An outbreak of a disease can lead to animal and production losses, expenses from veterinary services, quarantine and/or the cost of disinfection (Clark, 2002). The greatest threat from a disease occurs when introducing naïve flocks to a farm or from birds that have been in contact with other animals (Clark, 2002). Another risk includes traffic to and from the farm (personnel, vehicles, equipment, veterinarians, vaccination crews, and service representatives) which could carry a pathogen onto a farm (Clark, 2002). Rodents, infected or wild birds, insects, wind, water and improperly disposed carcasses are other vectors exposing farms to infection by pathogens (Carey et al., 1999).

To prevent disease exposure, biosecurity should be practiced to control levels of pathogens in animal facilities (Ruano et al., 2001). Biosecurity includes protocols and procedures put in place within an establishment to prevent the spread of pathogens between farms and between buildings within a farm (Poss, 1998). Physical barriers to farm entries, shower in/out facilities, and permanent on farm work forces are examples of procedures implemented to minimize disease exposure (Dekich, 1998). Other procedures include the construction of on-premise roads and walkways, which should be designed with all-weather materials to reduce the transport of organic matter on boots/shoes or vehicle tires (Carey et al., 1999). To further reduce the potential spread of disease-causing organisms, on-farm features should be designed to direct personnel,

vehicles and poultry from youngest birds to oldest birds, from "clean" areas to "dirty" areas (Carey et al., 1999). Spray wash stations are often used to spray the undercarriage and tires of vehicles entering and leaving a farm with disinfectant to prevent the transmission of pathogens on or off the farm. Disinfectants act on microorganisms at several target sites resulting in membrane disruption, macromolecule dysfunction, and metabolic inhibition (Maillard, 2002). For an effective disinfection protocol, consideration should be given to the microorganism being targeted, the characteristics of the disinfectant, and absence of organic matter (Dvorak, 2005). If a sound on-farm biosecurity program is followed, growers protect their economic interests, the local economy and the national poultry industry (Carey et al, 1999). Insufficient biosecurity may result in an epidemic affecting multiple flocks and/or growers, resulting in millions of dollars in eradication efforts (Carey et al., 1999).

Disinfectants

Disinfectants are commonly used in agricultural settings. They are an essential part of infection control practices and aid in the prevention of disease outbreaks on farms (Dvorak, 2005). Currently there are over 5000 antimicrobial products registered with the Environmental Protection Agency. Disinfecting agents are described by the Environmental Protection Agency as "antimicrobial pesticides", which are substances or mixtures of substances used to destroy or suppress the growth of harmful microorganisms such as bacteria, viruses, or fungi on inanimate objects and surfaces (EPA, 2008). Antimicrobial products may contain over 275 active ingredients and are available in several different applications (sprays, liquids, concentrated powders, and

gases). The main uses of antimicrobial pesticides are to disinfect, sanitize, reduce or mitigate growth or development of microbiological organisms, and/or protect inanimate objects (i.e. floors and walls), industrial processes or systems, surfaces, water, or other chemical substances from contamination, fouling or deterioration caused by bacteria, viruses, fungi, protozoa, algae, or slime (EPA, 2008). Antimicrobial activity can be influenced by many factors such as formulation effects, presence of organic material, synergy, temperature, and dilution (McDonnell and Russell, 1999).

Antimicrobial products include sanitizers, disinfectants, and sterilants (Dvorak, 2005). Information regarding the products chemistry, efficacy, toxicity to humans, plants, and animals must be evaluated and submitted to the agency before the product can be utilized.

Sterilizers or sporicides are generally used to completely kill or eliminate all forms of microbial life (fungi, viruses, bacteria, and their spores). Steam under pressure, dry heat ovens, low temperature gas, and liquid chemical sterilants are types of sterilizers used (EPA, 2008).

Disinfectants describe a product applied to the surface of an object to destroy or inactivate fungi and bacteria (Quinn, 2001). Disinfectants can be sporostatic but are not necessarily sporicidal (McDonnell and Russell, 1999). Antiseptics refer to the application to the surface of living organisms or tissues to prevent or stop the growth of microorganisms by inhibition or by killing them (Ewart, 2001). Specific information on the characteristics and specific use of each disinfectant can be found on the product label.

The product labels of these disinfectant agents include important information regarding the proper use and hazards of the chemical. When a disinfectant is chosen for use in a biosecurity program, strict attention should be given to the proper use of a product regarding its application, effectiveness, and hazards (Dvorak, 2005). Other information found on product labels include the claim of disinfection or germicidal activity against a specific microorganism (i.e. Gram negative or Gram positive bacteria), and its effectiveness of product under certain conditions such as dilution with hard water or in the presence of organic matter. Labels also include active and inert ingredients, precautionary statements, a first aid section, environmental, physical or chemical hazards and directions for use.

Disinfectant Testing

The efficacy of disinfectants is regulated by an intricate system of interactions between the microorganisms in question, along with the conditions and the specific disinfectant under review. General principles exist for disinfectant testing; these principles should be followed if useful information is to be obtained (Croshaw, 1981). Principles include the ability to yield information that can be interpreted in terms of practical use, report results that are repeatable and reproducible, and can be sufficiently controlled (Morgan-Jones, 1981). It may be necessary to standardize all equipment and protocols accordingly through experimentation. The primary function of any disinfectant is to destroy or irreversibly inactivate an unwanted microorganism. There are multiple types of disinfectant testing protocols (Croshaw, 1981). Tests can be placed into three main categories: tests that evaluate routine quality control of production

batches of a standard formulation (Rideal-Walker and other phenol coefficient tests), use-dilution tests which investigate a practical use-dilution for a disinfectant under certain defined conditions, and tests that study the efficiency of a disinfectant under simulated field conditions and uses (Morgan-Jones, 1981). These tests consist of a suspension of microorganism being tested, followed by a defined concentration of disinfectant (Croshaw, 1981). The disinfectant is dissolved in water of varying quality. The efficacy of these suspension tests was determined by calculating the difference between the initial microorganism concentration before the test and following the mixing of the suspension with the disinfectant. Approved and accepted methods of disinfectant tests can be found in the Association of Official Analytical Chemists (AOAC) (1984) handbook (Tomasino, 2005).

Classification of Chemical Disinfectants

The major classes of disinfectants include alcohols (ethyl alcohol, and isopropyl alcohol), aldehydes (formaldehydes, paraformaldehyde, and gluteraldehyde), alkalis (sodium or ammonium hydroxide, and sodium carbonate), biguanides (chlorhexidine), halogens (hypochlorite, and iodine), oxidizing agents (hydrogen peroxide, and peroxyacetic acid), phenolic compounds, and quaternary ammonium compounds (QAC) (Dvorak, 2005). Disinfectant classes commonly used in commercial agriculture include halogens (bleach and iodine compounds), oxidizing agents (Virkon-S®), phenols (TekTrol®, Pine Sol®), quaternary ammonia compounds (Roccal®) and chlorhexidine (Nolvasan®) (Dvorak, 2005).

Alcohol compounds are fast acting and highly effective against both Gram positive and Gram negative bacteria, but have no residual activity. Several alcohols have been shown to be effective antimicrobials; ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and *n*-propanol (McDonnell and Russell, 1999). Alcohols are limited in virucidal activity and are ineffective against spores. Other disadvantages of using alcohol include reduced efficacy in the presence of organic matter, flammability, and rapid evaporation (Dvorak, 2005). Ewart (2001) explained that alcohols affect microorganisms by denaturing proteins, which cause membrane damage and cell lysis. High concentrations (95%) of alcohol are less effective, because some degree of water activity is required to denature proteins; a concentration of alcohol consisting of 70-90% is optimal for bactericidal activity (Joklik, 1992).

Aldehydes are highly effective disinfectants, which act on a broad spectrum of microbes (bacteria and their spores, fungi, and viruses) (McDonnell and Russell, 1999). Examples of aldehydes include formaldehyde and gluteraldehyde. Aldehydes achieve bactericidal action by denaturing proteins and disrupting nucleic acids (Joklik, 1992; Ewart, 2001). Aldehydes are highly irritating, toxic to animals, and are potentially carcinogenic (Quinn, 2001).

Chlorhexidine compounds are the most widely used antimicrobial product in disinfecting animal premises (McDonnell and Russell, 1999). Chlorhexidine compounds kill microorganisms by damaging outer cell layers, and subsequently attacks the bacterial cytoplasmic or the yeast plasma membrane (Quinn, 2001). This compound has a broad antibacterial spectrum, but it is limited in its effectiveness against viruses, spore

forming bacteria, mycobacterium, and fungi (Quinn, 2001). Chlorhexidine compounds can only function in a limited pH range of 5-7 and are easily inactivated by soaps, detergents, and organic matter (McDonnell and Russell, 1999).

Halogen compounds are broad spectrum compounds that that have a low toxicity, low cost, and are easy to use (Dvorak, 2005). These compounds lose efficacy over time and are ineffective at temperatures above 110° F or pH levels greater than 9. Halogen compounds lose activity quickly in the presence of organic debris, sunlight and some metals (Shulaw et al., 2001). Chlorine and iodine based compounds are the most significant microbial halogens used in disinfection (McDonnell and Russell, 1999).

Chlorine compounds function by destroying the cellular activity of proteins (Maris, 1995). They are considered broad spectrum, and are efficacious against bacteria, enveloped and non-enveloped viruses, mycobacterium and fungi (Dvorak, 2005). At elevated concentrations these compounds can be sporicidal (Kennedy et al., 2000; Grooms, 2003). The most widely used chlorine based compounds are sodium hypochlorite (household bleach), and chlorine dioxide (McDonnell and Russell, 1999).

Iodine compounds are bactericidal, fungicidal, tuberculocidal, virucidal and sporicidal (Jeffrey, 1995). Iodines function by denaturing proteins associated with the enzymatic systems of microorganisms (Dvorak, 2005). Iodine agents are inactivated by quaternary ammonium compounds and organic debris (Shulaw et al., 2001).

Oxidizing agents are broad spectrum, peroxide based compounds that function by attacking the basic structural cell components, including lipids, proteins, and DNA (Maris, 1995). Peroxygen compounds are considered effective on hard surfaces and

equipment. Virkon-S® (potassium peroxymonosulfate and sodium chloride) is a peroxygen molecule, organic acid, and surfactant combination, with a wide microbial spectrum of activity and some efficacy in the presence of organic material (Dvorak, 2005).

Phenolics are broad spectrum disinfectants that function by denaturing proteins and inactivating membrane-bound enzymes to alter cell wall permeability of microorganisms (Dvorak, 2005). Phenols can be a coal-tar derivative or a synthetic formulation that usually have a milky or cloudy appearance when added to water, as well as a strong pine odor (Kennedy et al., 2000; Shulaw et al., 2001; Grooms, 2003). Phenols are typically formulated in soap solutions to increase their penetrative powers and are considered bactericidal, tuberculocidal, fungicidal, and virucidal for enveloped viruses (Jeffrey, 1995). Phenols are not effective against non-enveloped viruses and spores. Phenols do maintain activity in hard water and in the presence of organic matter and have some residual activity after drying.

Quaternary ammonium compounds are cationic detergents that are attracted to the negatively charged surfaces of microorganisms, where they irreversibly bind phospholipids in the cell membrane and denature proteins impairing permeability (Maris, 1995). Quaternary ammonium compounds are highly effective against Gram positive bacteria, and are effective against Gram-negative bacteria, fungi and enveloped viruses. They are not effective against non-enveloped viruses or mycobacteria and are considered sporostatic but not sporocidal (Kennedy et al., 2000; Grooms, 2003). They are more active at a neutral to a slightly alkaline pH but lose their activity at a pH of less

than 3.5. These compounds are easily inactivated by inorganic matter, detergent, soaps and hard water (Dvorak, 2005).

The use of heat, light and radiation may also be used to mitigate or eliminate microorganisms in the environment, in addition to chemical disinfectants. Heat is one of the oldest physical controls against microorganisms and is considered a reliable method of sterilization (Joklik, 1992). Moist heat (autoclave and steam) and dry heat (flame and baking) can both be used for inactivating pathogens but moist heat is more effective than dry heat (Quinn, 2001). Sunlight and ultraviolet light can have a damaging effect on pathogens and may be used as a method for inactivating viruses, mycoplasma, bacteria and fungi (Dvorak, 2005). The efficacy on ultraviolet light sterilization is limited to surface contact (Ewart, 2001).

Disinfectant Selection

The overall health and safety of humans and/or animals is of primary concern when a disinfectant is chosen for an application. All chemical disinfectants have some level of hazard and can pose a threat to human and animal health (aldehydes, phenols, sodium hydroxide) (Dvorak, 2005). Potential water discharge is another factor that should be considered when selecting a disinfectant. Disinfecting chemicals can be harmful for plants and aquatic life, such as sodium carbonate, hypochlorites, and phenolics (Quinn, 2001).

In addition to human and/or animal hazards there exist multiple factors that should be considered when a disinfectant is chosen for a biosecurity program. The appropriate disinfectant concentration is important to achieve the best results for each

situation. Some products may have more than one dilution depending on the situation. One dilution will kill the target organism while a weaker dilution will prevent multiplication of the organism (Ewart, 2001). The product label contains all necessary information regarding the appropriate dilution for the farm operation.

There are many ways to apply disinfectants, including spraying, brushing, and fumigation or wiping (EPA, 2008). The appropriate contact time is important to the effectiveness of a disinfectant (Ramesh et al., 2002). The contact time can be found on the product label clearly indicating the minimum contact time for optimal effectiveness against the target organism. The area being disinfected should be thoroughly soaked with the disinfectant to avoid drying or evaporation before the end of the recommended contact time (Ruano et al., 2001). The stability and storage of the disinfectant must also be monitored. Disinfectants, such as sodium hypochlorite lose efficacy when stored over long periods in a diluted form (Dvorak, 2005).

Environmental Considerations for Disinfectant Application

The ideal disinfectant would work against many types of microorganisms, in all types of conditions. Disinfectant effectiveness depends on the chemical category and formulation, correct dilution or dosage, contact time, absence of organic matter, target microorganism, temperature, pH, and water hardness (Doerning,1998). Organic matter (blood, fecal matter, litter) provides a physical barrier that protects microorganisms from contact with the disinfectant (Dvorak, 2005). Iodine and chlorine based disinfectants have been shown to be neutralized in the presence of debris and organic matter (Ewart, 2001). The type of surface to be disinfected can have a great impact on the effectiveness

of a disinfectant (Dvorak, 2005). Porous, uneven, cracked, pitted surfaces, wood and earthen floors, are difficult to disinfect. An ideal surface to be disinfected is smooth and non-porous (Ewart, 2001). Bacteria present on metal or other surfaces may form a biofilm, which is a slimy layer of an organic polymer matrix, adhering to a surface, in which microbes are embedded. Coops used to transport poultry to the processing plant may provide a source of contamination if not properly disinfected (Ramesh et al., 2002). If these coops are not cleaned and decontaminated efficiently, microorganisms deposited from the previous trip may contaminate subsequent flocks. Carr and colleagues (1999) found that a number of disinfectants were not as effective against *Salmonella*, possibly due to organic matter on the coops and protection provided by a biofilm. Ramesh and colleagues (2002) published a paper that examined the effects of thirteen commercial disinfectants for their capacity to destroy *Salmonella*, in the presence of organic matter, against a *Salmonella* biofilm. They found that of the thirteen disinfectants only sodium hypochlorite, sodium chlorite and alkaline peroxide compounds were effective in reducing *Salmonella*.

Moderate temperatures (68°F) allow for the best action of disinfectants. Dvorak (2005) stated that elevated temperatures may have the potential to increase the evaporation rate of the disinfectant which could have a negative effect on contact time. Water containing calcium and magnesium, is referred to as hard water. Calcium and magnesium can complex with cleaning compounds, leading to residue buildup which could negatively affect disinfectants (Dvorak, 2005).

Ruano and colleagues (2001) evaluated several commercially available disinfectants used by the poultry industry. They found that quaternary ammonium and phenolic compounds, mixed at the manufacturers' recommended concentration, were efficacious in the absence of organic matter against *Salmonella* Enteriditis and *Staphylococcus aureus*. However, they did not enumerate the cells. Product efficacy was determined by the presence or absence of microbial growth by observation of enrichment broth turbidity. In prior research, Rodgers and colleagues (2001) examined several commercially available disinfectants against *Staphylococcus aureus.* The data presented illustrated that when the disinfectants were mixed at the manufacturer's recommended concentrations, all the disinfectants were bactericidal against *Staphylococcus aureus*. However, with the addition of fluff, disinfectants were not as effective. Bacteria required an increase in contact time with the disinfectant and/or higher disinfectant dosage, in the presence of organic matter (Ruano et al., 2001). Many antimicrobial products are ineffective against spore-forming bacteria (Dvorak, 2005). *Clostridium perfringens* is an important bacterium negatively affecting the poultry industry. Due, to the ineffectiveness of disinfectants against this organism, alternative control methods need to be investigated.

Clostridium perfringens

Clostridium perfringens is the most significant cause of necrotic enteritis in poultry (Songer, 1996). *Clostridium perfringens* is a ubiquitous Gram-positive anaerobic spore-forming rod-shaped bacterium that is encapsulated and non-motile, producing multiple toxins and enzymes that affect the health of poultry flocks (Van Immerseel et al., 2004). Strains of *C. perfringens* are classified based on their production of toxinotypes; A, B, C, D, and E. These toxinotypes produce toxins α (alpha), β (beta), ε (epsilon), and ι (iota), respectively (Songer, 1996; Petit et al., 1999). *Clostridium* types A and C are responsible for lesions and symptoms affecting poultry (Songer, 1996). *Clostridium* type A strains produce alpha toxins, while *Clostridium* type C strains produce both alpha and beta toxins (Petit et al., 1999). Toxinotype A is a phospholipase C sphinagomyelinase which hydrolyzes phospholipids and promotes membrane disorganization, exhibit hemolytic, necrotic and vascular permeabilizing and platelet aggregating properties, leading to death (Titball, 1993). The exact mechanisms of beta toxins are largely unknown, but they are thought to stimulate hemorrhagic necrosis of the intestinal mucosa. *Clostridium perfringens* causes necrotic enteritis, ulcerative enteritis, necrotic dermatitis, botulism, necrotic hepatitis and cholangiohepatitis (Williams, 2005). Onset of these diseases occur when a high frequency of adhesion by *C. perfringens* result in damage to the intestinal mucosa (Williams, 2005).

Clostridium Perfringens in Poultry

Clostridium perfringens is a natural inhabitant of the chicken's gut, but onset of the disease does not occur unless predisposing factors are present. Colonization of *C. perfringens* is an event that occurs early in the life of poultry (Craven et al., 2000, 2001). The presence of *C. perfringens* in the poultry environment is high. It can be found in the dust, soil, feces, feed, poultry litter, eggshell fragments, fluff and in the intestinal tract of poultry (Craven et al., 2001). Feces of wild birds may also contain elevated numbers of *C. perfringens* which could further introduce the organism to poultry production facilities (Craven et al., 2000). When evaluating environmental samples on poultry farms, *C. perfringens* presence was detected on wall swabs (53%), fan swabs (46%), fly strips (43%), dirt outside the entrance (43%) and boot swabs (29%), indicating that the microorganism is ubiquitous throughout the environment (Craven et al., 2001).

Predisposing Factors for Necrotic Enteritis

It has been reported that *C. perfringens* is a naturally occurring bacteria in the environment of poultry production facilities. To cause the signs and symptoms of necrotic enteritis one or multiple predisposing factors may need to be present, such as damage to the intestinal mucosa. Intestinal damage, caused by coccidial pathogens, can result in the release of growth factors that may be utilized by *C. perfringens* in turn causing extensive proliferation in the lumen (Boyd et al., 1948; Petit et al., 1999). It has been documented that broiler chicks inoculated with sporulated *Eimeria* species coupled with the administration of *C. perfringens* contaminated feed had increased mortality when compared to contaminated feed alone (Van Immerseel et al., 2004). Additionally,

physical damage, possibly caused by litter-eating or fibrous material in the diet, may modify the mucosal lining (Williams, 2005).

It has been postulated that diet composition has a direct influence on the onset of necrotic enteritis in broilers. Diets rich in rye, wheat and barley in comparison to diets rich in corn, have high levels of indigestible, water-soluble non-starch polysaccharides (Branton et al., 1987; Ridell & Kong, 1992; Kocher, 2003). These diets are known to increase the viscosity of digesta, decreasing gut transit time, and have been shown to lead to an increase of intestinal anaerobic bacteria (Kocher, 2003). Additionally, slower passage rates may increase the nutrient availability of *C. perfringens* (Williams, 2005). When broilers were fed corn-based *C. perfringens* contaminated feed for three consecutive days compared to broilers fed diets with high amounts of wheat, rye or barley, overall mortality ranged from 0-12% in the corn based diet compared to 26-35% in the wheat, rye or barley diets (Ridell & Kong, 1992).

Another possible factor associated with feed can also be the feed form. It's been documented that there was an increase in mortality associated with finely ground rations when compared to coarsely ground feed (Branton et al., 1987). When birds were fed pellets instead of mash, Enberg and colleagues (2002) found that there was an increase in digestibility and a decrease in the number of *C. perfringens* in the intestine.

Diets high in protein, such as fishmeal, have been shown to increase numbers of ileal and cecal *C. perfringens*. Bone meal has also been associated with an increase in risk of necrotic enteritis (Kocher, 2003).

Immunosupression caused by exposure to infectious bursal disease, chicken anemia virus or Marek's disease may increase the incidence of *C. perfringens* in birds (Williams, 2005). McReynolds and colleagues (2004) developed a model for studying necrotic enteritis in broilers. The method consists of manipulating dietary components, which included feeding broilers a 55% wheat diet, along with administering a commercial coccidia vaccine and a commercial bursal disease vaccine, and challenging birds with *C. perfringens*. Results from this study found that there was an increase in *C. perfringens* in all treatment groups when compared to the negative controls.

Mucin is one of the first lines of defense against bacterial colonization. Altering mucin characteristics can affect the colonization of bacteria in the gut of a chicken, such as *Campylobacter jejuni* (Fernandez et al., 2000). It is possible that positively affecting mucin development in the chicken will inhibit *C. perfringens* colonization in the gut.

Mucin

Mucin is described as polymeric glycoproteins that comprise the main component of the mucus layer that covers the epithelium of the gastrointestinal tract at the interface between the external environment of the gut lumen and the gut epithelium (Montagne et al., 2004). Primary functions of mucus are to protect the epithelium from chemical, enzymatic, physical, and bacterial damage (Montagne et al., 2004).

Mucin functions as an attachment site and nutrient source for commensal bacteria, viruses, and parasites (Basbaum et al., 1999). Mucin does not kill bacteria, per say, but forms a viscoelastic gel (mucus) that traps bacteria along with other contaminants (Basbaum et al., 1999). Mucin functions to lubricate the gut epithelium,

protect the epithelium against acidic environments, protect against endogenous and bacterial proteases, and perform as a selective diffusion barrier permeable to nutrients (Montagne et al., 2004).

Bismuth compounds have been shown to modify mucin properties in humans, to effectively reduce *Helicobacter pylori* (Slominany et al., 1990). These compounds have also been demonstrated to increase mucin production, decrease acidic mucin and inhibit enzymes produced by *Helicobacter pylori* to degrade gastric mucin (Lee, 1991).

Bismuth

Bismuth (atomic number 83, weight 209) is the heaviest nonradioactive element found on the periodic table (Marshall, 1991). Bismuth salts originate from bismuth nitrate, which is subsequently produced by the action of nitric acid on free bismuth or bismuth ores. Further, the separation from the acid causes hydrolyses of bismuth nitrate to bismuth subnitrate, which reacts in solution with soluble basic salts forming bismuth subcarbonate, subgallate, subsalicylate or subcitrate. Due to the insoluble nature of bismuth in the presence of water, bismuth salts are considered effective following entrance into the stomach, an acidic environment. Upon entrance into the stomach, bismuth salts interact with hydrochloric acid forming a precipitate (an active metabolite). Frequently utilized forms of bismuth compounds include bismuth subsalicylate and bismuth subcitrate (Marshall, 1991). Sox and Olson (1989) investigated the binding and killing of *Escherichia coli* by bismuth subsalicylate and found that bismuth subsalicylate was able to bind and kill the organism. In 1999, Mahony and coworkers evaluated antimicrobial activities of bismuth compounds against *Clostridium difficle.* Evaluations

revealed that of the fourteen compounds tested, eight of the compounds showed significant antibacterial activity against *Clostridium difficle*. Specific brand names of bismuth compounds include Tritec, (Glaxo Wellcome, Research Triangle Park, NC), Pepto-Bismol (Proctor & Gamble, Cincinnati, OH) and De-Nol (Tri-Med Distributors P/L, Subiaco, Western Australia). Bismuth salts are also involved in gut protection (Larsen et al., 2003). Following bismuth citrate treatment of a duodenal ulcer, Moshal and colleagues (1979) microscopically observed an increase in mucus secretory granules in duodenal epithelial cells, suggesting that there is an enhancement of mucus glycoprotein secretion following bismuth subcitrate administration. Past research has shown that bismuth subsalicylate has antimicrobial activity (Manhart, 1990; Scarpignato and Pelosini, 1999); is effective in the treatment of traveler's diarrhea and has also been used to treat gastritis and ulcers caused by *Helicobacter pylori* (Gorbach, 1990). Similarly, bismuth subcitrate has been used to treat peptic ulceration (DuPont et al., 1987; Wagstaff et al., 1988). Bismuth compounds have been used effectively to reduce *Helicobacter pylori* in humans. These bacteria have morphological, physiological, and biochemical similarities to *Campylobacter jejuni* (Slomiany et al., 1990). Farnell and coworkers (2006) evaluated whether bismuth citrate or colloidal bismuth subcitrate, would reduce *Campylobacter jejuni* in broilers. Data from this study found that cecal *Campylobacter* colonization was reduced when birds were fed these compounds. It is possible that bismuth compounds may also reduce *C. perfringens.* To further enhance the effect of bismuth compounds acidifiers such as lactose or citric acid may be used to decrease the avian intestinal pH and improve efficacy of the compound.

Lactose

Lactose is a naturally occurring disaccharide found in mammalian milk. Several past investigations have shown that the addition of lactose to the diet or drinking water causes a reduction of *Salmonella* colonization in the lower intestine of chicks, promoting growth and reducing mortality (Corrier et al., 1990a; Hinton et al., 1990). An investigation in day-of-hatch chicks, inoculated with *Salmonella* Typhimurium, found that providing dietary lactose with normal cecal flora, decreased cecal pH, and increased the concentrations of bacteriostatic volatile fatty acids (Corrier et al., 1990a). The normal pH range in the intestinal tract of the chicken varies from 6.0 - 7.4. It has been hypothesized that the addition of lactose to the diets of chickens lowers the pH level due to fermentation of lactose by normal microflora in the intestine (Hinton et al., 1990). Facultative and obligate anaerobes found in the large intestine ferment lactose and produce byproducts which may lower pH in the intestine, as lactose is not readily absorbed by chickens (Hinton et al., 1990). A reduction in pH may enhance the activity of bacteriostatic volatile fatty acids, and reduce pathogen colonization.

Along with pH decreases, lactose may also enhance intestinal integrity. In recent studies, when the effects of dietary lactose on the disease condition of *C. perfringens'* associated necrotic enteritis was evaluated, scientists found that dietary lactose can significantly alter the severity of intestinal lesion development in broilers (McReynolds et al., 2007). They determined that when birds were fed a control diet that 100% had signs of clinical intestinal lesion development when compared with 70% of the birds fed a 2.5% lactose diet. This research compares similarly to a study conducted by Takeda

and coworkers (1995), who evaluated the effects of 2 or 10% dietary lactose on the reduction of C. *perfringens* in the ceca. They found that there was a significant reduction of bacteria in the ceca, presumably from the fermentation of lactose.

Organic Acids

Organic acids are used as food additives and preservatives for preventing food deterioration (Ricke, 2003). In poultry, organic acids are generally used to improve nutrient utilization in the feed. However, organic acids may also possess antimicrobial properties. It has been hypothesized that the undissociated form of organic acids are easily able to penetrate the lipid membrane of the bacterial cell. Following internalization, the organic acid dissociates into anions and protons (Deyner and Stewart, 1998). This causes the cytoplasm of the bacterial cell to utilize excessive energy to maintain a near neutral pH, which may result in depletion of cellular. Other speculations of the antibacterial activity include the interference with nutrient transport, cytoplasmic membrane damage resulting in leakage, disruption of outer membrane permeability, and influencing macromolecular synthesis (Deyner and Stewart, 1998).

Typically bacteria have optimal pH levels in which they favor for survival and growth. Acidic disinfectants may change the pH of the gut environment, making it detrimental to microorganisms (Ricke, 2003). Acidic disinfectants, such as citric acid function by destroying the bonds of nucleic acids and precipitating proteins (Maris, 1995). In chicks, citric acid may decrease the pH of the intestinal lumen contents by contributing hydrogen ions to the intestinal environment (Brown and Southern, 1985). Researchers found a reduction in *Salmonella* recovery, following administration of citric acid (10%) in the gut (Barnhart et al., 1999).

Conclusion

We expect that by illustrating the significance of disinfectant misuse in the field that biosecurity and sanitation programs may be improved. The objective of this research is to determine if temperature, storage, or organic matter negatively affects disinfectants. We will also investigate the effect of bismuth citrate, lactose or citric acid on necrotic enteritis in broilers. Intestinal lesion development of birds orally challenged with *C. perfringens* and intestinal *C. perfringens* colonization of the avian gut will be evaluated in these studies. These data will be utilized to help reduce incidence of foodborne pathogens and avian diseases through the effective use of these disinfectants and products.

CHAPTER III

EVALUATION OF DISINFECTANTS COMMONLY USED BY THE POULTRY INDUSTRY UNDER SIMULATED FIELD CONDITIONS

Introduction

Poultry diseases are costly to the poultry industry and are difficult to control (Fussell, 1998). During the last 30 yr, avian influenza (AI) has been introduced into the United States multiple times. Following outbreaks, poultry facilities are exceptionally difficult and expensive to clean, sanitize and disinfect. An outbreak of exotic Newcastle disease in California in 2002, cost $170 million dollars to eradicate (Hietala et al., 2005). The magnitude of this outbreak affected 21 poultry complexes, thousands of noncommercial entities (aviaries, rehabilitation centers), and allied industries from California, Nevada, Arizona and Texas (Crossley et al., 2005). Laryngotracheitis, chicken anemia virus and outbreaks of necrotic enteritis also cause increased morbidity and significant economic losses (Villegas, 1998; Van Immerseeel et al., 2004). *Staphylococcus aureus* infections increase morbidity and mortality from yolk sac infections and secondary infections affecting the bones, tendon sheaths, and leg joints (Moya, 1986). It is estimated that 1.4 million humans contract salmonellosis, caused by *Salmonella spp.*, costing $3 billion annually (Williams, 1988). *Salmonella* continues to be a predominant foodborne pathogen worldwide; with poultry and poultry products considered as a common vehicle for this pathogen.

The principal of disease prevention and control largely rely on biosecurity. Biosecurity includes protocols and procedures taken to prevent pathogens from infecting a farm and to prevent the transmission of disease by humans, insects, wild birds or other animals (Poss, 1998). Physical barriers, shower facilities, and on farm labor are examples of procedures employed in a biosecurity program to minimize disease exposure (Dekich, 1998). Tire wash stations and foot baths are also used to reduce pathogen transmission (Davison et al., 1999).

Disinfectants are important components of a biosecurity program. Classes of disinfectants include phenolics, quaternary ammonium compounds (QAC), halogens, oxidizing agents, chlorhexidine compounds, and alcohols (Smith and June, 1999; Dvorak, 2005). The objective of disinfection application is to reduce microbial populations (Eckman, 1994). Disinfectants act on microorganisms at several target sites resulting in membrane disruption, metabolic inhibition and or lysis of the cell (Denyer and Stewart, 1998; Maillard, 2002). In the field, disinfectants are often mixed with water in storage tanks and exposed to extreme environmental conditions prior to their actual application. Disinfectants may have a limited lifespan after their initial dilution and it's likely that heat, sunlight, time, organic matter (OM), or adulterants reduce their efficacy. Quaternary ammonium, binary, phenolic, and chlorhexidine compounds are commonly used in agricultural settings. In this experiment we used *Salmonella* Typhimurium and *Staphylococcus aureus* as our Gram negative and Gram positive organisms, respectively, commonly found in commercial poultry housing. The objective

of this experiment was to compare the effects of time, temperature, or OM on representative disinfectants used by a commercial poultry integrator.

Materials and Methods

Bacterial Culture

Staphylococcus aureus (SA) (American Type Culture Collection # 12600) and a primary poultry isolate of *Salmonella* Typhimurium (ST) were obtained from the USDA, Agriculture Research Service, Southern Plains Agricultural Research Center (College Station, TX).

Staphylococcus aureus was cultured in tryptic soy broth (Difco Laboratories, Detroit, MI) for 12 h. Cells were washed 3 times with Butterfield's solution by centrifugation (3,000 x g) and the approximate concentration of the stock solution was determined spectrophotometrically (625 nm). The bacterial stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL per replicate) that were spread-plated on mannitol salt agar (Becton, Dickinson and Co, Sparks, MD) plates.

Salmonella Typhimurium was cultured in tryptic soy broth (Difco Laboratories) for 8 h. The cells were washed 3 times with Butterfield's solution by centrifugation (3,000 x g) and the approximate concentration of the stock solution was determined spectrophotometrically (625 nm). The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL per replicate) that were spread-plated on brilliant green agar (Becton, Dickinson and Co) plates containing 25 ug/mL of novobiocin (Sigma Chemical Co., St. Louis, MO).

Disinfectants

Four commercial disinfectants were diluted to the manufacturers' recommended working concentrations with distilled water. The QAC (RXVeterinary products, Grapevine, TX) consisted of 10% alkyl dimethyl ammonium chloride, and 10% dimethyl ethyl benzyl ammonium chloride (8.8 mL/3.8 L). The phenolic compound (Biosentry Inc, Stone Mountain, GA) contained 7.92% O-phenylphenol, 9.97% O-benzyl-p-chlorophenol, and 1.95% p-tert-amyphenol (14.7 mL/3.8 L). The chlorhexidine compound (Fort Dodge Animal Health, Fort Dodge, IA) consisted of 2% chlorhexidine diacetate (29.5 mL/3.8 L). The binary compound (Wilson Manufacturing Company, Inc, Jefferson, GA) consisted of 13.02% didecyl dimethyl ammonium chloride, and 8.68% alkyl dimethyl benzyl ammonium chloride (7.3 mL/3.8 L). All cultures were carried out in 15 mL glass tubes in triplicate. Disinfectant activity was determined by comparing bacteria growth across time and concentrations for each disinfectant evaluated. Three independent experiments were conducted to evaluate the effect of time, temperature and OM on disinfectant efficacy.

Serial Dilutions

We used a modified technique derived from the Association of Official Analytical Chemists (1984) use-dilution test # 955.15 adapted from Robison et al. (1988). In each experiment, 0.5 mL of 10^8 cfu/mL of the test organism was added to 4.5 mL of diluted disinfectant (in experiments 2 and 3 disinfectants were supplemented with appropriate concentrations of chicken litter (organic matter)) and briefly vortexed at the lowest setting for 3 s. Following a 10 min incubation at room temperature, the tube was

vortexed and serially diluted into 4 subsequent tubes containing 4.5 mL of Butterfield's solution. One hundred microliters (µl) of each dilution tube was then spread plated onto selective agar. In experiment 2, Dey Engley agar (Difco Laboratories) was used as disinfectant neutralizing agar medium for both organisms**,** and incubated at 37°C for 24 h and enumerated. Dey Engley neutralizing agar contains ingredients that limit any residual activity. In preliminary studies (data not shown) we found that there was no difference in bacterial growth on Dey Engley agar compared to mannitol salt and brilliant green agar.

Enrichment

Staphylococcus broth (Difco Laboratories) and tetrathionate broth (Difco Laboratories) were used as enrichment for SA and ST, respectively. Dey Engley broth (Difco Laboratories) was used for both organisms in experiment 2. One hundred microliters (µl) of solution was collected from the initial incubation tube containing *Salmonella* or *Staphylococcus*, and inoculated into their respective enrichment broth. After 24 h incubation of the enrichment broth, 100 µl of each culture in experiments 1 and 3 were struck for isolation onto agar plates and incubated at 37°C for 24 h. In experiment 2, following the 24 h incubation in enrichment broth, a color change indicated a positive sample.

Samples that were negative at the 1:100 dilutions but positive after culture in enrichment broth were assigned an arbitrary value of 1.50 $\log_{10}$ of indicator organism according to Corrier et al. (1993).

Experimental Design

Experiment 1. Triplicate working (n=3) concentrations of disinfectants were stored at 4, 20, 32 and 43°C. Samples of diluted disinfectant were collected at 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, and 30 wk.

Experiment 2. Two trials (n=3) were conducted to evaluate the effect of OM on disinfectant efficacy of freshly made disinfectants stored at room temperature. Chicken litter was used as the source of OM. The litter was dried, finely ground and sterilized, prior to use. The OM was added to an incubation tube at concentrations of 0, 0.75, 1.5 or 3%.

Experiment 3. Two trials (n=3) were conducted to determine the effect of time and OM on 30 wk old and freshly made disinfectants stored at room temperature. Organic Matter was added to each incubation tube at 1.5%.

Statistical Analysis

Statistical analyses were completed with version 11.0 for Windows, SPSS statistical software package (Chicago, IL). Data in all experiments were analyzed via a one-way ANOVA using the GLM procedure due to the presence of significant interactions. Differences were deemed significant at $p \leq 0.05$ and means were separated using a Duncan's multiple range test. Significant interactions were as follows: experiment 1 – disinfectant and temperature, experiment 2- disinfectant and OM, experiment 3- disinfectant and storage time.

Results

In experiment 1, the effects of time and temperature on disinfectant efficacy were evaluated. Following incubation, all disinfectants retained total efficacy against ST for the duration of the experiment, except on the phenolic compound. A reduction ($p \leq 0.05$) of 5.5 logs of ST was observed with the phenolic compound after 6 wk of incubation at 43°C, and after 16 wk of incubation at 32°C compared to our stock concentration of ST (Fig. 3.1a,b). This reduced efficacy observed with the phenolic compound was indicative of a positive enrichment sample.

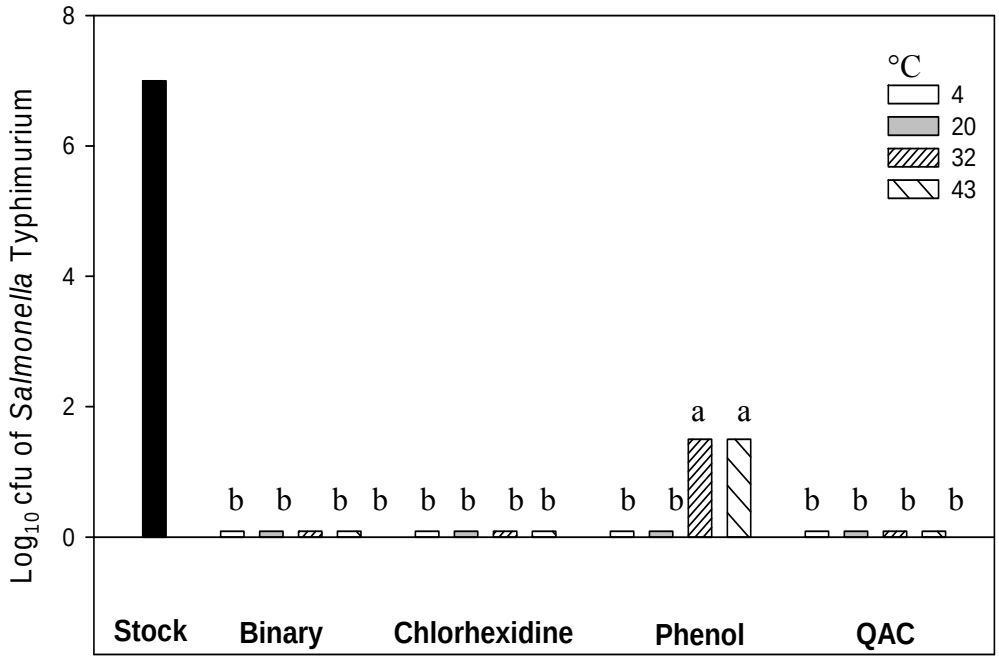

Figure 3.1a. Disinfectant efficacy following 30 wk of storage at treatment temperatures against *Salmonella* Typhimurium.

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

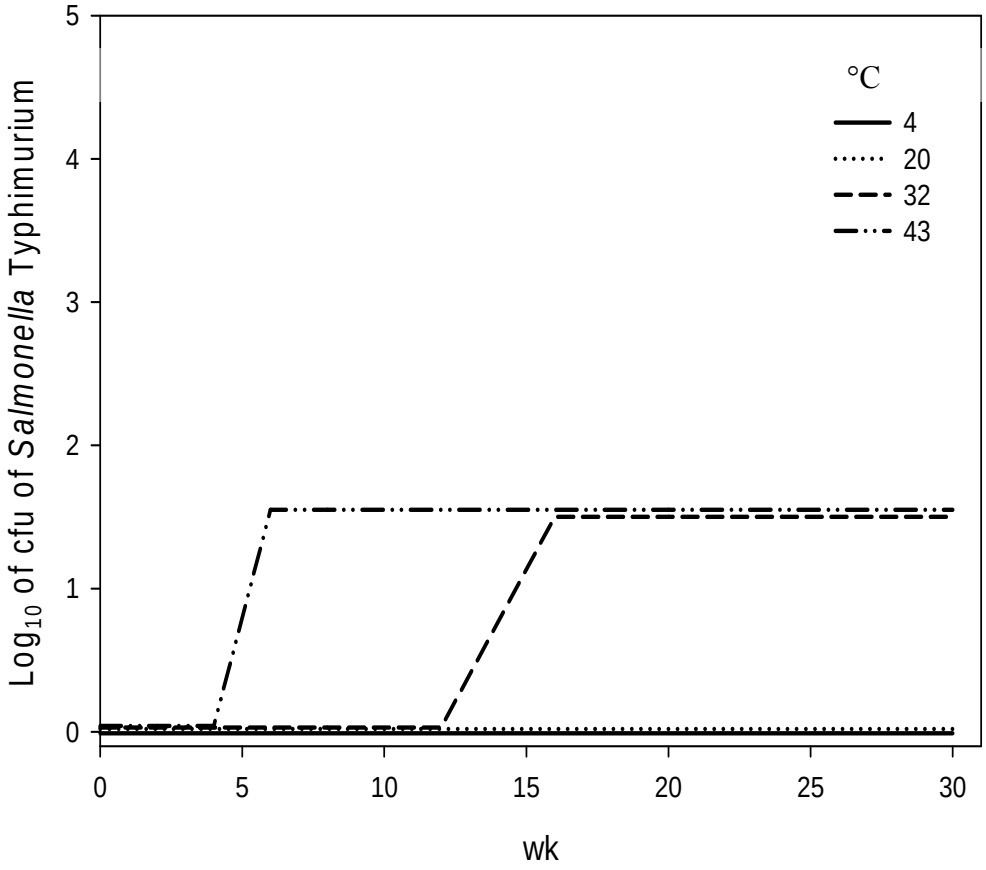

Figure 3.1b. Time point when the phenolic compound reduced effectiveness against *Salmonella* Typhimurium.

All disinfectants remained effective against SA regardless of temperature (Fig. 3.2).

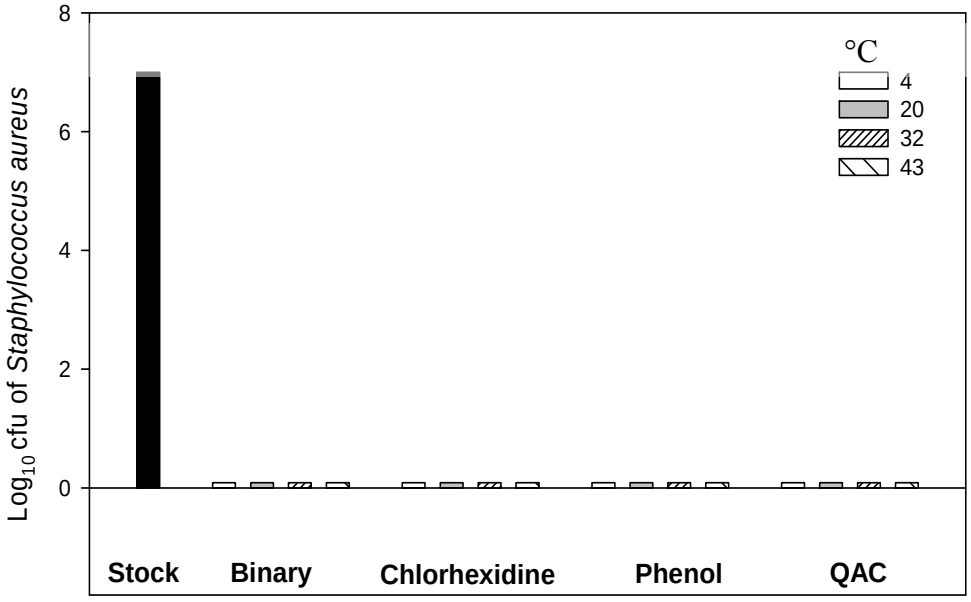

Figure 3.2. Disinfectant efficacy following 30 wk of storage at treatment temperatures against *Staphylococcus aureus*.

In experiment 2, decreases ($p \leq 0.05$) in disinfectant efficacy were observed in response to increasing levels of OM (Fig. 3.3). At 0% OM, all disinfectants reduced the stock concentration of the stock ST to undetectable limits. Following addition of OM, we observed reductions in efficacy on all disinfectants in a dose dependent manner. At 0.75% OM, cfu reductions were 1.5, 2.7, 3.0, and 5.8 logs of ST for chlorhexidine, QAC, binary and phenolic compounds, respectively. At 1.5% OM, cfu reductions were 0, 1.0, 3.1 and 3.0 logs of ST for chlorhexidine, QAC, binary and phenolic compounds, respectively. At 3% OM, cfu reductions were 0, 0.75, 1.5, and 3.0 logs of ST for chlorhexidine, QAC, binary and phenolic compounds, respectively. The phenolic compound was the most resistant disinfectant, while the chlorhexidine was the most susceptible.

All disinfectants were effective at a 0% ($p \leq 0.05$) concentration of OM against SA (Fig. 3.4). The binary and phenolic compounds reduced ($p \leq 0.05$) the total SA population at 0.75% and 1.5% OM, when compared to QAC and chlorhexidine compound.

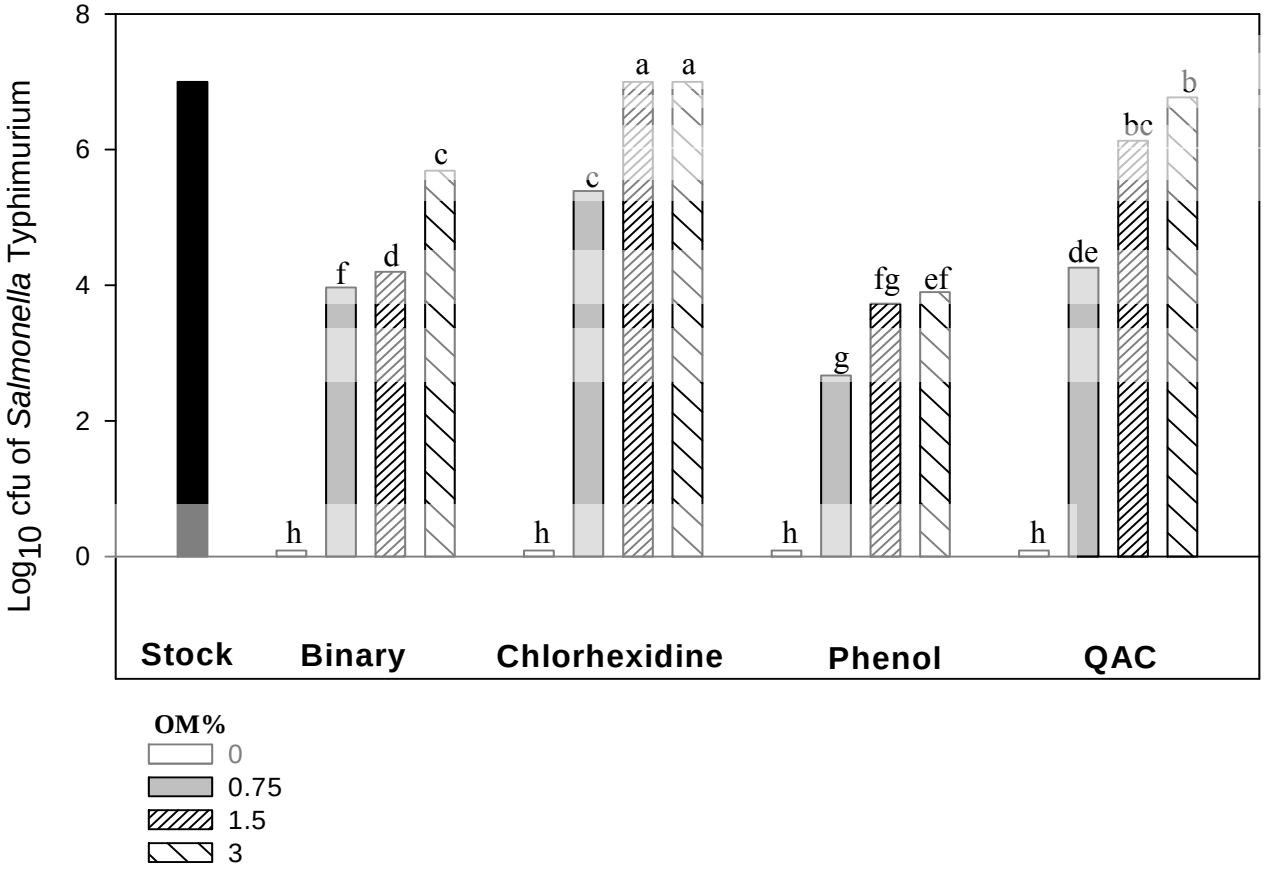

Figure 3.3 Effect of concentrations of organic matter (OM) on fresh disinfectants at room temperature against *Salmonella* Typhimurium.

[a-h] Means with different superscripts differ significantly ($p \leq 0.05$)

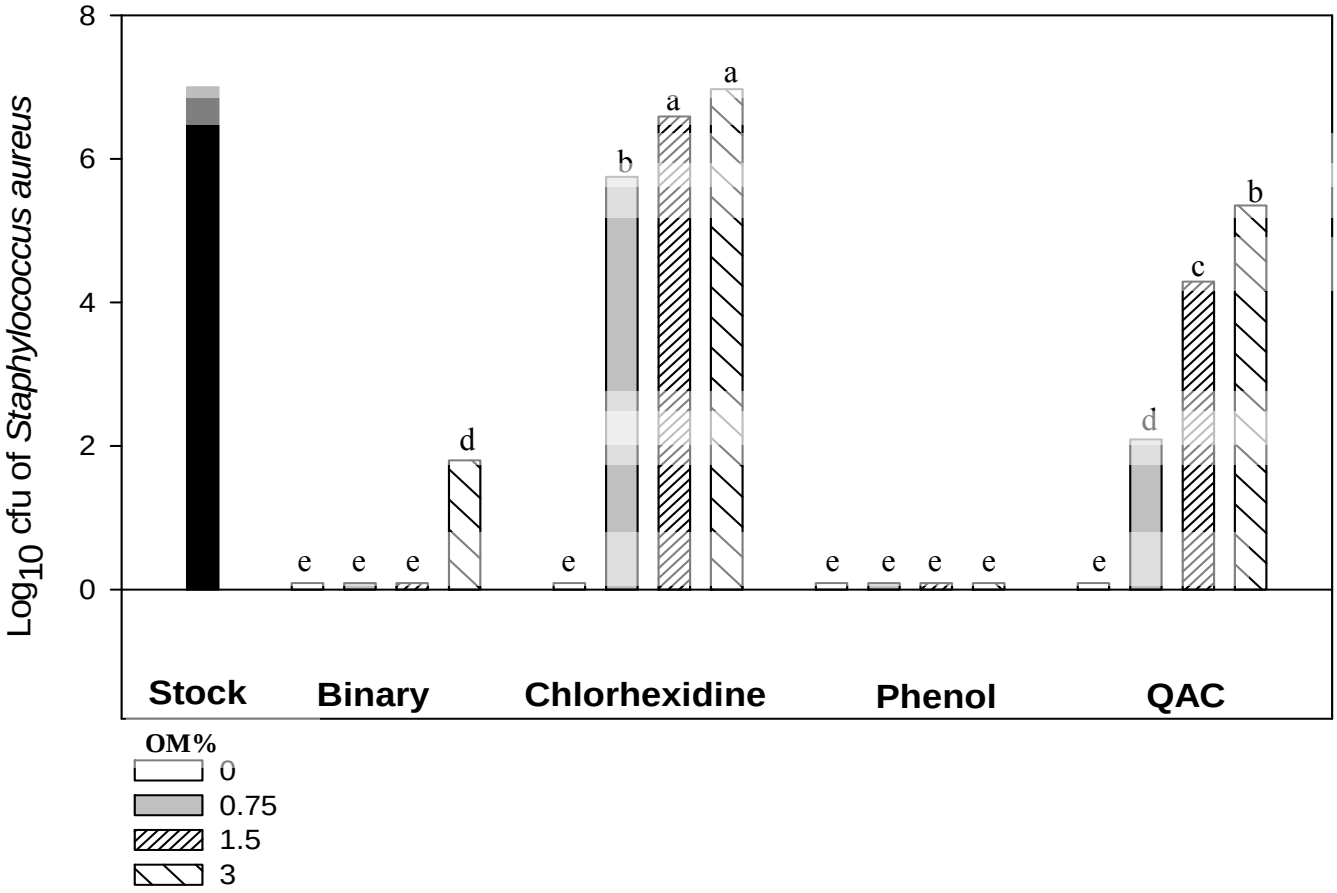

Figure 3.4 Effect of concentrations of organic matter (OM) on fresh disinfectants at room temperature against *Staphylococcus aureus*.

[a-e] Means with different superscripts differ significantly ($p \leq 0.05$)

At 3% OM, the phenolic compound reduced ($p \leq 0.05$) the total SA population, the chlorhexidine compound was ineffective, but the QAC and binary compound were able to reduce cfu of the stock solution of SA by 2.6 and 5.2 logs, respectively.

In experiment 3, the consequences of long term storage on disinfectant efficacy in the presence of OM against ST were evaluated (Fig. 3.5 and 3.6). The efficacy of the 30 wk old QAC was significantly reduced when compared to freshly prepared disinfectant against ST (Fig. 3.5). The 30 wk old phenolic compounds were significantly reduced in efficacy when compared to freshly prepared disinfectant against ST. There were no significant differences between the fresh and 30 wk old solutions for the chlorhexidine and binary treatments against ST. The fresh and 30 wk old chlorhexidine compound was ineffective against ST.

A significant decrease was observed in the efficacy of the 30 wk old QAC and binary compound against SA (Fig. 3.6), relative to freshly prepared solutions of the disinfectant. There were no differences between fresh and 30 wk old phenolic and chlorhexidine compounds.

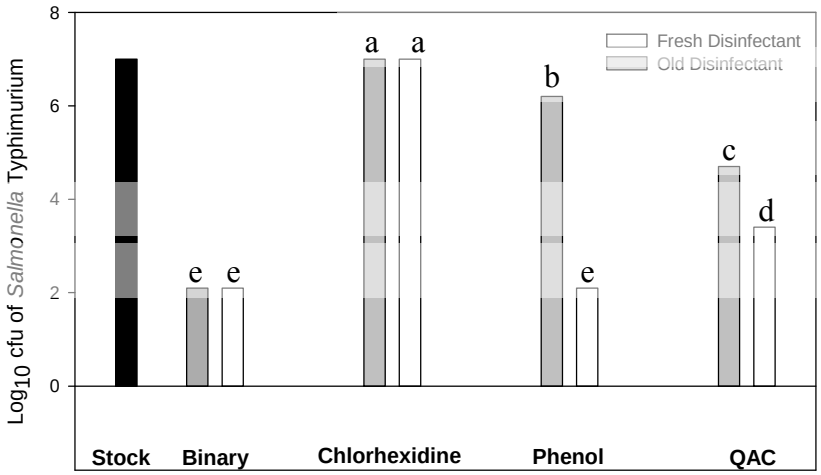

Figure 3.5. Effect of 30 wk old disinfectants compared to fresh disinfectant at room temperature against *Salmonella* Typhimurium with the addition of 1.5% organic matter.

[a-e] Means with different superscripts differ significantly ($p \leq 0.05$)

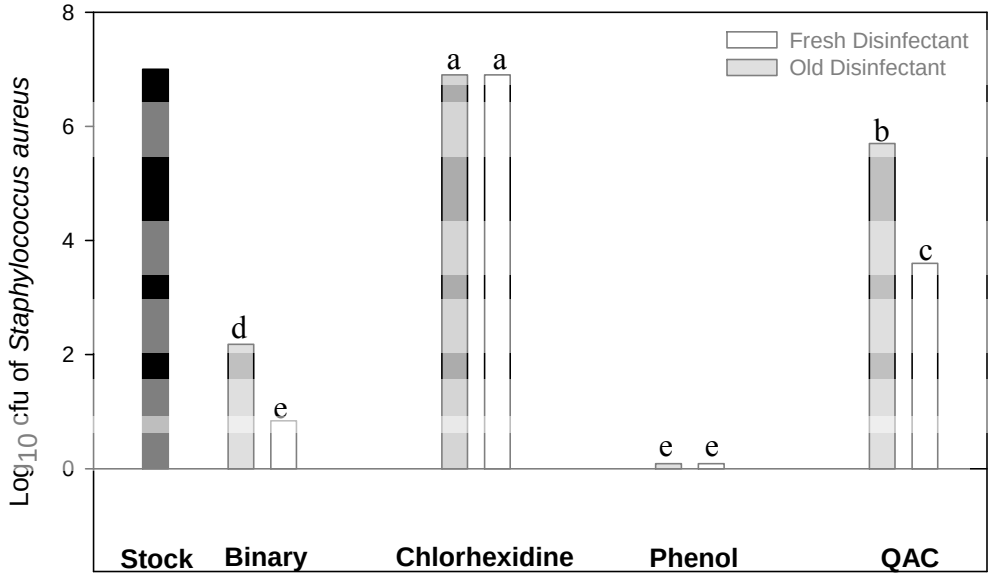

Figure 3.6. Effect of 30 wk old disinfectants compared to fresh disinfectant at room temperature against *Staphylococcus aureus* with the addition of 1.5% organic matter.

[a-e] Means with different superscripts differ significantly ($p \leq 0.05$)

Discussion

Once disinfectants are diluted they are often stored and exposed to less than optimal storage conditions, such as ultraviolet light exposure or extreme temperatures prior to their actual application. These conditions can negatively affect the antimicrobial properties of disinfectants. The use of an inactive or outdated disinfectant may result in a false sense of security for production personnel (Shulaw et al., 2001). Disinfectants should ideally be stored in a dark, cool location and undiluted (Dvorak, 2005). This study demonstrates that working concentrations of disinfectants may be stored for up to 30 wk at less than optimal temperatures without loss of effectiveness against ST and SA (Fig. 3.1a,b). However, when exposed to OM, long term storage of disinfectants may have a detrimental effect relative to freshly prepared disinfectants.

Disinfectants should be used following the cleaning and removal of excessive OM (blood, fecal matter, litter, fat, and hatchery fluff). Organic matter provides a physical barrier that protects microorganisms from contact with the disinfectant (Dvorak, 2005). Foot baths should be placed in the entry way of houses in an effort to avoid transferring disease agents into the flock and to prevent trafficking of pathogens into vehicles and off the farm to other operations (Poss, 1998). Insufficient removal of organic debris prior to stepping into the disinfectant solution, inappropriate contact time allowed for disinfectants, and irregular changes of disinfectant solution are typical problems associated with boot baths (Dvorak, 2005). These problems increase the incidence of pathogenic microorganisms on footwear and affect the amount of debris accumulated in the foot bath solution. Quinn (2001) suggests, similar to our

observations (Fig. 3.3 and 3.4), that phenolic compounds should be used for foot baths and any application where excessive OM may be present, due to their better efficacy in the presence of OM. Rodgers and colleagues (2001) evaluated the effects of 18 commercial disinfectants against SA. They found that when disinfectants were mixed at the manufacturers' recommended concentrations, all the disinfectants were bactericidal against SA. However, with the addition of OM (fluff) the disinfectants were not as effective. Payne and colleagues (2005) evaluated commonly used poultry house disinfectants on reducing total aerobic bacteria, yeast, mold, *Campylobacter*, and *Salmonella* populations on poultry house floors. Results of their study suggest that application rate, disinfectant type, time of exposure and the presence of OM are all important considerations when including a chemical disinfectant application in a sanitation program. An insufficient concentration of a disinfectant may cause organisms to enter a viable but nonculture able state (Roszak et al., 1983; Mckay, 1992) or possibly develop antimicrobial resistance (Gismondo et al., 1995).

Commercially available disinfectants are not all classified as broad spectrum. Multiple factors should be considered when a disinfectant is chosen, such as organic matter on the surface to be treated, presence of OM in the diluent, quality of water, corrosiveness or toxicity of the product, application method, temperature, porosity of the surface being treated, length of contact time, infectious organisms targeted, susceptibility of the infectious organisms and correct dilution (Prince et al., 1991; Quinn, 2001; Dvorak, 2005; Payne et al., 2005).

In conclusion, long term storage of disinfectants at 4, 20, 32 or 43°C did not reduce efficacy in the absence of OM against SA. However, a reduction in efficacy was observed over time with the phenolic compound against ST. Following the inclusion of OM, reduced efficacy was observed in a dose dependent manner against both organisms, excluding the phenolic compound against SA. Fresh disinfectant performed better in the presence of OM than 30 wk old disinfectant. These results emphasize the need to use fresh disinfectants and that OM should be removed prior to disinfection. Appropriate use of disinfectants should be considered as an important intervention strategy to control avian diseases in poultry. Biosecurity and an effective disinfectant program will reduce foodborne pathogens, immunosuppressive viruses, reportable diseases, and opportunistic infections.

CHAPTER IV

EFFECT OF BISMUTH CITRATE, LACTOSE AND CITRIC ACID ON NECROTIC ENTERITIS IN BROILERS

Introduction

Necrotic enteritis (NE), commonly caused by *Clostridium perfringens* (CP), is an economically important avian enteric disease (Williams, 2005). The incidence of NE has recently increased because of the withdrawal of in-feed antibiotic growth promoters with anti-clostridial activity (Knarreborg et al., 2002). *Clostridium perfringens* is ubiquitous in nature and is considered an opportunistic pathogen (Craven et al., 2001). For infection to occur one or more predisposing factors must be present, including mucosal damage (commonly caused by coccidiosis), diets with high levels of indigestible water-soluble non-starch polysaccharides or immunosupression (Van Immerseel et al., 2004; Williams, 2005). Disease occurs when high numbers of CP adhere to damaged intestinal mucosa, proliferate, and then produce toxins. Toxin production results in damage to the small intestine, leading to lesions and necrosis (Van Immerseel et al., 2004). Infected chickens appear to be depressed, anorexic, and stationary, leading to a reduction in performance and/or death (Van Immerseel et al., 2004). Outbreaks of this disease may result in downgraded or rendered carcasses, and mortality rates may reach up to 1% per day (Kaldhusal and Lovland, 2000). Alternative feeding strategies such as probiotics or prebiotics may be used to reduce the incidence of

NE. One strategy that may be worth pursuing is modifying the avian gastrointestinal mucosal environment, in which *C. perfringens* flourish, with bismuth compounds.

Bismuth compounds, primarily colloidal bismuth subcitrate (CBS) and bismuth subsalicylate (BSS) have been used to treat gastric disorders in humans for over 300 years (Marshall, 1991). These compounds have treated duodenal ulcers, gastritis, chronic diarrhea, traveler's diarrhea, and acute diarrhea in young children (DuPont et al., 1987; Soriano-Brucher et al., 1990; Steffen, 1990). Bismuth subsalicylate and other bismuth compounds (acid bismuth subsalicylate, bismuth sulfate, bismuth citrate, and bismuth oxychloride) have been shown to inhibit the growth of *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter* (Manhart, 1990). The administration of bismuth compounds have also been shown to protect the gastric mucosa. In past investigations CBS has been to reduce *H. pylori*, by altering mucin characteristics in humans (Slomiany et al., 1990; Tillman et al., 1996; Rauws et al., 1988; Fraser, 2004). These studies suggest that the modification of chicken mucin with bismuth compounds may also reduce *C. perfringens* colonization in chickens. Bismuth citrate and colloidal bismuth subcitrate have been used to reduce cecal colonization by *Campylobacter jejuni* in broilers (Farnell et al., 2006). In addition to bismuth compounds, lactose has been used to reduce pathogens in poultry.

Lactose or milk sugar is a naturally occurring disaccharide found in mammalian milk. Lactose fed to broilers at 2.5%, has been shown to significantly reduce intestinal lesions and mortality rates associated with NE (McReynolds et al., 2007). Addition of lactose to the diet or drinking water has been shown to reduce *Salmonella* in the lower

intestine, reduce mortality, and promote growth (Corrier et al., 1990b; Hinton et al., 1990). Along with this protective effect in the gut, milk sugars have been shown to decrease the pH of the chicken intestinal tract from 6.0 - 7.4 to 4.4 - 5.6 (Hinton et al., 1990). Feeding lactose to chickens resulted in an increase in bacteriostatic acetic and propionic acids causing a decrease in cecal pH (Corrier et al., 1990a). Organic acids may be another method of lowering the pH of the avian gut environment and offer protection against *C. perfringens* colonization.

Organic acids can change the pH of the gut, creating an inhospitable environment for some microorganisms (Ricke, 2003). Citric acid is a weak organic acid and has been speculated to cause a decrease in the pH of intestinal contents by contributing hydrogen ions to the intestinal environment in chickens (Brown and Southern, 1985). Similar to lactose, citric acid may also have anti- *Salmonella* properties. In an evaluation of potential disinfectants for preslaughter broiler crop decontamination, researchers found that citric acid at or greater than 10% caused a reduction in *Salmonella* in a simulated crop environment, suggesting that citric acid may have antimicrobial affects (Barnhart et al., 1999).

Due to the insoluble nature of bismuth compounds in the presence of water, bismuth compounds are considered most effective following entrance into the acidic environment of the stomach. Research demonstrates that bismuth compounds form a precipitate (active metabolite) at a pH of less than 5, facilitating the infiltration of bismuth into human microvilli (Wagstaff et al., 1988). It is possible that the addition of lactose or citric acid may reduce intestinal pH to an optimal range for enhancing bismuth

efficacy in broilers. The purpose of this study was to determine if bismuth citrate can reduce gut colonization by CP and reduce intestinal lesion development in broilers challenged with our NE model. We will also determine if the addition of lactose or citric acid enhance the efficacy of bismuth citrate.

Materials and Methods

Experimental Birds

Day of hatch chicks were obtained from a local commercial hatchery and placed in floor pens with pine shavings and supplemental heat. Chicks were provided water and a commercial whole wheat-corn based broiler ration *ad libitum* that met or exceeded the NRC (1994) guidelines. Elevated concentrations of wheat in the diet have been shown to intensify the occurrence of NE (Riddell and Kong, 1992).

Immunosupression Vaccine Administration

As previously described, a commercial bursal disease vaccine (Schering Plough Animal Health, Millsboro, DE) was used as an immunosuppressant in the current investigation (McReynolds et al., 2004). All experimental birds were administered the vaccine on d 14 at a level 10 times the recommended dose of the manufacturer via an ocular route.

Clostridium perfringens *Administration*

Multiple isolates of CP (type A) obtained from active field cases in Virginia, North Carolina and Georgia were used in this investigation (McReynolds et al., 2004). The isolate was grown in thioglycollate medium (Becton Dickinson Co., Sparks, MD)

for 12 h. Birds were challenged once a day for 3 days by oral gavage (1.5 mL/bird) with a stock culture of 10^7 cfu of CP/mL.

Bacterial Culture

To measure the colonization of CP, a 6 in /15.24 cm section of the small intestine, 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 thioglycollate medium (Becton Dickinson Co). Three ten-fold serial dilutions were performed and plated onto thioglycollate agar (Becton Dickinson Co) and incubated anaerobically (24 h at 37°C). Colonies exhibiting typical colony morphology were counted and recorded. Colony forming units were transformed into Log_{10} values.

NE Lesion Scores

The jejunum and ileum of the small intestine were examined for gross lesion scores associated with NE. 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; and 4 = severe and extensive necrosis, marked hemorrhage, much gas in intestine (Prescott et al., 1978).

pH Analysis

On the last day of the second experiment, all birds were sacrificed and upper ileum pH values were determined. Intestinal pH was determined by the insertion of a

sterile glass pH electrode(Model 05669-20; Cole Palmer, Niles, IL) through an incision in the intestinal wall ensuring that the electrode remained in contact with the gut contents.

Experimental Design

Three independent experiments were conducted to evaluate the effect of bismuth citrate (Sigma Chemical Co., St. Louis, MO), lactose (Sigma Chemical Co) or citric acid (Agri Laboratories LTD, St. Joseph, MO) on NE in broilers.

Experiment 1. Birds were randomly assigned treatments consisting of: 0, 50, 100, or 200 ppm bismuth citrate. Birds were fed respective diets from day-of-hatch until termination of the experiment. All birds were challenged as previously described (McReynolds et al., 2007). Two replicate trials were conducted to evaluate CP colonization and the development of intestinal lesions.

Experiment 2. Birds were randomly assigned to treatment groups consisting of negative control, bismuth negative control, or a challenged treatment group. Challenged treatments consisted of a positive control, 2.5% dietary lactose, citric acid, bismuth, a combination of bismuth with 2.5% dietary lactose or a combination of bismuth with citric acid. This experiment evaluated intestinal pH and the development of intestinal lesions.

Experiment 3. Birds were randomly assigned to treatment groups consisting of negative control, bismuth negative control, or a challenged treatment group. Challenged treatments consisted of a positive control, 2.5% dietary lactose, bismuth or a

combination of 2.5% dietary lactose with bismuth treatment. This experiment evaluated CP colonization and intestinal lesion development.

Statistical Analysis

Statistical analysis was completed with the SPSS statistical software package (Chicago, IL). Data in all experiments were analyzed via a one-way ANOVA using the GLM procedure. Differences were deemed significant at $p \leq 0.05$ and means were separated using Duncan's multiple range test.

Results

When evaluating CP colonization in the first trial of experiment 1, there were no significant differences between the treatments. A reducing trend in lesion scores associated with CP was observed in birds fed 200 ppm bismuth citrate when compared with the positive controls (Fig. 4.1.). In trial 2, reductions ($p \leq 0.05$) in CP colonization were observed in birds fed 100 ppm or 200 ppm bismuth citrate when compared with the 0 ppm bismuth citrate treatment (Fig. 4.2.).

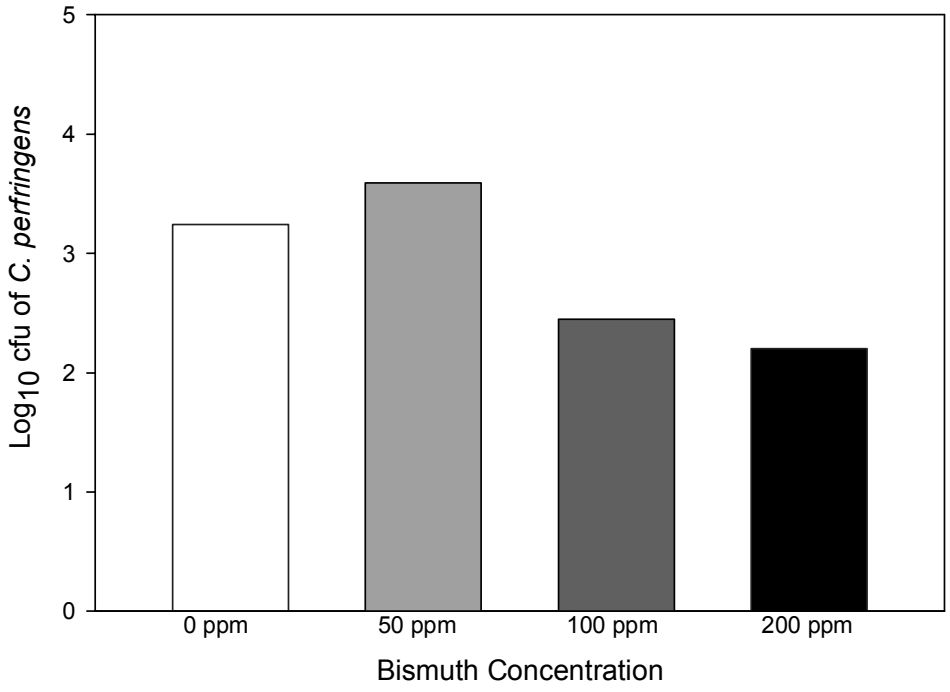

Figure 4.1. Intestinal *Clostridium perfringens* colonization of broilers treated with 0, 50, 100, or 200 ppm bismuth citrate (Trial 1).

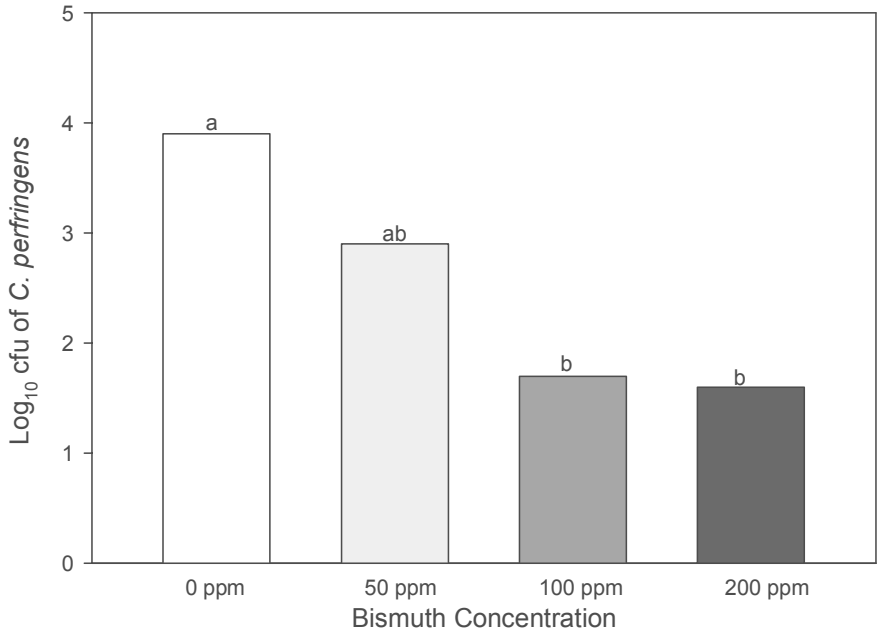

Figure 4.2. Intestinal *Clostridium perfringens* colonization of broilers treated with 0, 50, 100, or 200 ppm bismuth citrate (Trial 2).

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

Following CP challenge, lesion scores for the 50 ppm, 100 ppm and 200 ppm bismuth citrate treatment groups were reduced ($p \leq 0.05$) when compared with birds fed 0 ppm bismuth (Fig. 4.3). Birds fed the 100 ppm or 200 ppm diet resulted in a significant reduction in lesion score when compared with the 0 ppm treatment group (Fig.4. 4). There were no differences between the positive control (0 ppm bismuth citrate) and the 50 ppm bismuth citrate treatment. In experiment 2, we evaluated dietary lactose and citric acid for enhancing the efficacy of bismuth citrate in reducing intestinal pH and lesion development. We found no significant differences, following CP

challenge, between birds fed bismuth citrate, relative to birds fed a combination of bismuth citrate with dietary lactose or citric acid in lesion development (Fig. 4.5). The intestinal pH of birds fed a combination of bismuth citrate with dietary lactose or citric acid was not significantly reduced when compared with birds fed bismuth citrate alone (Fig. 4.6). A significant reduction in pH was observed in birds fed bismuth citrate and lactose relative to the negative control. In experiment 3, we evaluated the effect of dietary lactose and bismuth citrate on NE intestinal lesion development and CP colonization, birds were fed 2.5% lactose with 100 ppm bismuth citrate. A significant positive effect between, dietary lactose and bismuth citrate was not observed when evaluating CP colonization (Fig. 4.7). A reducing trend in CP colonization was observed in challenged birds fed bismuth citrate, when compared with challenged birds fed a combination of bismuth and lactose. The addition of 2.5% dietary lactose with 100 ppm bismuth citrate reduced ($p \leq 0.05$) intestinal lesion development relative to the positive control and lactose positive control treatment (Fig. 4.8).

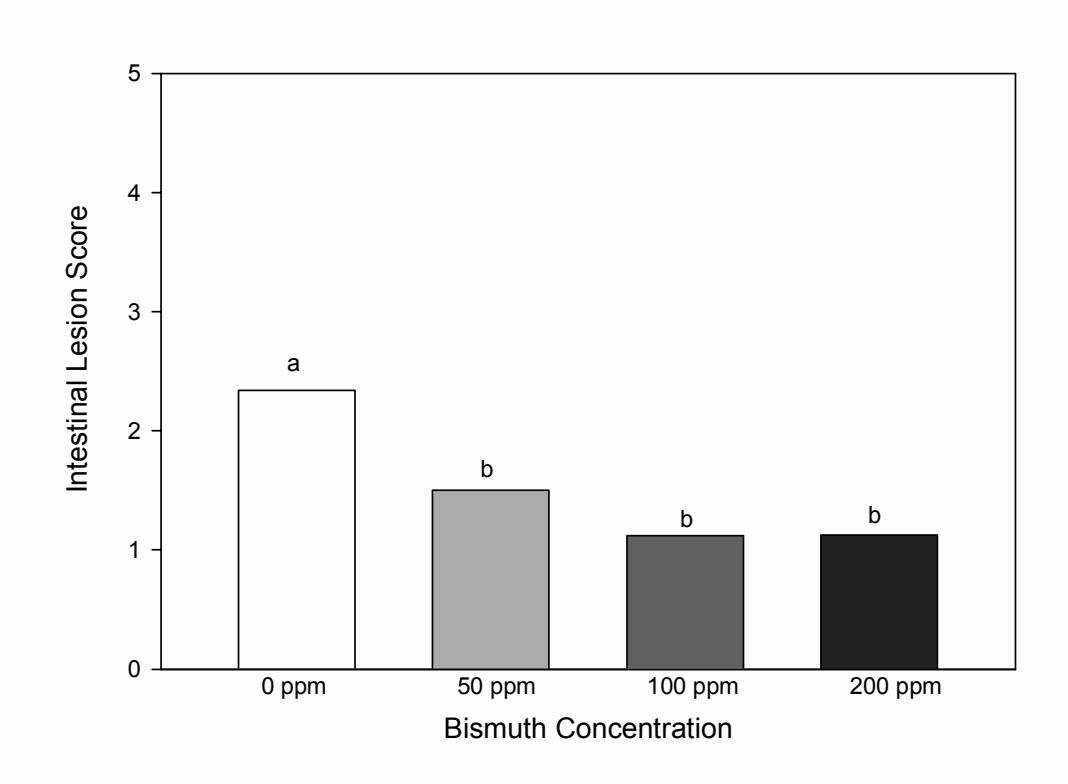

Figure 4.3. Intestinal lesion development in broilers treated with 0, 50, 100, or 200 ppm bismuth citrate (Trial 1).

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

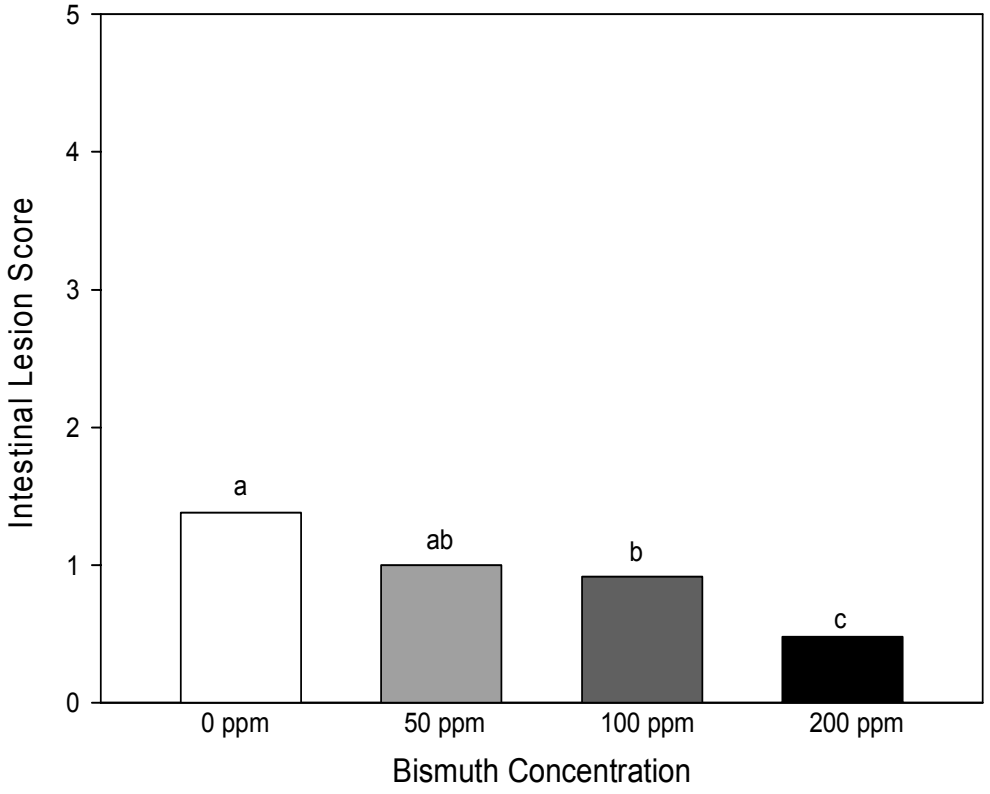

Figure 4.4. Intestinal lesion development in broilers treated with 0, 50, 100, or 200 ppm bismuth citrate (Trial 2).

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

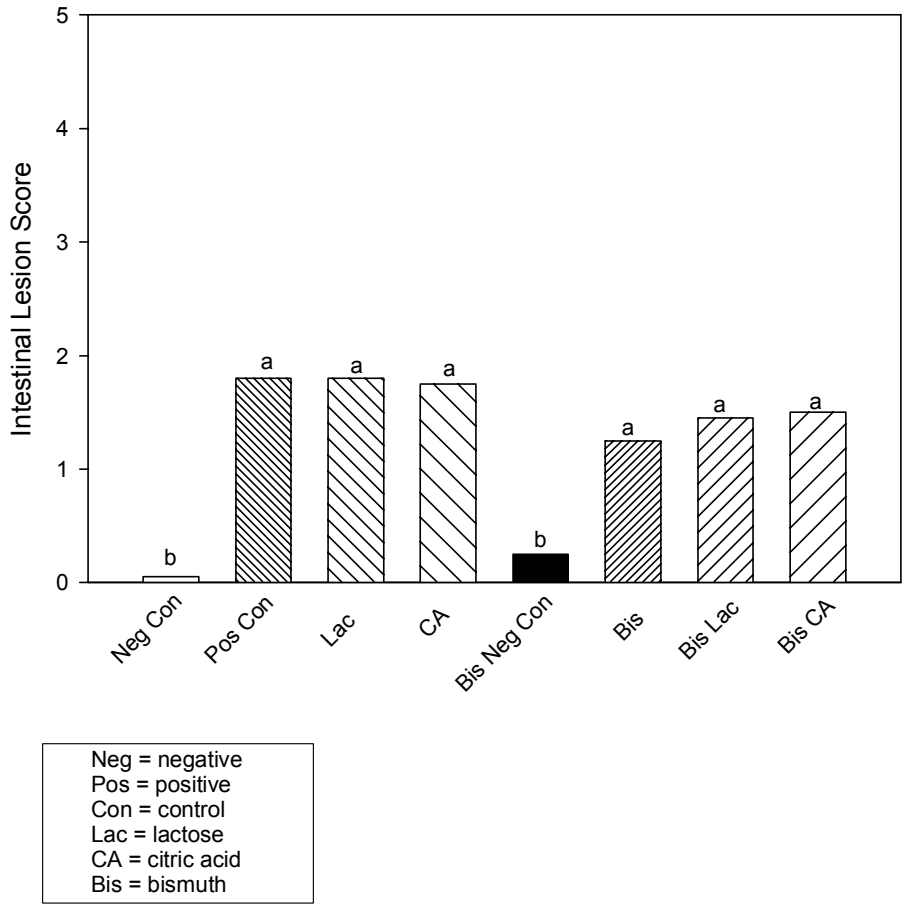

Figure 4.5. Evaluation of bismuth citrate and lactose or citric acid on intestinal lesion development.

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

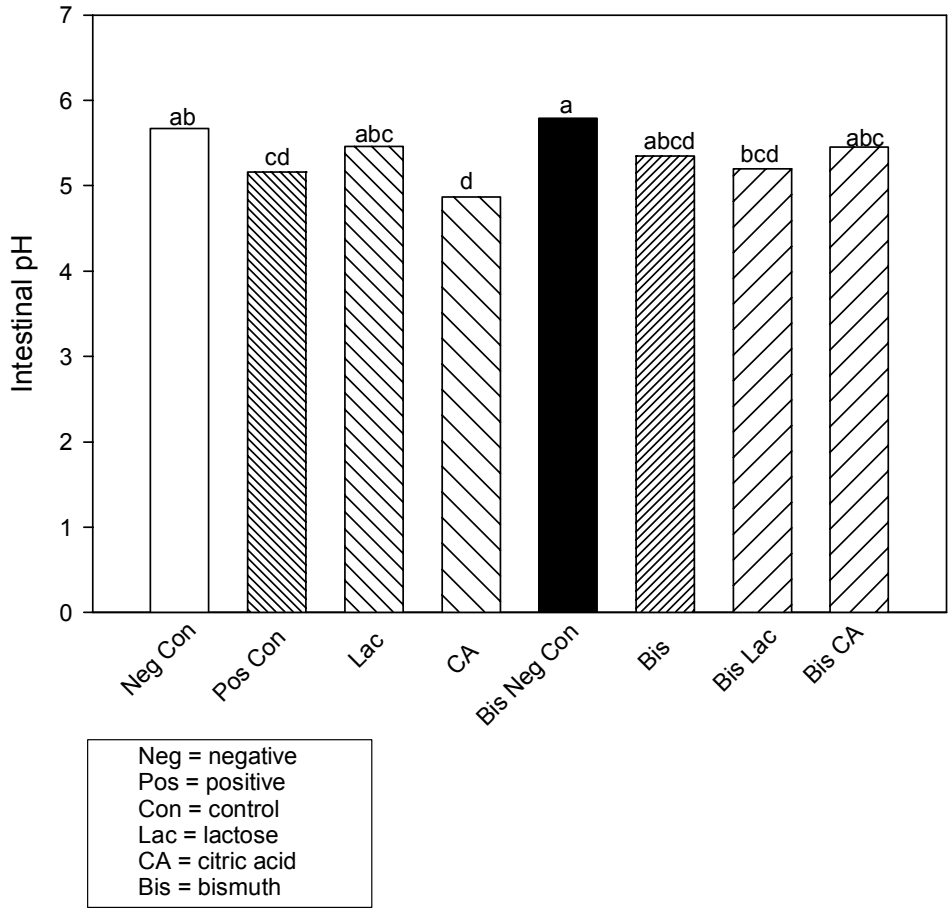

Figure 4.6. Evaluation of bismuth citrate and lactose or citric acid on intestinal pH..

[a-d] Means with different superscripts differ significantly ($p \leq 0.05$)

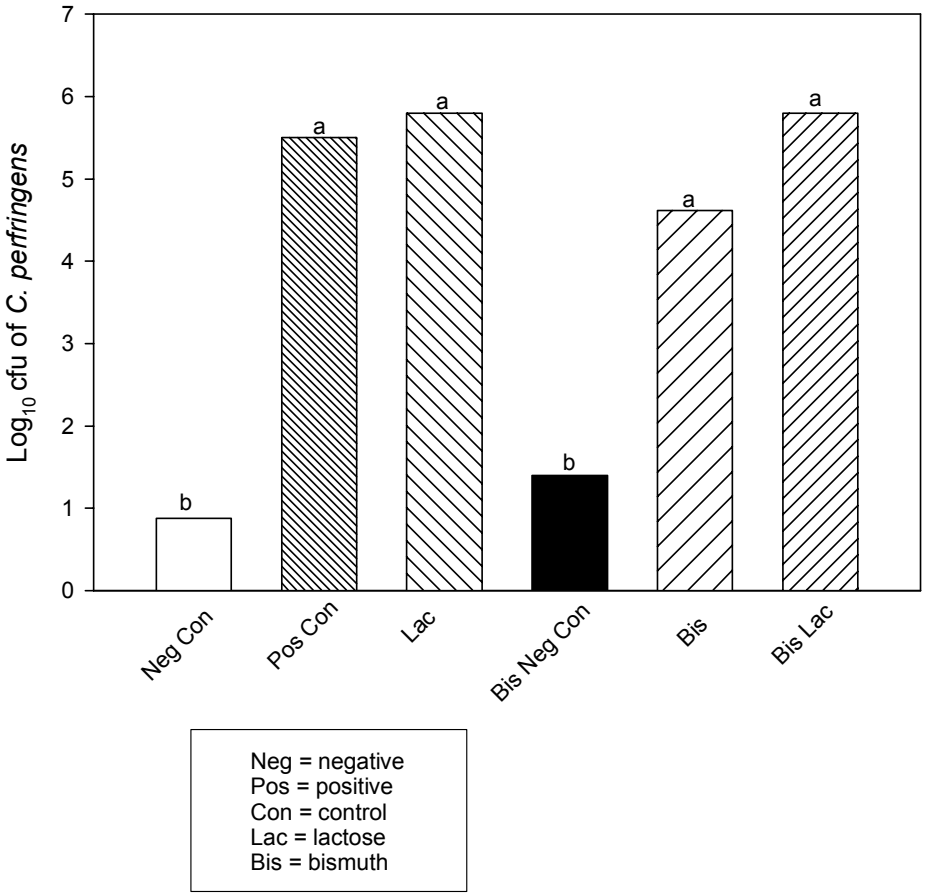

Figure 4.7. Evaluation of lactose and bismuth citrate on intestinal *Clostridium perfringens* colonization.

[a-b] Means with different superscripts differ significantly ($p \leq 0.05$)

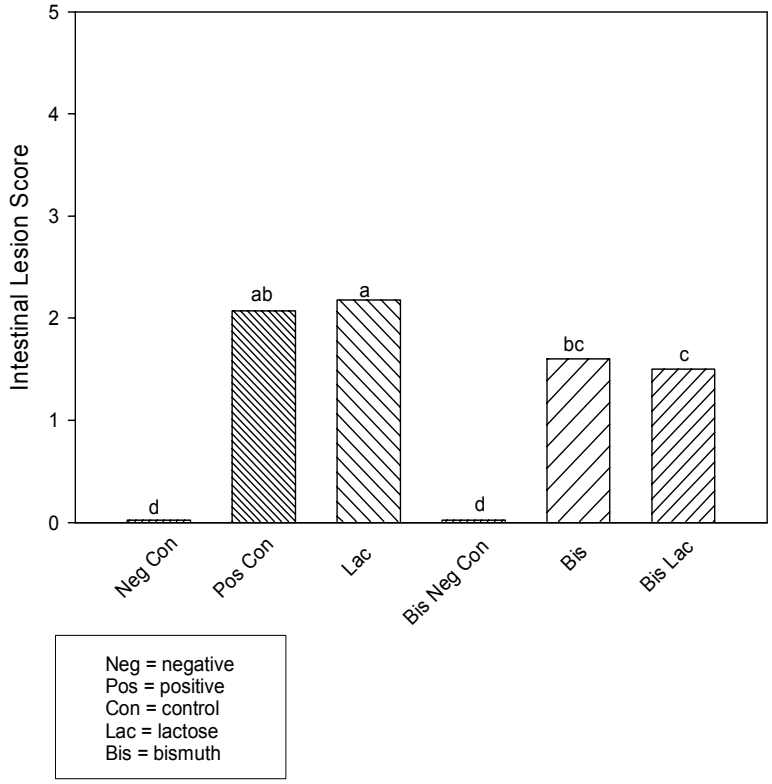

Figure 4.8. Evaluation of lactose and bismuth citrate on intestinal lesion development.

[a-d] Means with different superscripts differ significantly ($p \leq 0.05$)

Discussion

Necrotic enteritis negatively affects broiler production worldwide. The disease can be subclinical or fatal, leading to increased morbidity and mortality (Williams, 2005). In-feed antibiotic growth promoters (AGPs) have been an effective means of controlling NE (Williams, 2005). The risk of NE has increased due to the voluntary or involuntary withdrawal of certain AGPs in the European Union, due to the perceived risk of antibiotic resistance in humans (Van Immerseel et al., 2004). Following an involuntary ban in Scandinavia, broiler flocks began to experience increased health problems, with CP infections being the most significant (Kaldhusal and Lovland, 2000). In the U.S., the use of AGPs has been under consumer scrutiny due to the similarity of drugs used in humans to treat bacterial infections. Two well-known corporations in the U.S. (KFC and McDonalds) have both made statements saying that chicken meat grown with AGPs will not be accepted (Kentucky Fried Chicken, 2002; McDonald's Corporation, 2003). Some consumers may feel that antimicrobial drug use in poultry can cause drug resistance in humans. There are no regulatory guidelines in the U.S., however pressure from consumers has caused some producers to voluntarily remove AGPs from poultry feed.

Bismuth compounds have been used to treat gastric medical disorders since at least the 1700's in human (Marshall, 1991). Bismuth compounds are anti-microbial and improve gut health in man (Larsen et al., 2003). Specific brands of bismuth compounds include Tritec (Glaxo Wellcome, Research Triangle Park, NC), Pepto-Bismol (Proctor &

Gamble, Cincinnati, OH) and De-Nol (Tri-Med Distributors P/L, Subiaco, Western Australia). Data from experiment 1 suggest that the addition of 100 or 200 ppm bismuth citrate reduced ($p \leq 0.05$) CP colonization and intestinal lesion development. Similar results have been reported when bismuth citrate and colloidal bismuth subcitrate were fed to day-of-hatch chicks, challenged with *Campylobacter jejuni* (Farnell et al., 2006).

We hypothesized that the addition of lactose or citric acid would enhance the efficacy of bismuth citrate in broilers. A decrease in pH has been proposed to further enhance the anti-microbiological activity of bismuth compounds (Wagstaff et al., 1988). Colloidal bismuth subcitrate forms a precipitate (an active metabolite) at pH levels of less than 5 aiding in infiltration of gastrointestinal microvilli with the compound (Wagstaff et al., 1988). A study conducted by Tasman-Jones and colleagues (1987) found that as pH increases, adherence of CBS to the gut epithelium decreases. We found that birds fed citric acid had a reduced ($p \leq 0.05$) intestinal pH relative to the lactose or positive control. The normal pH range of the intestinal tract of chickens is 6.0 - 7.4. Citric acid reduced the pH from 5.67 (negative control) to 4.87.

An investigation in day of hatch broilers found that dietary lactose and probiotics decreased cecal pH and increased concentrations of bacteriostatic volatile fatty acids (Corrier et al., 1990a).

In experiment 3, we hypothesized that dietary lactose would enhance the efficacy of bismuth citrate. Dietary lactose fed to broilers has been shown to significantly reduce intestinal lesions associated with NE (McReynolds et al., 2007). Takeda and coworkers (1995) have also shown that birds fed dietary lactose had a significant reduction of cecal CP.

In conclusion, bismuth citrate treatments of 100 ppm and 200 ppm reduced CP colonization and intestinal lesion development. The combination of bismuth citrate and lactose offered protection against intestinal lesions associated with NE. Due to the reduction of AGPs in the commercial poultry industry, alternative feeding strategies need to be investigated to mitigate the incidence of NE. Bismuth citrate and lactose treatments may provide a cost-effective approach in controlling this disease.

CHAPTER V

CONCLUSION

Chapter III: In experiment 1 where the effect of long term storage and temperature (4, 20, 32 or 43°C) were evaluated on the efficacy of working concentrations of disinfectants, a reduction in disinfectant efficacy was not observed against SA. A reduction in efficacy was observed with the phenolic compound at the two highest temperatures. This reduction was indicative following a negative cfu data, but positive after samples were enriched in tetrathionate broth. When the effect of the inclusion of organic matter was similarly evaluated in experiment 2, a reduction ($p < 0.05$) in disinfectant efficacy was observed in a dose dependent manner against ST and SA, with the exception of the phenolic compound against SA.

In experiment 3 when comparing the effects of fresh and 30 wk old disinfectant solutions, we found that fresh disinfectants performed better in the presence of organic matter. This experiment was conducted to validate the assay in experiment 1. Following the 30 wk incubation in the first experiment we failed to find any significant affects on disinfectant efficacy in the absence of organic matter, with the exception of the phenolic compound.

These disinfectants were diluted and stored in a "clean" environment. Following the addition of organic matter, a simulated field environment was created and disinfectants were challenged. In support of these finding of the present manuscript, correct disinfectant use should be considered to reduce avian diseases on the farm.

In retrospect, we feel that we should have taken into consideration the stress exerted on the bacteria as a result of the selective agar. This stress may have been sub lethal, causing some cells to be alive but not culturable.

Chapter IV: We evaluated the effect of bismuth citrate, lactose or citric acid on necrotic enteritis in broilers. In experiment 1 (trial 1), the administration of 50, 100 or 200 ppm bismuth citrate reduced ($p \leq 0.05$) intestinal lesion development when compared to 0 ppm bismuth citrate. In trial 2, reductions ($p \leq 0.05$) in lesion scores was observed in birds administered 100 or 200 ppm bismuth citrate. When evaluating CP colonization, reductions in colonization was not observed in trial 1. In trial 2, a reduction ($p \leq 0.05$) was observed in birds fed 100 ppm or 200 ppm bismuth citrate when compared to birds fed 0 ppm bismuth citrate.

In experiment 2, we evaluated dietary lactose and citric acid on enhancing the efficacy of bismuth citrate in reducing intestinal pH and lesion development. We found no significant differences, following CP challenge, between birds fed bismuth citrate, relative to birds fed a combination of bismuth citrate with dietary lactose or citric acid in lesion development.

The intestinal pH of birds fed a combination of bismuth citrate with dietary lactose or citric acid was not significantly reduced when compared with birds fed bismuth citrate alone. A significant reduction in pH was observed in birds fed a combination of bismuth citrate and lactose relative to the negative controls.

When evaluating the effect of dietary lactose and bismuth citrate on necrotic enteritis intestinal lesion development and CP colonization in experiment 3, the addition of dietary lactose with 100 ppm bismuth citrate reduced ($p \leq 0.05$) intestinal lesion development relative to the positive control and lactose positive control. When evaluating CP colonization we did not observe any significant reductions on colonization.

Taken together, these data indicate that bismuth citrate alone or bismuth citrate with dietary lactose may be considered as an alternative feed additive in controlling necrotic enteritis. Future investigations will look at evaluating the feed conversion ratio and/or weight gains.

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