THE EFFECT OF 2-NITRO-1-PROPANOL SUPPLEMENTATION ON *SALMONELLA* COLONIZATION IN LAYING HENS AND BROILERS

by

OSMAN YASIR KOYUN

(Under the Direction of WOO KYUN KIM)

ABSTRACT

The presence of *Salmonella* in poultry continues to be a problem in the industry. We evaluated the effect of 2-nitro-1-propanol (NP) on the recovery of *Salmonella* from the internal organs of layers and broilers as well as on the ileum immune response to *Salmonella* infections. In the first experiment, laying hens were orally challenged with a nalidixic acid resistant *Salmonella* Enteritidis (SE^{NR}) and supplemented with different levels of NP. Although there were numerical decreases in the number and prevalence of SE^{NR} in the L/GB, spleen, ovary, ceca and fecal samples, the difference among the treatments was not statistically significant. NP supplementation at both levels downregulated ($P < 0.05$) the mRNA expressions of TLR-4 and IL-6 in the ileum of the hens. In the second experiment, broilers were challenged with a nalidixic acid resistant *Salmonella* Typhimurium (ST^{NAR}) and supplemented with different levels of NP. Bird performance was not significantly different among the treatments at any point during the 21-day trial. Supplementation with 200 ppm NP resulted in a decline ($P < 0.05$) in fecal shedding at 6 dpi. No significant difference in ST^{NAR} prevalence was detected in the L/GB and spleen samples, but 200 ppm NP supplementation showed a ($P < 0.05$) reduction in ST^{NAR} numbers in

ceca collected on day 11. Although the mRNA expressions of TLR-4, IL-1β and IL-10 were detected in all treatment groups at the end of the study, there was no statistically significant difference in the expression of these genes by 100 ppm of NP supplementation. However, the mRNA expression of IL-6 was upregulated ($P < 0.05$) by 200 NP of supplementation compared to positive control. Overall, NP showed a bactericidal effect against *Salmonella* by decreasing ($P < 0.05$) ST^{NAR} presence in the ceca and feces of broilers, and it downregulated ($P < 0.05$) the mRNA expressions of TLR-4, IL-6, INF-Ɣ and IL-10 playing vital roles in the ileum immune response to SE^{NR} infection in hens. However, the mechanism of actions and effective dose needs to be determined by further research in order to provide the industry with another method to inhibit *Salmonella* infections in poultry.

INDEX WORDS: *Salmonella* Enteritidis, *Salmonella* Typhimurium, 2-nitro-1-propanol

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DEDICATION

I would like to dedicate this thesis to my parents. It would not be a complete achievement without your continuous support and endless love.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Salmonellosis is a worldwide health concern and the majority of human salmonellosis has been linked to the consumption of contaminated products including meat and eggs from poultry. Although numerous studies have been conducted to improve our understanding of *Salmonella* ecology and pathogenicity, the microorganism continuously gives rise to new food safety challenges and remains one of the major food-borne pathogens in the world. The natural microflora of animal's intestinal tract can contain *Salmonella*; therefore, controlling *Salmonella* in food-producing animals is problematic and leads to the high rate of food contamination. Although vaccination and hygienic precautions can decrease the contamination rates, a major concern is contamination of chicken houses on commercial farms. Prebiotics, probiotics and organic acids are commonly used to reduce *Salmonella* infections in poultry, with the rate of success based on the additive used; however, alternative feed additives are still needed to create a sustainable reduction or even elimination of *Salmonella* in the industry. The additive dietary effects of nitrocompounds against food-borne pathogens such as *Salmonella, Campylobacter, Listeria* and *E. coli* have been tested in previous *in vivo* and *in vitro* studies. These compounds have shown a great potential due to its bactericidal effect against above-mentioned pathogens. We report a variety of effects of NP supplementation of diets against *Salmonella* colonization in laying hens and broiler chicks.

LITERATURE REVIEW

Salmonella **history and taxonomy**

Salmonella was named after Daniel E. Salmon when he first isolated "*Bacillus choleraesuis*" from porcine intestines in 1884. Lignieres changed this name to "*Salmonella choleraesuis*" in 1900 (John-Brooks, 1934; Salmon and Smith, 1885; Smith, 1894). *Salmonella*, a genus in the family of Enterobacteriaceae, are gram-negative, facultative anaerobe, oxidase negative, catalase positive, non-spore forming motile (peritrichous flagella) rods which cause human illness- except the two poultry pathogens *Salmonella enterica* serovar Gallinarium and *Salmonella enterica* serovar Pullorum (Andrews, 1993; D'Aoust and Purvis, 1998; Lopes et al., 2016; Schofield, 1945). Although the optimal growth temperature for *Salmonella* spp. is 37 °C, growth has been recorded at 54°C as well (Adley and Ryan, 2016). Hydrogen sulfide production, inability to hydrolyze urea, lysine and ornithine decarboxylation are some of the biochemical characteristics of *Salmonella* (D'Aoust and Maurer, 2007).

The *Salmonella* genus consists of two species: *S. enterica* and *S. bongori*, with *S. enterica* being divided into six subspecies (I, *S. enterica* subsp. e*nterica*; II, *S. enterica* subsp. salamae; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica)* (Brenner et al., 2000; Issenhuth-Jeanjean et al., 2014; Popoff et al., 1998). Salmonellae leading to human diseases are split into a limited number of human-restricted typhoidal serotypes (*S.* Typhi and *S.* Paratyphi A) and over 2600 non-typhoidal *Salmonella* (NTS) serotypes with a wide range of hosts, causing diarrheal diseases. Some NTS serotypes such as *S.* Gallinarium or *S.* Pullorum are host-specific as is seen in poultry (Feasey et al., 2012).

Serotyping is a procedure that differentiates strains of microorganisms into various groups according to their antigenic composition. Antigenic classification of *Salmonella* is based on antibody reaction with 3 types of surface antigens: somatic O antigens, flagellar H antigens and Vi capsular antigens. The O antigen designates the serogroup the *Salmonella* isolate belongs to and the H antigen specifies the serovar. The O antigen, a heat stable polysaccharide, is attached on the outer surface of the lipopolysaccharide. The Vi capsular antigen commonly presents in *S.* Typhi, but it was also identified in *S.* Dublin and *S.* Paratyphi C. H antigens are comprised of flagellin subunits and are filamentous part of the flagella (Agasan et al., 2002; D'Aoust and Maurer, 2007; Nataro et al., 2011).

Epidemiology of Non-Typhoidal *Salmonella* (NTS)

NTS infections continue to be a critical concern in the public health of industrialized and developing countries. *Salmonella* infections can cause severe gastrointestinal problems such as gastroenteritis, typhoid fever and paratyphoid fever, or result in death of children and elderly people (Cui et al., 2008; Li et al., 2014; Liang et al., 2015). Over 93 million cases of gastroenteritis because of *Salmonella* spp. result in approximately 155,000 deaths every year. As reported by Salm-Surv (a food-borne disease surveillance network supported by World Health Organization), *S.* Enteritidis was the most common serotype (65% of the isolates), followed by *S.* Typhimurium (12%) and *S.* Newport (4%) in the world from 2001 to 2005. *S.* Enteritidis and *S.* Typhimurium accounted for 26% and 25% of the isolates, respectively in Africa. In Asia, Europe and Latin America/Caribbean, *S.* Enteritidis was the most common isolate (38%, 87% and 31%, respectively). *S.* Typhimurium represented 29% of the isolates, followed by *S.* Enteritidis (21%) and other *Salmonella* spp. (21%) in North America (Galanis et al., 2006; Majowicz et al., 2010; Su et al., 2002; Westrell et al., 2009).

According to data reported by FoodNet from 1996 to 2005, NTS infections have been the major cause of death (39%) from foodborne bacterial pathogens and produced the highest mortality in adults over 65 years old and children under 5 years old (Barton Behravesh et al., 2011). CDC reported many cases of salmonellosis in the US between 2005 and 2010 due to *Salmonella* outbreaks related to contaminated raw ingredients, poor storage or inadequate cooking of foods (Lynch et al., 2006). Chickens, ducks, sheep, goats, pigs, reptiles, amphibians, birds, rodents, dogs, cats and various wild animals can be a reservoir for NTS (Dione et al., 2011; Jardine et al., 2011; Ombui et al., 2017; Siembieda et al., 2011; Swanson et al., 2007; Wacheck et al., 2010). One of the most serious multistate outbreaks occurred in January 2010, caused by *S.* Enteritidis-contaminated eggs and lasted one year with approximately 1939 reported illnesses (CDC, 2010). In 2017, CDC declared another multistate outbreak report related to *Salmonella* infections (caused by *S.* Hadar, *S.* Braenderup, *S.* I 4, [5],12: i-, *S.* Enteritidis, *S.* Infantis, *S.* Indiana, *S.* Litchfield, *S.* Mbandaka, *S.* Muenchen and *S.* Typhimurium) linked to live poultry in backyard flocks, resulting in 1120 cases, 249 hospitalizations and 1 death (CDC, 2017).

Pathogenicity

The main characteristics of the pathogenesis of salmonellae are host cell invasion and survival as an intracellular microorganism. As infection localization develops, salmonellae are usually restricted to the mesenteric lymph nodes. The gastric acidity and the bile salts of the small intestine, which have bactericidal properties, are the first defense mechanisms of the host against infection. *Salmonella* attach to the intestinal mucosa in the small intestine and move through the lymphoid follicles of the ileum. The presence of M cells and the absence of the mucus-secreting cells characterize this part of the intestine. Fimbriae (adhesins) favor the recognition and attaching of *Salmonella* to Peyer's plaques (Thorns and Woodward, 2000; Vimal et al., 2000).

The type III secretion systems mediate the entrance of *Salmonella* into the Peyer plaques. The pathogenesis of *Salmonella* spp. is characterized by virulence factors that are expressed by several genes clustered on the chromosome and classified as *Salmonella* pathogenicity islands (SPI) (Bonny et al., 2011). The primary virulence characteristics of *S. enterica* are provided by SPI1 and SPI2 encode type III secretion systems (Boko et al., 2013). SPI1 takes part in the invasion of host cells and inflammation of phagocytic or non-phagocytic cells of the intestinal mucosa as well as hosting the invasion *inv*A gene found in most *Salmonella* strains. SPI2 is required to encode the proteins which function in intracellular survival and replication in phagocytes. The *spi*C gene in SPI2 encodes for structural parts and secretion, contributes to the production of mediators with a significant role in the virulence of *Salmonella* and in the expression of flagella components. *Salmonella* has other virulence factors that are not present on the SPI and are found on other mobile genetic elements such as plasmids. The *Salmonella* virulence plasmid includes five genes (*spv*RABCD) that contribute to the systemic spread and facilitate the replication of the microorganism in extra-intestinal areas (Brenner et al., 2000; Brisabois, 2001).

Salmonella in poultry and poultry products

Salmonella has been known as a causative agent for human diseases such as food poisoning, typhoid, paratyphoid, septicemia, and sequelae. Although a considerable amount of research has been completed to enhance the understanding of *Salmonella* ecology and pathogenicity in humans, the microorganism continuously displays new food safety challenges and remains one of the major food-borne pathogens throughout the world. Ingested bacteria

proliferate in the small intestine, colonize and invade intestinal tissues, inducing an inflammatory response. After a 12 to 72-hour incubation period, infected individuals may have abdominal pains, vomiting, diarrhea, and fever. Healthy individuals recover from salmonellosis within 2 to 7 days; however, the disease can lead to more severe prognoses in young children, the elderly, pregnant women, and immunocompromised people (Hannah, 2007; Humphrey, 2004).

Food producing animals are a common reservoirs for many zoonotic pathogens, including *Salmonella* (Korsgaard et al., 2009; Santos et al., 2008), because of the ability of salmonellae to persist in the intestine or translocate to and invade other internal tissues (Humphrey, 2004). Invasive diseases pose a massive food safety threat because bacteria reach the interior of food products such as with the salmonellosis pandemic caused by *S*. Enteritidis contamination in table eggs (Humphrey, 2004). The egg related salmonellosis is mainly due to the consumption of raw or undercooked eggs (De Buck et al., 2004; Louis et al., 1988; Lynch et al., 2006; Palmer et al., 2000) that are contaminated with *S*. Enteritidis (De Reu et al., 2006; Gantois et al., 2008; Greig and Ravel, 2009; Guard-Petter, 1998). Egg contents may be contaminated with *S*. Enteritidis through either the trans-shell or -ovarian route, even though it is not clear which is most critical (Gantois et al., 2009; Humphrey, 1994). Infection of the reproductive tissues (ovary and oviduct) is widely considered as an underlying cause of contaminated eggs (De Buck et al., 2004; Keller et al., 1995; Miyamoto et al., 1997). Laying hens are generally infected with *S.* Enteritidis by oral ingestion from an environmental source which leads to colonization of the reproductive tract (Gast et al., 2007; Kinde et al., 1996). After intestinal colonization, the bacteria can invade the reproductive tissues through systemic infection or from the cloaca. Although *Salmonella* has been recovered from the ovary and oviduct of laying hens, the specific site and mechanism of bacterial colonization is not clearly known (De Buck et al., 2004; Gantois et al., 2009). *Salmonella* has been recovered from the reproductive tissues of experimentally infected hens in various studies (Gast et al., 2007; Howard et al., 2005; Miyamoto et al., 1997). The permeability of the capillary endothelia in the ovary has been linked to bacterial colonization (Bell and Freeman, 1971).

S. Enteritidis is a critical food safety problem for the table egg industry (Garber et al., 2003; Mollenhorst et al., 2005), since it is a major human pathogen contaminating eggs, and the relationship between egg contamination and laying hen infection has been well elucidated. However, controlling *S*. Enteritidis is an issue for producers as there are numerous factors

including environmental contamination, niches for bacterial proliferation, and horizontal transmission within a facility (Guard-Petter, 2001; Mollenhorst et al., 2005). Laying hens infected with *S*. Enteritidis typically show no clinical signs of illness; therefore, efforts by producers are not able to reveal whether the eggs are contaminated (Guard-Petter, 2001). The infection route of *S*. Enteritidis begins with environmental contamination of the housing facility. Flies (Holt et al., 2007; Olsen and Hammack, 2000), rodents (Garber et al., 2003; Meerburg and Kijlstra, 2007), humans (Guard-Petter, 2001), and wild birds (Craven et al., 2000) have been identified as potential sources for *Salmonella* transmission. After contaminating a housing facility, *S*. Enteritidis must adapt to and proliferate within the environment. It has been reported that the survival of *Salmonella* through the food chain is likely due to its ability to, with the aid of a complex regulatory system, respond efficiently to environmental changes (Humphrey, 2004). Upon these stages, laying hens ingest the bacteria and become infected with *S*. Enteritidis. Following colonization, *S*. Enteritidis can be shed through the feces and contaminate an entire flock. Risk factors linked to horizontal transmission of *S*. Enteritidis infection are housing system, flock size, and airborne transmission (Mollenhorst et al., 2005). The National Animal Health Monitoring System conducted the Layer '99 study to estimate the prevalence of *S*. Enteritidis in commercial laying houses in the U.S., and 7% of the surveyed houses were positive for *S*. Enteritidis (Garber et al., 2003).

The prevalence of *S*. Enteritidis in eggs produced by naturally infected hens is relatively low. Studies reported *S*. Enteritidis prevalence in egg content as 0.55 and less than 0.06%, respectively (Humphrey et al., 1991; Poppe et al., 1992). In U.S. laying hens and eggs, it was estimated that one in every 20,000 (0.005%) eggs produced annually would be contaminated with *S*. Enteritidis. According to previous estimation, of the 77.7 billion table eggs produced in the U.S. in 2009, approximately 3.88 million eggs would have been contaminated with *S*. Enteritidis (Ebel and Schlosser, 2000). There were 997 outbreaks of *S*. Enteritidis reported in the U.S. from 1985-2003, which resulted in 33,687 illnesses, 3,281 hospitalizations, and 82 deaths. Of the 439 (44%) cases, 329 (75%) were egg based or contained egg ingredients (Braden, 2006). Since 2012, reduction in *S*. Enteritidis infection has been one of five high-priority objectives for the U.S. Department of Health and Human Services (Crim et al., 2014).

S. Enteritidis is not the only serovar known to colonize the reproductive tissues of laying hens and contaminate the internal content of eggs. *S*. Typhimurium, *S*. Hadar, *S*. Gallinarium,

and *S*. Pullorum have also been recovered from the reproductive tissues and eggs of infected hens (Keller et al., 1997; Okamura et al., 2001a; Okamura et al., 2001b; Snoeyenbos et al., 1969). When laying hens were intravenously inoculated with *S*. Enteritidis, *S*. Typhimurium, *S*. Infantis, *S*. Hadar, *S*. Heidelberg, and *S*. Montevideo, *S*. Enteritidis was recovered from the ovary and preovulatory follicles (7 dpi) at significantly greater ($P < 0.05$) rates than *S*. Typhimurium and *S*. Hadar (Okamura et al., 2001a).

Salmonella Typhimurium (*S*. Typhimurium) is one of the main causes of self-limiting diarrhea. *S*. Typhimurium has a broad-host range and can cause disease in humans, cattle, pigs, horses, sheep, poultry, and rodents (Salyers and Whitt, 2002). Additionally, *S*. Typhimurium is found in water, soil, insects, food plants, animal feces, and raw foods (Food and Administration, 2004). The natural microflora of an animal's intestinal tract can contain *S*. Typhimurium; therefore, controlling *Salmonella* in food-producing animals is a huge concern due to the high rate of contamination. *S.* Typhimurium was the most common serotype associated with foodborne disease outbreaks in the United States before 1970 (Olsen et al., 2001; Tauxe, 1991). Nowadays, besides *S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg, *S*. Newport, and *S*. Hadar are common *Salmonella* serotypes recovered from commercially produced chickens, turkeys, quail and ducks. The Centers for Disease Control (CDC) has reported that the aforementioned *Salmonella* serotypes are responsible for human salmonellosis in the United States (Olsen et al., 2000). Since chicken is commonly purchased as cut-up parts, USDA-FSIS conducted a survey of raw chicken parts in 2012 and reported an estimated 24% prevalence of *Salmonella*. In 2013, USDA-FSIS announced its *Salmonella Action Plan* indicating that USDA-FSIS will conduct a risk assessment and improve performance standards for poultry parts during 2014, among other key activities (Crim et al., 2014). To reduce the transmission of *Salmonella* to humans through the food chain, it is crucial to determine potential sources of contamination within the poultry production system.

The presence of *Salmonella* at the farm level has been investigated in varied studies to determine prevalence in breeder houses, hatcheries, and broiler farms (Bailey et al., 2001; Byrd et al., 1999; Liljebjelke et al., 2005). In a study that focused on *Salmonella* prevalence in Arkansas, Alabama, Georgia, and North Carolina, it was reported that 88% of the 49 breeder farms sampled were *Salmonella* positive (Berghaus et al., 2012). In another study conducted from July 1995 to May 1996 in Texas, researchers found that *S.* Heidelberg and *S.* Kentucky

accounted for 50% of the *Salmonella* isolates (n=30) from 5 hatcheries and 59.6% of the *Salmonella* isolates (n=94) from 13 broiler houses (Byrd et al., 1999).

Immune response against *Salmonella* infections in chickens

Bacterial infections of chicken continue to be a concern of the poultry industry due to their impact on both public and animal health. There are various bacterial pathogens in the poultry industry and our understanding of the immune response to these pathogens is insufficient. However, the foodborne pathogens, particularly *S. enterica*, have been studied in great detail (Wigley, 2013). The history, taxonomy, pathogenicity, prevalence and global burden of *Salmonella* infections were mentioned in previous sections; therefore, this section will focus on how the avian immune system develops a response to *Salmonella* infections.

The early response of innate immunity and the subsequent adaptive immune response mediates the defense against microbial infections in mammals and birds. Innate immunity, also called native immunity, is the initial step in the defense against microbes and is composed of cellular and biochemical mechanisms occurring even before infections (Abbas et al., 2015). The major elements of innate immunity are physical or chemical barriers, such as epithelial surfaces; phagocytic cells (neutrophils, macrophages) and natural killer (NK) cells; blood proteins, including parts of the complement system and other mediators of inflammation; and proteins called cytokines that mediate most of the activities in the cells of innate immunity (Liu, 2012).

Salmonella commonly infects chickens via the fecal–oral route with spread starting from the intestinal tract at the distal ileum and ceca of the bird (Barrow et al., 2012). Invasion is an inflammatory process stimulating the expression of proinflammatory cytokines and the chemokines CXCLi1 and CXCLi2, regarded as the equivalent of mammalian IL-8 (Matulova et al., 2013; Setta et al., 2012; Withanage et al., 2004; Withanage et al., 2005). This induces an influx of heterophils and monocytic phagocytes to the gut causing inflammation and deterioration such as fusion and flattening of the villi. Although there is an enteropathogenic response, diarrhea rarely develops. While the bacterium itself leads to cellular changes and inflammation by its SPI1 Type III secretion system, the main event in the process seems to be the recognition of flagellin via toll-like receptor 5 (TLR 5). This is elucidated by the fact that the non-flagellate avian-adapted serovars produce less inflammation during epithelial invasion *in vitro* or *in vivo*, and that mutations in the flagellin gene of *S.* Typhimurium results in a more immediate invasion with lower initial levels of inflammatory signal. (Chappell et al., 2009; Iqbal

et al., 2005; Kaiser et al., 2000). The result of stimulation of innate immunity is mainly an influx of heterophils, the avian polymorphonuclear cell, and macrophages to the intestine. These not only can cause inflammatory damage, but also greatly limit the invasive disease. Our comprehension of the biology and role of heterophils is essentially from *Salmonella* infection studies. Depletion of heterophils affects *S.* Enteritidis by changing the type of the infection, from a gastrointestinal to systemic which emphasizes the key role of heterophils in early immunity (Kogut et al., 1994).

Polymorphonuclear leukocytes (PMNs) are key cellular elements of innate immunity and their function is killing pathogenic microbes by phagocytosis. Heterophil, the avian equivalent to the mammalian neutrophils, are the main PMNs in poultry and plays a role in the phagocytosis of invasive microbes and unknown particles or molecules (Kogut et al., 2003). Heterophils have an array of TLRs (Kogut et al., 2012) and are effective phagocytes which develops extracellular traps to facilitate this process (Chuammitri et al., 2009). Eleven TLRs have been identified in humans and mice and each of them recognize and respond to different microbial elements. Birds have 10 known TLRs; 5 of them are orthologous to both birds and mammals. Chicken TLRs are linked to varied functions: TLR-2, TLR-5 and TLR-21 recognizes peptidoglycan, flagellin and unmethylated cytidine phosphate guanosine (CpG) DNA, respectively. In addition, TLR-4 binds lipopolysaccharides (LPS) and has been attributed to resistance to *S.* Typhimurium infections (Keestra et al., 2010; Leveque et al., 2003; Temperley et al., 2008). In addition, avian heterophils depend more on antimicrobial peptides for bacterial killing unlike mammalian neutrophils (Kannan et al., 2009), and even though they generate nitric oxide and oxidative responses to *Salmonella*, they do not have the myeloperoxidase pathway (Maxwell and Robertson, 1998). Research on the interaction of *Salmonella* with primary cultures of heterophils along with primary and continuous macrophage lines has been crucial in our comprehension of pattern recognition receptors in the chicken, including TLR5 as explained above. This can be clearly observed in TLR4 where variation in macrophage responses to *S.* Typhimurium challenge has identified both changes in levels of TLR4 expression and polymorphism in the receptor sequences between chicken lines. This can indicate that responsiveness to LPS in chicken, which is generally lower than in mammals, is managed by variation in both levels of expression of the receptor and its structure (He et al., 2006; Higgs et al., 2006). There is no mammalian equivalent to chicken TLR21, though it functions similar to mammalian TLR9 in recognition of

unmethylated (or CpG) sequences. Although the identification of the role of TLR21 was discovered in the studies related to the response to *Campylobacter jejuni* infections (Keestra et al., 2010), our understanding of the response to CpG motifs became clear with the developments of these sequences as immunostimulatory molecules to help control *Salmonella* (He et al., 2007; Xie et al., 2003).

Monocytes/macrophages are the sensors and scavengers of microbial invasion, have roles in cytokine release and T-cell activation, and trigger nitric oxide (NO) production which is crucial for bactericidal activity; therefore, they are essential mediators in both innate and acquired immunity (Bogdan et al., 2000; O'Mahony et al., 2008; Yamate et al., 2000). Avian macrophages differ slightly from mammalian macrophages in terms of structure and/or function by displaying a series of TLRs; expression of MHC Class II; and phagocytic action; and antimicrobial activity. It has not been elucidated whether avian macrophages are M1 or M2 phenotypic. The interaction between macrophages, dendritic cells and *Salmonella* is a crucial step in the progression of systemic infection (Chappell et al., 2009). The use of chicken models has concluded that the genetic locus *SAL1* displayed a phenotype of resistance to systemic salmonellosis (Mariani et al., 2001). Macrophages obtained from these birds showed enhanced oxidative killing and immediate expression of key inflammatory and TH1-associated cytokines (Wigley et al., 2006; Wigley et al., 2002).

Cytokines are soluble, intermediary and low-molecular-weight proteins that are secreted by many cells, particularly by T cells, of the innate and adaptive immune systems. Cytokines can facilitate homeostasis by acting as chemical mediators within the immune system as well as communicating with cells in other systems (Coico and Sunshine, 2015). Various names are used to describe cytokines such as lymphokine (produced by lymphocytes), monokine (produced by monocytes), chemokine (cytokines with chemotactic functions), and interleukin (produced by one leukocyte and interacting with other leukocytes) (Zhang and An, 2007).

Studies of cytokines and chemokines expression *in vitro* have shown that paratyphoid species give rise to significant mRNA expression levels of proinflammatory IL-6, inducible nitric oxide synthase (iNOS) and chemokines (Setta, 2011). In a study, it was proposed that host gene expression and differences between chicken lines, with respect to the host responses to the *Salmonella* infection, are host dependent (van Hemert et al., 2006).

Low quantities of enteric bacteria have been detected inside macrophages when the chicken cecal immune response was studied. These results suggest that paratyphoid *Salmonella* serovars have the capability to invade the cecal mucosa, influencing the level and character of the immune response. The expression of IL-12, IL-18, TNF-α (tumor necrosis factor alfa), and iNOS in the cecum was associated with the invasiveness of the serovars in the lamina propria. In contrast, IL-2 mRNA expression, differences in the numbers of TCR2 (T-cell receptor 2) and CD4+ cells seem to be more dependent on the infection in the intestinal epithelial cells (Berndt et al., 2007). Researchers developed the idea that chickens respond to natural colonization of cecum by an elevated expression of IL-8 and IL-17 in the first week of life. It was also determined in this study that chickens infected with *S.* Enteritidis before, during and after the IL-8 and IL-17 induction, responded by Th1 (T helper cell subset 1) stimulating IL-8 and IL-17, while birds infected after this point responded through the Th17 (T helper cells subset 17) branch of the immune response. These results suggest that the gut microbiota and expression of some cytokines advance the resistance to *S.* Enteritidis infection (Crhanova et al., 2011).

The effects of Salmonella infection on gene expression in poultry

The biology of avian *Salmonella* infection is very diverse even though common broad-host range *Salmonella* serovars colonize the lower gastrointestinal tract of chickens. Some serovars, especially *S.* Typhimurium and *S.* Enteritidis, may manifest a weak systemic infection mitigated by cellular immunity within two to three weeks (Barrow et al., 2004; Beal et al., 2004). Colonization by these serovars usually triggers an inflammatory response in the ileum and ceca (Setta et al., 2012; Withanage et al., 2005). Studies concluded that salmonellosis promotes a strong Th1 response which is, along with the clearance of *Salmonella*, dependent on age and cellular development of chickens. *Salmonella* infection leads to an increase of γδ lymphocytes and upregulation of IFN-γ, IL-12, and IL-18, inducing activation of the TH1 response (Berndt and Methner, 2001; Berndt et al., 2006).

Studies have shown that *Salmonella* infections in birds can result in the expression of proinflammatory cytokines and the chemokines CXCLi1 and CXCLi2, which are regarded as the equivalent of mammalian IL-8 (Matulova et al., 2013; Setta et al., 2012; Withanage et al., 2004; Withanage et al., 2005). This leads to an increased number of heterophils and monocytic phagocytes in the gut producing inflammation and damage to the villi. The genetic locus SAL1, identified in chickens (Mariani et al., 2001), displayed phenotypic resistance to systemic

Salmonella infection because the derived macrophages generate immediate expression of inflammatory and TH1-associated cytokines (Wigley et al., 2006; Wigley et al., 2002). Induction of pro-inflammatory cytokines upon *Salmonella* infection has been well elucidated (Hu et al., 2015). Early studies reported that infection with *Salmonella* could substantially elevate the expression of inflammatory cytokines and other innate immune genes in the chicken cecal tonsils (Haghighi et al., 2008), spleen (Zhou and Lamont, 2007), macrophages (Zhang et al., 2008) and heterophils (Chiang et al., 2008).

Researchers reported that *S.* Typhimurium infection in chickens up-regulated some cytokines and chemokines in macrophages, the cecal tonsils, ileum, spleen and liver (Beal et al., 2004; Withanage et al., 2004). IL-6, a well-known pro-inflammatory cytokine, was up-regulated in the ileum and spleen on days one and three dpi with *S.* Typhimurium. However, IL-6 was up-regulated in cecal tonsils by 21 dpi. This late expression during the infection may demonstrate that this cytokine does not have a role in producing an inflammatory response to *S.* Typhimurium (Withanage et al., 2005). The up-regulation of IL-10 cytokine has been detected in the tissues of *Salmonella* infected birds (Cheeseman et al., 2007). IL-12 gene expression in cecal tonsils of chickens was significantly elevated on days one and five post infection with *S.* Typhimurium (Haghighi et al., 2008).

Based on these studies, it has been shown that there is a correlation between cytokine expression profiles in the bird and resistance to *S.* Typhimurium (Haghighi et al., 2008). The authors concluded that there is a relationship between Th1 cytokines, IFN-γ down-regulation and IL-12 up-regulation and inhibition of *S.* Typhimurium colonization in the intestinal lumen. Among these cytokines, IL-12, IFN-γ and IL-18 had a role in *Salmonella* inhibition, while IL-4 and IL-10 are crucial to lessen subsequent inflammatory responses (Beal et al., 2004; Eckmann and Kagnoff, 2001; Withanage et al., 2005).

Use of Nitrocompounds as an intervention strategy

Nitrocompounds, with a structural formula described as R-NO_2, can be described as the derivative of hydrocarbons, alcohols, and/or fatty acids, in which there is separation for one or more hydrogen atoms by a nitro (-NO_2) group. Nitrocompounds have been divided into aliphatic and aromatic nitrocompounds based on the presence of different alkyls; they have also been categorized into unitary, binary, and multiple nitrocompounds according to their number of nitro groups (Zhang et al., 2017).

2-nitro-1-propanol (NP) is an alternative electron acceptor utilized by *Denitrobacterium detoxificans* (Anderson et al., 2000) and has been safely administered intraruminally to cattle (Majak and Clark, 1980) and chicks (Jung et al., 2004b). The toxicity of NP is relatively unknown. In monogastric animals such as rats, the LD_{50} of the regiochemical isomer 3-nitro-1-propanol is 77 mg/kg body weight (BW) when administered orally (Majak et al., 1983). Secondary nitroalkanes such as 2-nitropropane and 2-nitrobutane have been shown to cause damage to rat liver DNA and RNA and to be mutagenic in their ionized form when tested by the Ames *Salmonella* assay; however, primary nitroalkanes and nitrocarbinols such as NP were not reported to be carcinogenic or mutagenic (Conaway et al., 1991a; Conaway et al., 1991b). Furthermore, toxic effects were not observed in rats following a 2-year chronic inhalation exposure to 100- or 200-ppm nitroethane (Griffin et al., 1988). The position of the nitro group greatly affects the toxicity of the nitrocompounds since the oral LD_{50} of NP to 1- week-old chicks were shown to be more than 1,300 mg/ kg BW. Although adverse biological effects were not seen in the chick when administered orally, no lethal dose of NP for poultry has not been determined (Jung et al., 2004b). In addition, these compounds are not registered as feed additives or in the list of GRAS (Generally Recognized as Safe) due to certain characteristics such as disturbing odor and skin irritation.

Nitrocompounds such as nitroethane, 2-nitroethanol, 2 nitro-1-propanol **(NP)**, 3-nitrooxypropanol, 3- nitro-1-propanol, and 3-nitro-1-propionic acid have been reported to show an inhibitory effect on methanogenesis in ruminants by as much as 90% *in vitro* (Anderson et al., 2003; Anderson et al., 2010; Anderson et al., 2008; Božic et al., 2009; Saengkerdsub et al., 2006; Zhang and Yang, 2011a; Zhang and Yang, 2011b) and more than 69% *in vivo* (Anderson et al., 2006; Brown et al., 2011). The effect of the addition of nitroethane and nitroethanol at 21.8 mM in swine fecal slurries decreased the production of skatole, an odor pollutant in livestock waste, after 24 h (Beier et al., 2009). NP and 2-nitroethanol exhibited bactericidal activity against *S.* Typhimurium and *E. coli* during 24 h of incubation in porcine fecal suspensions (Anderson et al., 2007).

It has been revealed that selected short chain nitrocompounds exhibited antimicrobial activity against populations of *Salmonella*, *E. coli* and total coliforms in layer hen manure (Ruiz-Barrera et al., 2017). A similar approach used in another study indicated that supplementation with nitroethanol or NP in broiler diets (up to 33.3 mg/kg) influenced the uric acid degradation

and ammonia production in broiler manure while maintaining optimal growth performance (Mowrer et al., 2016). Nitrocompounds exhibit potential to reduce ammonia volatilization in poultry manure by inhibiting the growth of uric acid–utilizing microorganisms (Kim et al., 2006).

Short-chain nitrocompounds such as NP, 2-nitroethanol, nitroethane, and 2-nitro-methyl propionate showed an inhibitory activity against *Campylobacter jejuni* and *C. coli* in vitro (Horrocks et al., 2007). 2-nitroethanol, nitroethane, and, in particular, NP exhibited inhibitory activity against *L. monocytogenes in vitro* (Dimitrijevic et al., 2006). Select food-borne pathogens such as *S.* Typhimurium, *E. coli* O157:H7 and *Enterococcus faecalis* were inhibited by NP in vitro (Jung et al., 2004a) and *S.* Typhimurium in the ceca of broiler chicks was reduced by NP administration as well (Jung et al., 2004b). Dietary supplementation with nitrocompounds (nitroethane and NP) reduced *Salmonella* colonization in internal organs of laying hens (Adhikari et al., 2017). In this study, addition of 200 ppm of NP to the diet resulted in a reduction of the cecal colonization by *S.* Enteritidis.

In the light of these studies, nitrocompounds appear to be valuable for use either as feed additives against *Salmonella*, *Campylobacter*, *Listeria* and *E. coli* or as strategies to reduce ammonia volatilization and methanogenesis in poultry litter. Their mechanism of inhibition, toxicity and, possible adverse effects on organisms needs to be elucidated by further *in vivo* and *in vitro* studies in order to determine if these compounds can safely be used in the poultry industry.

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CHAPTER 2

THE EFFECT OF 2-NITRO-1-PROPANOL SUPPLEMENTATION ON *SALMONELLA* COLONIZATION IN LAYING HENS [1]

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ABSTRACT

The presence of *Salmonella* in laying hens continues to be a problem in the industry. A study was conducted to evaluate the effect of 2-nitro-1-propanol (NP) on recovery of *Salmonella* from internal organs of laying hens. Thirty-four White Leghorns were orally challenged with a nalidixic acid resistant *Salmonella* Enteritidis (SE^{NR}). Hens were housed individually in wire cages and randomly allocated to one of seven dietary treatments: T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP, T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP, and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP. Fecal samples were collected at 3 and 6 days post inoculation **(dpi)** and assayed for recovery of SE^{NR}. Fecal shedding in T3 was not different from T2 and, T4 had the least positivity among the low inoculum treatments at 3 and 6 dpi. Also, T6 and T7 were not different from T5 on either day, respectively. Ceca, liver with gall bladder **(L/GB)**, spleen and ovary samples were collected for recovery of SE^{NR} at 7 dpi. T3 and T4 reduced cecal SE^{NR} count numerically ($P > 0.05$) compared to T2. SE^{NR} numbers in ceca were same (4.5 log10 cfu) in both T6 and T7 and greater than T5. There was no significant difference in SE^{NR} prevalence in the L/GB, spleen and ovary samples. In L/GB, 40% of the samples (n=5) from T2 and T7, and 20% of the samples from T6 were SE^{NR} positive. When the spleens were sampled, 60%, 20% and 40% of the samples from T2, T3 and T4 were SE^{NR} positive, respectively. All the spleen samples collected from T6 were SE^{NR} positive, while 40% of the samples were SE^{NR} positive in both T5 and T7. As for the ovaries, no SE^{NR} was detected in the samples collected from T4 and T6, and 20% of the samples were SE^{NR} positive in T2, T3, T5 and T7. Pro- and anti-inflammatory cytokines playing roles in the immune response to *Salmonella* infection such as interferon (IFN)-Ɣ, interleukin (IL)-6, IL-10 and toll like receptors (TLR)-4 mRNA expressions were analyzed in the ileum of hens collected at 7 dpi. After SE^{NR} challenge, the expression of INF-Ɣ in T2 and T5 were not different from T1. However, the mRNA expressions of both IL-6 and IL-10 in T2 and T5 were downregulated ($P < 0.05$), and the TLR-4 mRNA expression was lower ($P < 0.05$) only in T5 compared to T1, respectively. NP supplementation at both levels downregulated ($P < 0.05$) the mRNA expressions of TLR-4 and IL-6 in both T3 and T4, but IL-10 mRNA expression was

downregulated in only T3 when compared to T2. The mRNA expression of INF-Ɣ was downregulated ($P < 0.05$) in T6 compared to T5. On the other hand, TLR-4 and IL-10 mRNA expressions were upregulated ($P < 0.05$) in T7 compared to T5. Overall, inclusion of NP into laying hen diets was effective as an intervention strategy by reducing the cecal count of the bacteria and altering the prevalence of SE^{NR} in internal organs while stimulating an immune response in the ileum by modulating the expressions of pro- and anti-inflammatory cytokines. However, the mechanism of effect needs to be elucidated by further research.

Key words: laying hens, 2-nitro-1-propanol, *Salmonella* Enteritidis

INTRODUCTION

Foodborne disease continues to be a health concern in the United States. The Foodborne Diseases Active Surveillance Network (Food Net) reported that 19,056 infections, 4,200 hospitalizations, and 80 deaths were caused by foodborne pathogen infections from 2006 to 2013. *Salmonella* Enteritidis was the top serotype isolated among these pathogens (Crim et al., 2014). Chickens are the most abundantly produced food animal in the world today, and *S.* Enteritidis is one of the most common serotypes found in commercially produced chickens, turkeys, quail and ducks in the United States (Lukefahr, 1999; Olsen et al., 2000). Poultry can become infected by *Salmonella* through varied sources such as contaminated feed and water (Corry et al., 2002; Heyndrickx et al., 2002; Rose et al., 1999). Although vaccination and hygienic precautions can decrease the contamination rates in flocks resulting in a reduction in vertical transmission, a major concern is contaminated chicken houses. Routine cleaning and disinfection can reduce the risk, but not eradicate *Salmonella* from the environment completely (Van Immerseel et al., 2004). Thus, fecal shedding of *Salmonella* from laying hens should be investigated to reduce the horizontal contamination.

Feed additives such as prebiotics, probiotics and organic acids are commonly used to control *Salmonella* infections in poultry, with the rate of success based on the additive used (Adhikari et al., 2017b; Corrier et al., 1993; Van Immerseel et al., 2005). Inconsistent results from anti-*Salmonella* strategies have been reported in studies over the years. Experiments focusing on the efficacy of these strategies often include *in vivo* bacterial challenges designed in different ways. There are varied parameters affecting the outcome of the challenge such as the breed and age of the birds, housing facilities, route or dose of inoculation and challenge serovar (Marcq et al., 2011). To investigate how anti-*Salmonella* strategies can provide pathogen control in the industry, reliable oral challenge models are needed. A model of *Salmonella*-challenged mature laying hens can be useful to study the efficiency of promising feed additives such as nitrocompounds that can find a place in the poultry industry.

The additive dietary effects of nitrocompounds such as 2-nitro-1-propanol **(NP)** and 2-nitroethanol have been studied to reduce *Salmonella* colonization in the internal organs of laying hens ($P < 0.05$) (Adhikari et al., 2017b). Potential effects of varied nitrocompounds (nitroethane, 2-nitroethanol, NP and nitropropionic acid) were evaluated on ammonia volatilization in poultry manure (Kim et al., 2006). According to the results from this study, nitrocompounds exhibited

reduction in ammonia volatilization in poultry manure by inhibiting growth of uric acid–utilizing microorganisms. A similar study was conducted to reveal the effects of dietary nitrocompounds on bird performance, ammonia volatilization, and changes in manure nitrogen. It demonstrated that supplementation of 2-nitroethanol or NP into broiler diets up to 33.3 mg/ kg affects uric acid degradation and ammonia production in broiler manure while sustaining optimal growth performance (Mowrer et al., 2016).

The effects of pH on the bactericidal activity of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-nitro-proprionate were studied against *C. jejuni* and *C. coli in vitro* (Horrocks et al., 2007). Results from this study suggested that growth inhibition of *C. jejuni* and *C. coli* by the nitrocompounds was pH and concentration dependent. *In vitro* effects of incubating an experimental chlorate product, nitrate, or select short-chain nitrocompounds, alone or in combination, against experimentally inoculated *Salmonella enterica* serovar Typhimurium and indigenous *E. coli* in porcine fecal suspensions were tested (Anderson et al., 2007). In this experiment, NP and 2-nitroethanol showed bactericidal activity against *S.* Typhimurium and *E. coli* during 24 h of incubation. Inhibitory effect of select nitrocompounds (2-nitroethanol, nitroethane and NP) on growth and survivability of *L. monocytogenes in vitro* was confirmed (Dimitrijevic et al., 2006). Inhibitory activity of NP against *S.* Typhimurium, *E. coli* O157:H7 and *Enterococcus faecalis* has been reported (Jung et al., 2004a). Same author reported in a similar study that NP reduced *Salmonella* in the ceca of broiler chicks (Jung et al., 2004b).

The biology of avian *Salmonella* infection is very diverse even though common broad-host range *Salmonella* serovars colonize the lower gastrointestinal tract of chickens. Some serovars, especially *S.* Typhimurium and *S.* Enteritidis, may manifest a weak systemic infection mitigated by cellular immunity within two to three weeks (Barrow et al., 2004; Beal et al., 2004). The intestinal epithelium is a physiological and immunological barrier against enteric pathogens. The innate immune system is regarded as scavenger system which is in charge of fighting against the invading pathogens. Stimulation of the innate immune system is described by the production of inflammatory cytokines; however, it is now clear that innate effector cells mediate a specific immune response, directing the advanced adaptive immune response (Kaiser, 2010). This complicated interaction between the innate and adaptive immune responses is crucial for the clearance of *Salmonella* infection. Previous studies have concluded that cellular immune responses are more vital for tissue clearance of *Salmonella* infection in poultry (Beal et al.,

2006). Cytokines are immunoregulatory proteins that have a vital role in both innate and adaptive immune responses. Cytokine production can be regulated by commensal bacteria in the gastrointestinal tracts (Corthay, 2006). In chickens, pro-inflammatory, Th1, and Th2 cytokines have also been reported to contribute to the immune response after *Salmonella* infection in chickens (Withanage et al., 2004; Withanage et al., 2005). In the light of these studies, we hypothesized that NP can reduce *S.* Enteritidis in the ceca and in other internal organs and decrease the prevalence of the microorganism in feces as well as leading to an immune response in the ileum of laying hens. The objectives of the study were first to test a SE challenge model in mature laying hens, second to evaluate the inhibitory effect of NP on SE and third to promote an immune response to SE infection in the ileum.

MATERIAL AND METHODS

Salmonella strain and inoculum preparation

Nalidixic acid resistant *Salmonella* Enteritidis (SE^{NR}) was used to challenge the organism. SE^{NR} was stored at -80°C in Nutrient Broth (Acumedia, East Lansing, MI; NB) with 16% glycerol. SE^{NR} was grown and maintained on brilliant green with sulphapyridine agar plates (Acumedia, East Lansing, MI; BGS) containing 200 ppm of nalidixic acid (NAL-Sigma Chemical Co., St. Louis, MO; BGS Nal) for 24 h at 37°C. Individual colonies were suspended into a sterile 0.85% saline solution. The absorbance was adjusted to 0.20 ± 0.01 OD540nm using a spectrophotometer (Spect 20, Milton-Roy, Thermo Spectronics, Madison, WI). Culture solution was serially diluted and plated onto BGS-NAL plates for enumeration. Hens were orally challenged with a 1.0 mL of approximately 1.1×10^6 and 1.8×10^8 cfu SE^{NR} based on their treatment groups.

Hens, housing and dietary treatments

Thirty-four Single-Comb White Leghorns hens (44-week old at the beginning of the experiment) were used for the study. Hens were housed individually in wire cages under a 16h light: 8h dark lightening program. All hens were fed a corn-soybean standard layer ration for one week and then randomly allocated to one of seven dietary treatments: T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP, T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP, and T7 =

SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP (Table 2.1). The diet was formulated to provide 2, 600 kg/kcal metabolizable energy (**ME**), 16% crude protein (**CP**), 4.4% Ca and 0.5% available P (NRC, 1994). After one week adaptation period, each hen except the ones in T1 was orally challenged with 1.0 mL of 10^6 or 10^8 cfu SE^{NR} based on treatments. Feed was withdrawn from all hens 10 h before challenge and provided right after SE^{NR} challenge. Hens were divided into five replicates per treatment diet except T1 (n=4). Hens were provided water (automatic nipple-type drinkers) and mash feed *ad libitum* throughout the experiment period. The experiment protocol was approved by the Institutional Animal Care and Use Committee of University of Georgia.

Sampling protocol and analyses

Feces, ceca, L/GB and ovary (bacteriological)

Fresh fecal samples were collected from each cage at 3 and 6 dpi to be screened for fecal shedding. The fecal samples were collected separately into 50 ml-centrifuged tubes, transported in an ice chest and processed at Poultry Microbiological Safety and Processing Research Laboratory, USDA, Athens, GA. The samples were individually weighed and diluted with buffered peptone water (BPW; 3X volume/weight). Afterwards, the samples were pre-enriched overnight at 37°C for 24h for *S.* Enteritidis growth before being streaked for isolation onto BGS-NAL. The following day, the samples were streaked onto BGS-NAL plates and incubated overnight at 37°C for 24h for enrichment. Growth of SE^{NR} was observed and recorded as positive or negative for the samples.

All hens were humanely euthanized on 7 dpi. Ceca, L/GB, spleen and ovary samples were collected aseptically into sterile stomacher bags (VWR, Radnor, PA). All the samples were macerated by a rubber mallet, individually weighed before dilution with buffered peptone water (BPW; 3X volume/weight), and stomached (Techmar Company, Cincinnati, Ohio) for 60 s. L/GB, spleen and ovaries were pre-enriched overnight at 37°C for 24h before being streaked onto BGS-NAL plates for isolation and incubated overnight at 37°C for 24h for enrichment. Growth of SE^{NAR} was observed and recorded as positive or negative for the samples. Cecal samples were analyzed using a modification of Blanchfield method (Blanchfield, et al., 1984). In a concise manner, after stomaching for 60 s, two cotton-tipped swabs were dipped and moistened in the cecal material for approximately 5 s. One BGS-NAL plate was surface-swabbed (plate A). The second swab was transferred into a sterile 9.9 mL BPW dilution tube. The tube was vortexed

for approximately 10 s, and a third swab was used to surface-swab a second BGS-NAL plate (plate B). The contents of dilution tube were returned to the stomacher bag and incubated with the plates at 37°C overnight. All plates together with the cecal samples were incubated overnight at 37°C. Negative samples were re-streaked from the overnight pre-enrichments onto a fresh BGS-NAL plate (plate C) and incubated overnight at 37°C. Counts were approximated and converted to log10 cfu SE^{NR}/g of cecal contents.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Ileum sections were aseptically excised, immediately frozen in liquid nitrogen and stored at -80°C until analyzed for inflammatory cytokines. Total RNA was extracted from 100 mg of tissues using Qiazol lysis reagent (Qiazen, Valencia, CA) according to the manufacturer's instruction. The RNA concentration was measured at an optical density of 260_{nm} using a NanoDrop 2000 spectrophotometer (Thermo Scienctific, MA, USA). RNA samples were normalized to a concentration of 2 µg/µl, and purity was verified by evaluating the optical density ratio of 260_{nm} to 280_{nm}. The normalized RNA was reverse- transcribed using a High Capacity cDNA synthesis kit (Applied Biosystems, Life Technologies, CA, USA). The house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the immune cytokines. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using a Step One thermo cycler (Applied Biosystems, Foster City, CA). Primers for chicken immune genes such as toll-like receptor (TLR-4), interleukins (IL-6, and IL-10) and interferon (IFN**)**-Ɣ were designed according to National Center for Biotechnology Information (NCBI). Pairs of primers used in our study are shown in Table 2.4. Gene expression data were analyzed by difference in cycle threshold (ΔΔCT) method (Livak and Schmittgen, 2001).

Statistical analyses

For L/GB, spleen, ovary and feces SE^{NR} recovery, the prevalence was analyzed with Fisher's exact test. The mean of $\log_{10}$ viable SE^{NR} counts obtained from the ceca was subjected to one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS, 2001). Significant differences between the means of different treatment groups were determined by Duncan's multiple-range test and significant differences were assessed at $P < 0.05$.

RESULTS AND DISCUSSION

SE^{NR} numbers and prevalence

Ceca and feces

The SE^{NR} numbers in ceca were counted as log_{10} cfu/g of cecal contents and are shown in Figure 1.1. T3 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 100 ppm NP) and T4 diets (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 200 ppm NP) reduced the cecal SE^{NR} count numerically ($P > 0.05$) compared to T2 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml). However, the SE^{NR} numbers in ceca were same (4.5 log10 cfu) in both T6 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 100 ppm NP) and T7 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 200 ppm NP) and higher than in the T5 diet (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml).

Fecal samples were collected at 3 and 6 days post inoculation (dpi) and assayed for recovery of SE^{NR} (Table 2.3). There was no significant difference among all treatments in terms of SE^{NR} prevalence in the feces both sampling days. At 3 dpi, although T3 was not different from T2, T4 had the lowest prevalence (40%) among all treatments in terms of shedding. T6 and T7 were not different from T5 and all the samples collected from these treatments were SE^{NR} positive. At 6 dpi, 80% and 60% of the fecal samples from T3 and T4 were SE^{NR} positive, respectively. While all of the fecal samples were SE^{NR} positive in T6 as in T5, 80% of the samples were SE^{NR} positive in T7.

Salmonella commonly infects chickens via the fecal–oral route with spread starting initially from the intestinal tract at the distal ileum and ceca of the bird (Barrow et al., 2012). Oral ingestion of *Salmonella* leads to intestinal colonization (especially in the ceca) and shedding of the pathogen in excreted feces. The frequency and duration of intestinal colonization in poultry is affected by varied factors such as age, genetic line, immune status of the birds, by the strain and dose of *Salmonella* to which they are exposed. While young birds are more susceptible to the infection that can lead to early mortality, mature birds are more resistant and can host *Salmonella* in their intestinal tract without showing clinical signs (Brown et al., 1976). When mature laying hens are infected with *Salmonella*, even at very high doses, the frequency of intestinal colonization has a tendency to decrease drastically over time more than is the case for young chicks (Gast et al., 2004), but it can sometimes persist for several months (Gast and Beard, 1990). We compared our results with previous studies, either *in vitro* or *in vivo*, focused

on the bactericidal effect of nitrocompounds against *Salmonella* colonization in the ceca. The inhibitory activity of NP against *S*. Typhimurium in the ceca of 6 days of age broiler chicks was tested (Jung et al., 2004b). Two experiments were conducted with 6-day-old broiler chicks challenged via oral gavage with approximately 10^6 cfu (exact inoculum dose was not stated) of a novobiocin- and nalidixic acid–resistant *S*. Typhimurium. In experiment 1, chicks were divided into three groups: 0 (control), 6.5, and 13 mg NP per bird. In experiment 2, chicks were divided into four treatment groups: 0 (control), 13, 65, and 130 mg NP per bird. *S*. Typhimurium concentrations were reduced by up to 2 log units in the group treated with NP at the 13 mg per bird dose at both 24 and 48 h post-treatment relative to untreated controls. When compared with controls, mean *S*. Typhimurium concentrations were similarly reduced in all groups receiving NP regardless of dose level (Jung et al., 2004b). In another study, NP and 2-nitroethanol were supplemented into diets to reduce *S*. Enteritidis colonization in internal organs of mature laying hens. Inclusion of 100 ppm nitroethanol and 200 ppm NP into laying hen diets reduced cecal SE^{NR} count (Adhikari et al., 2017b). In the current study, addition of NP at both levels to the diets of hens challenged with low inoculum dose (10^6 cfu/ml) reduced cecal SE^{NR} count numerically ($P > 0.05$). As for T6 and T7, the counts of SE^{NR} were identical (4.5 log10 cfu) in both treatments and higher than T5. In brief, 200 ppm of NP showed its inhibitory activity numerically better when the hens were challenged with low inoculum dose of SE^{NR}, but NP was not effective on the hens challenged with high inoculum dose of SE^{NR}. Our results conflicted with previous studies; therefore, it can be suggested that the high inoculum dose of SE^{NR} (10^8 cfu/ml) used in the study might be excessive for NP to exhibit any bactericidal activity against *Salmonella* colonization in the ceca.

In vitro studies were conducted to reveal the inhibitory effect of nitrocompounds applied on fecal suspensions collected from different species. Fecal samples were collected from a Holstein cow and analyzed to reveal the inhibitory effect of NP against *S*. Typhimurium. After 24 h, concentrations of *S*. Typhimurium in fecal suspensions (already inoculated with 10^5- 10^6 cfu/ml of the microorganism) containing 10 mM NP were 2.7 log10 cfu lower ($P < 0.05$) than concentrations in control incubations containing no NP. *S*. Typhimurium concentrations in fecal fluid containing 2.5 mM NP were 1.75 log10 cfu lower than concentrations in control incubations but this reduction was not significant (Jung et al., 2004a). *In vitro* effects of incubating an experimental chlorate product, nitrate, or select short-chain nitrocompounds, alone

or in combination, against experimentally inoculated *S.* Typhimurium and indigenous *E. coli* in porcine fecal suspensions were tested. In this experiment, 2-nitro-1-propanol and 2-nitroethanol, but not necessarily nitroethane, exhibited bactericidal activity against *Salmonella* Typhimurium and *E. coli* during 24 h of incubation in porcine fecal suspensions. When nitrocompounds are incubated with added chlorate, the combined activity of the compounds is markedly enhanced. Coincubation of the fecal suspensions with nitrate also markedly enhances the bactericidal effect of chlorate against these test bacteria (Anderson et al., 2007). The effect of the addition of nitroethane and nitroethanol at 21.8 mM in swine fecal slurries decreased the production of skatole, an odor pollutant in livestock waste, in swine fecal slurries at 24 h incubation (Beier et al., 2009). In the current study, although NP supplementation showed a potential to reduce SE^{NR} in feces, there was no significant difference among all treatments on both sampling days. It can be concluded that NP supplementation of the diets was not effective to reduce the fecal shedding of SE^{NR} in our study. It can be suggested that the number of hens per treatment or sample size should be increased to obtain better results in order to see the effect of NP against SE^{NR}.

L/GB, spleen and ovary

There was no significant difference in SE^{NR} reduction in the L/GB, spleen and ovary samples (Table 2.2). In L/GB, 40% of the samples (n=5) from T2 and T7, and 20% of the samples from T6 were SE^{NR} positive, but no SE^{NR} was detected in T3, T4 and T5. When the spleens were sampled, 60%, 20% and 40% of the samples from T2, T3 and T4 were SE^{NR} positive, respectively. All the spleen samples collected from T6 were SE^{NR} positive, while both T5 and T7 were 40% positive for SE^{NR}. In the ovaries, no recovery of SE^{NR} was detected in T4 or T6, however 20% were positive in T2, T3, T5 and T7, respectively.

Once *Salmonella* have been ingested by the chicken, it encounters the acidic conditions of the proventriculus, the first barrier which is readily overcome due to the immediate adaptation to lower pH, for instance, *S.* Typhimurium can survive acidic conditions as low as pH 3 (Lee et al., 1994). Upon this action, *Salmonella* is able to colonize the small intestine and the underlying lymph tissue; however, the specific site of colonization along the intestinal tract based on the serovar (Carter and Collins, 1974; Henderson et al., 1999). As the number of the organism increases, *Salmonella* can invade all tissues of the body due to the bacteria's ability to proliferate within the liver and spleen (Henderson et al., 1999). After intestinal colonization, the bacteria can invade the reproductive tissues through systemic infection or infection can be originated

from the cloaca. *Salmonella* has been recovered from the reproductive tissues of experimentally infected hens in various studies (Gast et al., 2007; Howard et al., 2005; Miyamoto et al., 1997) and it was determined that the permeability of the capillary endothelia in the ovary has a role in bacterial colonization (Bell and Freeman, 1971). In the end, *Salmonella* can be reintroduced via the gall-bladder into the small intestine over the course of the infection. (Carter and Collins, 1974).

One recent study evaluated the oral (OR) versus intracloacal (IC) challenge route of SE^{NR} (1 ml of 10^8 cfu/ml) in mature White Leghorn laying hens (44 wk old) to consistently colonize the internal organs (Adhikari et al., 2017a). The frequencies of recovery were greater in hens challenged by OR in spleen samples vs. IC, and greater in hens challenged by IC for L/GB vs. OR. The frequency of SE^{NR} did not differ between the 2 routes for ovaries (Adhikari et al., 2017a). As for the supplementation of nitrocompounds to diets of hens as an intervention strategy, nitroethanol and NP were used to reduce *Salmonella* infection in hens (45 wk old) challenged with 1.0 mL of approximately 1.9 x 10^8 cfu SE^{NR} (Adhikari et al., 2017b). There was no difference in SE^{NR} reduction in the L/GB or ovary after supplementation with either nitroethanol or NP. In L/GB, 50% of cases (n=6) were positive in T2 (SE^{NR} challenged control) and T6 (SE^{NR} challenged + 200 ppm NP), whereas 66% of cases were positive in T4 (SE^{NR} challenged + 200 ppm Nitroethanol), and 33% in both T3 (SE^{NR} challenged + 100 ppm Nitroethanol) and T5 (SE^{NR} challenged + 100 ppm NP). The recovery of SE^{NR} in the ovary by 7 dpi was 0 except for T3 which had 16.7% positive cases (Adhikari et al., 2017b). In the current study, when the L/GB samples were analyzed, 40% of the hens were positive in T2 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml), but no SE^{NR} was detected in T3 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml) + 100 ppm NP) or T4 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 200 ppm NP). Also, 20% and 40% of the hens were positive in T6 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 100 ppm NP) and T7 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 200 ppm NP), respectively. Surprisingly, SE^{NR} was detected in T5 (SE^{NR} challenged with high inoculum dose -10^8 cfu/ml). When the spleens were sampled, 60%, 20% and 40% of them were SE^{NR} positive in T2, T3 and T4. All the spleen samples collected from T6 were SE^{NR} positive while only 40% were SE^{NR} positive in both T5 and T7. In the ovaries, no SE^{NR} was detected in T4 or T6, and 20% of samples were positive in T2, T3, T5 and T7. Based on our results, it seems that NP supplementation of the diets gave

rise to changes in the prevalence of SE^{NR} in the above-mentioned internal organs. However, as the results were inconsistent and the differences among treatments were statistically insignificant, further research is necessary to determine the bactericidal dose of NP by designing a different challenge model capable of creating a consistent colonization of SE^{NR} in the internal organs of mature laying hens. In addition, instead of evaluating the organ samples as SE^{NR} positive or negative to determine the prevalence of the pathogen in the internal organs, other microbiological or molecular methods might be used to better determine the microbial load in the internal organs in order to find out the inhibitory effect of NP supplementation.

Ileum immune gene expression

Pro- and anti-inflammatory cytokines such as interferon (IFN)-Ɣ, interleukin (IL)-6 and IL-10 and toll-like receptors (TLR)-4 gene expressions were analyzed in order to determine the effects of SE^{NR} challenge with or without NP supplementation (Figure 2.2, 2.3 and 2.4). Ileum samples from each treatment were collected at 7 dpi. The house keeping gene, GAPDH, was used to normalize the immune cytokines. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed. Gene expression data were analyzed by difference in cycle threshold (ΔΔCT) method (Livak and Schmittgen, 2001).

There was a numerical increase ($P > 0.05$) in the mRNA expression of INF-Ɣ in T2 and T5 after *Salmonella* challenge compared to T1. As for the effect of NP supplementation on this cytokine, the expressions of INF-Ɣ in T3 and T4 were numerically lower ($P > 0.05$) than T2. INF-Ɣ mRNA expression was downregulated ($P < 0.05$) in T6 as compared to T5. IFN-Ɣ, produced by T cells, is an indicator cytokine for the activation of macrophages and has a crucial role in control of infection and elimination of *Salmonella*. After *Salmonella* challenge, upregulation of IFN-Ɣ has been reported in experiments conducted *in vitro* and *in vivo* (Bao et al., 2000; Kano et al., 2009; Kogut et al., 2005). Various studies have evaluated the expression of this cytokine after supplementation of diets with feed additives. IFN-Ɣ was upregulated by *Salmonella* challenge, but reduced by supplementing NP to the diets of laying hens (Adhikari et al., 2017b). In a study, probiotic treatment to control *Salmonella* infection downregulated the expression of IFN-Ɣ in chicken cecal tonsils (Haghighi et al., 2008). In the current study, the INF-Ɣ mRNA expression was downregulated ($P < 0.05$) in T6 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 100 ppm) compared to T5. This reduction in T6 can be attributed

to the potential bactericidal effect of NP against *Salmonella* so that INF-Ɣ expression decreases in the ileum of the hens.

The expression of IL-10 was downregulated ($P < 0.05$) in T2 and T5 after *Salmonella* challenge compared to T1. T3 downregulated ($P < 0.05$) the expression of IL-10 compared to T2. In T7, IL-10 mRNA expression was upregulated ($P < 0.05$) compared to T5. *S.* Enteritidis has lipopolysaccharide (LPS) as a component of its outer membrane. LPS triggers inflammation through a variety of immunological changes that stimulate different cells (MacKay and Lester, 1992; Nakamura et al., 1998) as well as by stimulating IL-10 gene expression in chickens (Ghebremicael et al., 2008). IL-10 is a regulatory cytokine having a role in reduction of the inflammatory response to *Salmonella* infection (Eckmann and Kagnoff, 2001), exhibiting a negative effect on IFN-Ɣ expression by Th1 cells and promoting the proliferation of Th2 cells (Rothwell et al., 2004), and deactivating macrophages (O'Farrell et al., 1998). It was reported that oral administration of various lactic acid bacteria reduced the *Salmonella* invasion and inflammation of broiler chicks by elevating the expression of IL-10 in cecal tonsils (Chen et al., 2012). Effects of supplementing nitrocompounds to the diets on the IL-10 mRNA expression in the ileum of laying hens challenged with *Salmonella* were tested (Adhikari et al., 2017b). In the study, IL-10 mRNA expression was upregulated by adding 200 ppm of nitroethanol to the diets of laying hens compared to unchallenged control treatment. However, the IL-10 mRNA expression was not different from unchallenged control treatment when the diets were supplemented with 100 and 200 ppm of NP, respectively (Adhikari et al., 2017b). Although it was stated that *S.* Enteritidis LPS stimulates IL-10 gene expression in chickens (Ghebremicael et al., 2008), the expression in T2 and T5 were lower ($P < 0.05$) than T1 after *Salmonella* challenge in the current study. This difference might be due to the age of the birds. Ghebremicael et al. (2008) inoculated 1 d of age birds. These young birds would be more susceptible to infection while mature birds would be more resistant and can actually host *Salmonella* in the intestinal tract without clinical symptoms (Brown et al., 1976). In the current study, hens were 46-week old at inoculation with and therefore, it can be hypothesized that the inflammation in the ileum was not sufficient to stimulate IL-10 expression in the cells at 7 dpi. T3 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 100 ppm NP) downregulated ($P < 0.05$) the expression of IL-10 compared to T2; however, the IL-10 mRNA expression was upregulated ($P < 0.05$) in T7 compared to T5. It may be deduced from these results that NP as a potential feed additive

exhibited a better bactericidal activity when it was used against a low inoculum dose (10^6 cfu/ml) of SE^{NR} which resulted in less inflammation to trigger the IL-10 expression in the ileum.

The expression of IL-6 in T2 and T5 was lower ($P < 0.05$) than T1, respectively. NP supplementation at both levels downregulated ($P < 0.05$) the expression of IL-6 in T3 and T4 compared to T2, respectively. NP did not cause any significant difference in the expression of IL-6 in T6 or T7 when compared to T5, respectively. IL-6 is a multifunctional cytokine produced by different types of cells and has a role in acute-phase responses and immune regulation (Wigley and Kaiser, 2003). IL-6 activity has been found in various infectious diseases of chickens including salmonellosis and it was reported that induction of IL-6 plays vital role in the response to different serovars of *Salmonella* in chickens (Kaiser et al., 2000). Invasion of chicken cells by *S.* Typhimurium or *S.* Enteritidis leads to an 8-fold increase of IL-6 mRNA which can trigger a strong inflammatory and immune response, limiting the infections to the gut and preventing development of systemic disease (Wigley and Kaiser, 2003). It has been reported that *Salmonella* challenge increased the mRNA expression of IL-6 mRNA level in the cecal tonsils of hens at 7 dpi (Bai et al., 2014). A significant increase in the IL-6 mRNA expression was detected in the ileum of chickens challenged with *Salmonella* at 1 dpi when compared to an uninfected control group (Hu et al., 2015). Supplementation of nitroethanol and NP resulted in a downregulation of IL-6 expression in the ileum of laying hens challenged with *Salmonella*. (Adhikari et al., 2017b). In the current study, NP supplementation at both levels downregulated ($P < 0.05$) the expression of IL-6 in T3 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 100 ppm NP) and T4 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 200 ppm NP) compared to T2, respectively. However, IL-6 expressions in T6 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 100 ppm NP) and T7 (SE^{NR} challenged with high inoculum dose = 10^8 cfu/ml + 200 ppm NP) was not statistically different from T5. In brief, addition of NP to the diets showed a down-regulatory effect on the expression of IL-6 in the hens challenged with a low inoculum dose of SE^{NR}. Our data compares favorably to a previous study (Adhikari et al., 2017b) in which it was stated that NP has the potential to inhibit the expression of pro-inflammatory cytokines and interact with the host either by modulating the gut microbiome or direct interaction with *Salmonella*.

TLR-4 mRNA expression in T5 was lower ($P < 0.05$) than T1 after *Salmonella* challenge. NP supplementation at both levels downregulated ($P < 0.05$) the expression of TLR-4 in T3 and T4 compared to T2. TLR-4 mRNA expression was upregulated ($P < 0.05$) in T7 compared to T5. The primary function of the immune system is to identify and fight against pathogens. In the presence of microorganisms in the gut, toll-like receptor (TLR), also known as pattern recognition receptors, may stimulate expression of proinflammatory cytokines such as IL-6 (Kaiser, 2010) as well as recognizing microbial-associated molecular patterns resulting in a chain of reaction that triggers the immune system (Aderem and Ulevitch, 2000). In addition, resistance to *Salmonella* has closely been associated with an upregulation of TLR-4 and different chemokines and cytokines (Chaussé et al., 2011; Sadeyen et al., 2006) and aberrant expression of TLR-4 has been linked to susceptibility to *S.* Enteritidis infection in chickens (Gou et al., 2012). Addition of 100 ppm NP to the diets of laying hens downregulated the TLR-4 mRNA expression in the ileum (Adhikari et al., 2017b). In the current study, NP supplementation at both levels downregulated ($P < 0.05$) the expression of TLR-4 in T3 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 100 ppm NP) and T4 (SE^{NR} challenged with low inoculum dose = 10^6 cfu/ml + 200 ppm NP) compared to T2. This downregulation might be caused by the expected inhibitory effect of NP against *Salmonella* infection resulting in a decrease in the expression of TLR-4 in the ileum.

CONCLUSION

Challenging mature laying hens with two different inoculum doses of SE^{NR} did not serve the purpose of revealing the inhibitory effect of NP as a potential feed additive. NP supplementation of the diets numerically reduced the counts of SE^{NR} in the ceca and affected the prevalence of SE^{NR} in other internal organs and feces, as well as downregulating the mRNA expressions of varied cytokines which play vital roles in the immune response to *Salmonella* infection in the ileum. Further research is needed to determine the mechanism of NP and its effects against other *Salmonella* serovars in order to provide this promising feed additive as an intervention strategy for use in the poultry industry.

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Table 2.1 Diet composition of layer ration supplemented with different levels 2 nitro-1-propanol (NP)[1]

Item	Diets		
Ingredient (% of the diet)	**T1/T2/T5**	**T3/T6**	**T4/T7**
Corn, Grain	59.45	59.44	59.43
Soybean Meal -48%	25.03	25.03	25.03
Limestone	9.62	9.62	9.62
Soybean Oil	2.71	2.71	2.71
Defluor. Phos.	2.10	2.1	2.1
DL-Methionine	0.13	0.13	0.13
Common Salt	0.30	0.30	0.30
Vitamin Premix[2]	0.50	0.50	0.50
Mineral Premix[3]	0.15	0.15	0.15
NP	0.00	0.01	0.02
Calculated composition[4]			
ME (kcal/kg)	2.85	2.85	2.85
CP (%)	16	16	16
Ca (%)	4.4	4.4	4.4
Available P (%)	0.5	0.5	0.5

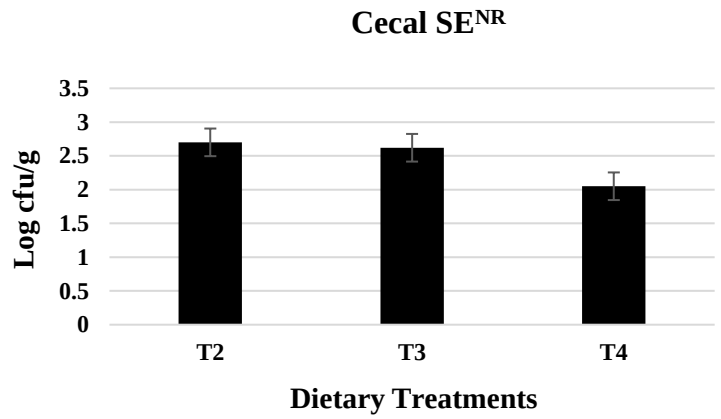
[1] *T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP, T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP, and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP.*

[2] *Supplemented per kg of diet: thiamin mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0 g; pyridoxine HCl, 4.7 mg; Dbiotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 g; transretinyl acetate, 1,892 g; α tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.*

[3] *Supplemented as per kg of diet: manganese (MnSO4.H2O), 60 mg; iron (FeSO4.7H2O), 30 mg; zinc (ZnO), 50 mg; copper (CuSO4.5H2O), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium (NaSe03), 0.3 mg.*

[4] *ME = Metabolizable energy; CD = Crude protein*

a)



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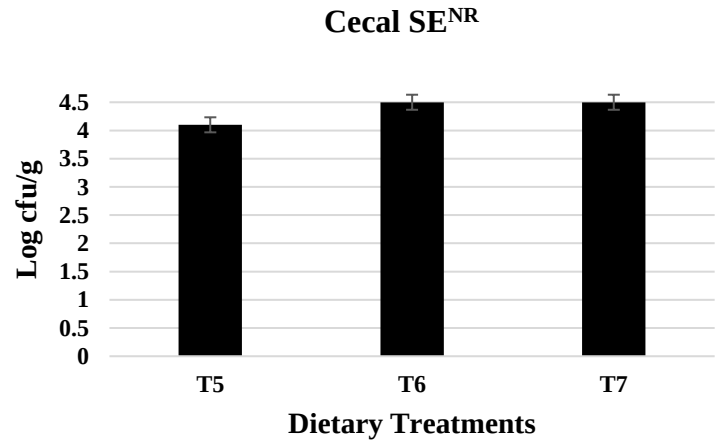

Figure 2.1 Presence of Salmonella number in ceca collected at 7 days post-infection (dpi) from laying hens fed diets supplemented with different levels of 2 nitro-1-propanol (NP). Hens (n=5/treatment) were challenged with Salmonella Enteritidis (SE^{NR}). Error bars represent standard errors.
a) T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP. b) T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP.

Table 2.2 Presence and absence of SE^{NR} in liver with gall bladder (L/GB), spleen and ovary in laying hens supplemented with 2 nitro-1-propanol (NP)[1].

	Incidence (%) of SE^{NR} – positives[2]						
Organs	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
L/GB	0	40	0	0	0	20	40
Spleen	0	60	20	40	40	100	40
Ovary	0	20	20	0	20	0	20

[1] *T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP, T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP, and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP.*

[2] *Hens were 5 per treatment group (n=5) except Treatment 1 (n=4).*

Table 2.3 Presence and absence of SE^{NR} in feces collected from laying hens supplemented with 2 nitro-1-propanol (NP)[1].

	Incidence (%) of SE^{NR} – positives[2]						
Days	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Day 3	50	80	80	40	100	100	100
Day 6	50	100	80	60	100	100	80

[1] *T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP, T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP, T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP, and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP.*

[2] *Hens were 5 per treatment group (n=5) except Treatment 1 (n=4).*

Table 2.4 Chicken cytokines and toll-like receptor primer sequences

Gene[2]	Primer sequence (5′–3′)	Gene bank accession no.	Fragment size, bp	Annealing temperature, °C
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	K01458	116	56
TLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	190	56
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	AJ309540	233	59
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	NM001004414	103	56
IFN-Y	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	NM205149	159	58

[2] IL = interleukin; IFN = interferon; TLR = Toll-like receptor; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

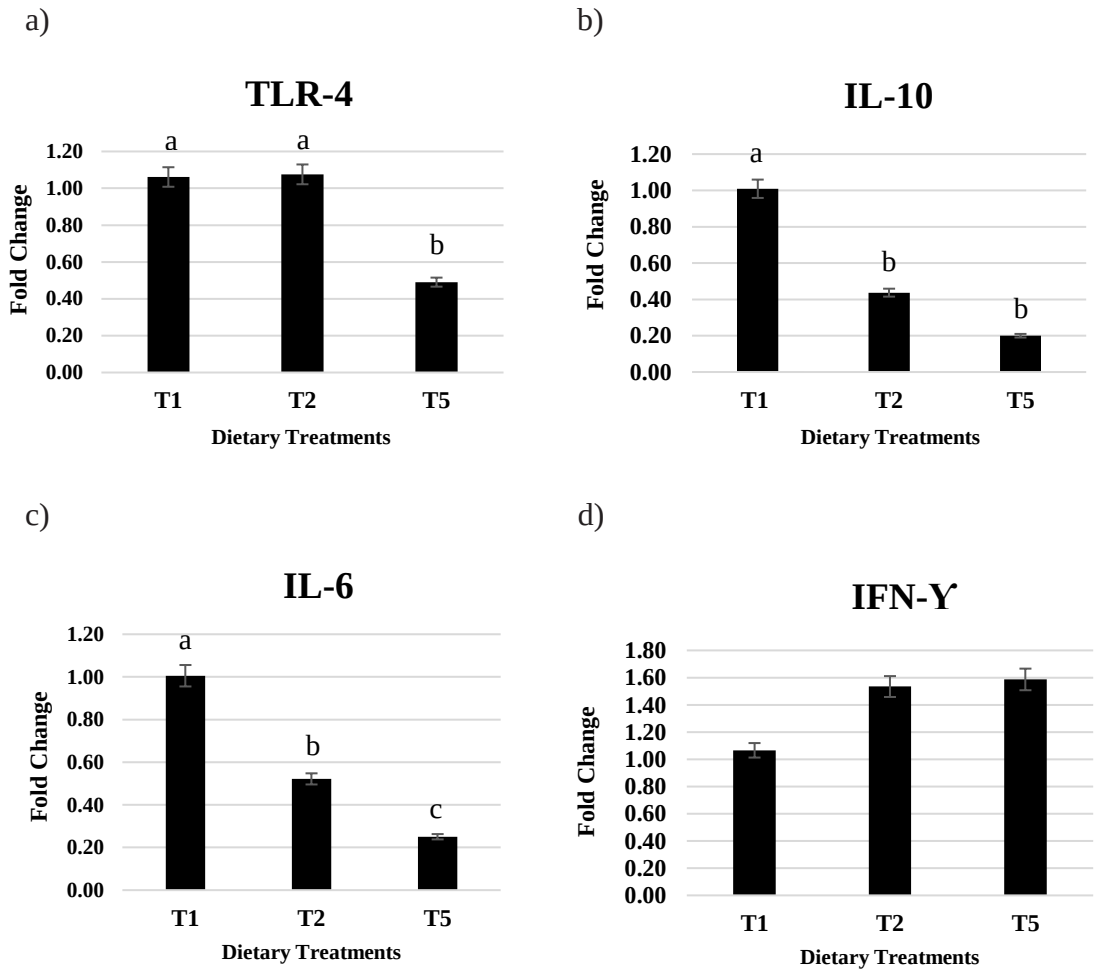

Figure 2.2 *The effect of low and high inoculum dose of SE^{NR} challenge on ileal immune gene expression of cytokines: a) Toll-like receptor (TLR)-4, b) Interleukin (IL)-10, c) IL-6 and d) Interferon (IFN)-Y.* Hens *were challenged with SE^{NR} (n = 5/treatment except T1=4/treatment). Gene expressions were calculated relative to housekeeping gene, GAPDH. Error bars represent standard errors. Bars with different letters (a, b to c) differ significantly across the treatment groups ($P < 0.05$). T1 = SE^{NR} unchallenged (negative control), T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) and T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml).*

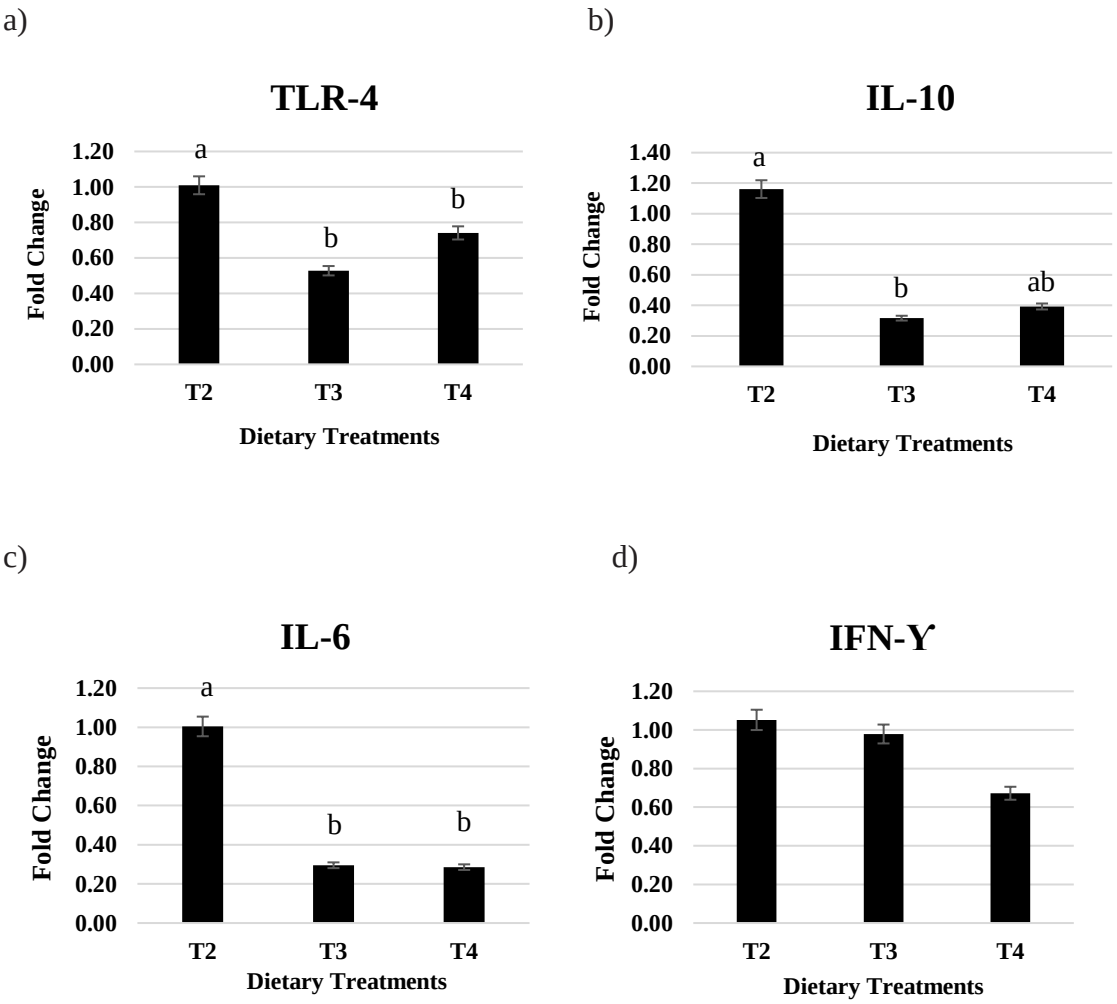

Figure 2.3 The effect of NP supplementation of diets on ileal immune gene expression of cytokines in hens challenged with low inoculum dose of SE^{NR}: a) Toll-like receptor (TLR)-4, b) Interleukin (IL)-10, c) IL-6 and d) Interferon (IFN)-Y. Hens were challenged with SE^{NR} *(n = 5/treatment). Gene expressions were calculated relative to housekeeping gene, GAPDH. Error bars represent standard errors. Bars with different letters (a to b) differ significantly across the treatment groups (P < 0.05). T2 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml), T3 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 100 ppm NP and T4 = SE^{NR} challenged with low inoculum dose (10^6 cfu/ml) + 200 ppm NP.*

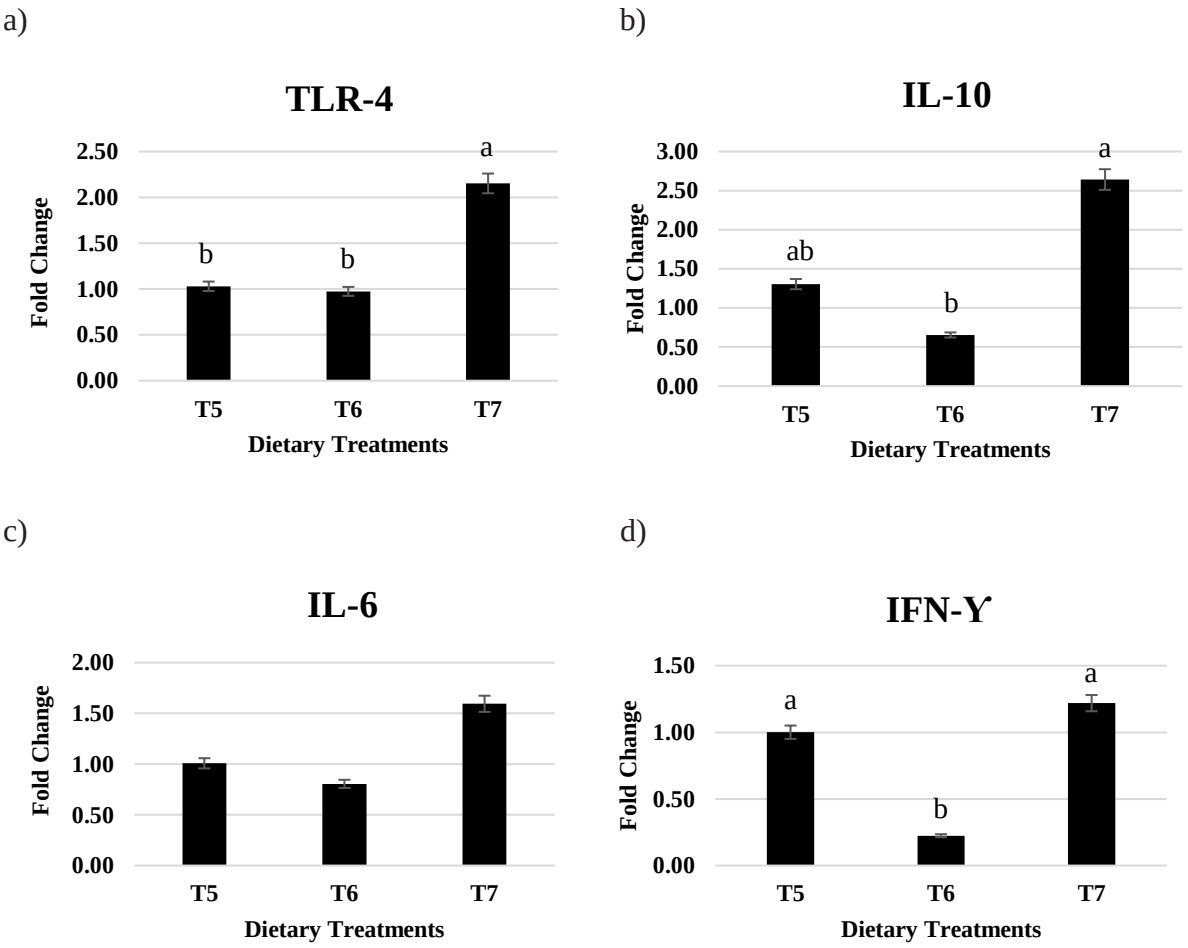

Figure 2.4 The effect of NP supplementation of diets on ileal immune gene expression of cytokines in hens challenged with high inoculum dose of SE^{NR}: a) Toll-like receptor (TLR)-4, b) Interleukin (IL)-10, c) IL-6 and d) Interferon (IFN)-Y. Hens were challenged with SE^{NR} (n = 5/treatment). Gene expressions were calculated relative to housekeeping gene, GAPDH. Error bars represent standard errors. Bars with different letters (a to b) differ significantly across the treatment groups (P < 0.05). T5 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml), T6 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 100 ppm NP and T7 = SE^{NR} challenged with high inoculum dose (10^8 cfu/ml) + 200 ppm NP.

CHAPTER 3

THE EFFECT OF 2-NITRO-1-PROPANOL SUPPLEMENTATION ON *SALMONELLA* COLONIZATION IN BROILERS [2]

[2] O. Yasir Koyun, D.E. Cosby, N.A. Cox and W.K. Kim. To be submitted to *Poultry Science Journal*

ABSTRACT

The presence of *Salmonella* in broilers continues to be a problem for the industry. A study was conducted to evaluate the effect of 2-nitro-1-propanol (NP) on *Salmonella* recovery of internal organs of broiler chicks. Two-hundred and forty chicks were obtained from a local hatchery and 180 were orally challenged with a nalidixic acid resistant *Salmonella* Typhimurium (ST^{NAR}). Chicks were housed in battery cages, 60 were assigned to T1= ST^{NAR} unchallenged (negative control), and the remaining were randomly allocated to one of the three dietary treatments: T2 = ST^{NAR} challenged (positive control), T3 = ST^{NAR} challenged + 100 ppm NP, T4 = ST^{NAR} challenged + 200 ppm NP. Bird weight and feed consumption was recorded on days 7,14 and 21, and mortality recorded daily. Fecal samples were collected at 3, 6, 9 and 12 days post inoculation (dpi) and assayed for recovery of ST^{NAR}. Ceca, liver with gall bladder (L/GB) and spleen were collected for recovery of ST^{NAR} at 4,7,11 and 17 dpi. The mean of log_{10} viable ST^{NAR} counts from the ceca and growth performance data were subjected to ANOVA using the GLM procedure of SAS. Significant differences between the means of different treatment groups were determined by Duncan's multiple-range test ($P < 0.05$). The performance of birds was not significantly different compared to the control group at any point during the 21-day trial. For fecal shedding, L/GB and spleen ST^{NAR} recovery, the prevalence was analyzed with Fisher's exact test. At 3 dpi, there was a numerical decrease ($P > 0.05$) in T4 (ST^{NAR} challenged + 200 ppm NP) compared to T2 (positive control) in terms of fecal shedding. At 6 dpi, a decline ($P < 0.05$) in fecal shedding occurred in T4 compared to T2. At 9 and 12 dpi, the fecal samples from T2, T3 and T4 were 100% ST^{NAR} positive. No significant difference in ST^{NAR} prevalence in the L/GB and spleen samples was observed. At day 7 and 15, there were numerical decreases in prevalence in L/GB samples collected from T3 compared to T2. As for the spleen samples, T3 showed a reduction at day 7 and, T4 decreased the prevalence at day 21 compared to T2, but no significance was observed. All cecal ST^{NAR} counts in T2, T3 and T4 showed a trend; peaking at day 11 and then going down at days 15 and 21. T4 showed a significant ($P < 0.05$) reduction, from 3.98 to 2.84 log10 cfu/g, in ST^{NAR} number in ceca collected on day 11 compared to T2. The pro- and anti-inflammatory cytokines, interleukin (IL)-6, IL-1β and IL-10 and toll-like receptors (TLR)-4 gene expressions were analyzed. Expression of mRNA for TLR-4, IL-1β and IL-10 was detected in all treatment groups at the end of the study, no statistically significant difference in the expression rate of these genes in T1 compared to T2 or in T2 compared to T3 was noted. The

mRNA expression of IL-6 in T2 was downregulated ($P < 0.05$) compared to T1 and upregulated ($P < 0.05$) by both T3 and T4 compared to T2 at the end of the study. Overall, NP supplementation of the diets did not have an adverse effect on growth performance for the 21 day grow-out period but did decrease the prevalence of ST^{NAR} in feces and reduced the colonization in ceca at specific sampling days during the study. NP did not downregulate the mRNA expression of cytokines having vital roles in the ileal response to ST^{NAR} infection. Further studies need to be carried out in order to find out the effective dose of NP for use in reducing and/or eliminating bacterial contamination in the poultry industry.

INTRODUCTION

Salmonella has been accepted as a causative agent for human diseases such as food poisoning, typhoid, paratyphoid, septicemia, and sequelae. Despite numerous studies and researches conducted to improve our understanding of *Salmonella* ecology and pathogenicity in humans, the microorganism continuously gives rise to new food safety challenges and remains one of the major food-borne pathogens in the entire world. (Hannah, 2007; Humphrey, 2004). Salmonellosis is a worldwide health concern and approximately 95% of human salmonellosis cases are linked to the consumption of contaminated products such as meat and eggs from poultry (Foley and Lynne, 2008). The US population is one of the large consumers of poultry meat, about 12 million tons of broiler meat every year, with a consistent increase of approximately 3% per year. The relationship of *Salmonella* with raw foods obtained from animal products, especially poultry products, has become clear. For the industry, poultry products have been accepted as one of the major *Salmonella* reservoirs for decades (Bohorquez, 2007). The Center for Disease Control and Prevention reported that more than 2,300 types of *Salmonella* have been identified; however, the two most common are *S.* Enteritidis and *S.* Typhimurium, which are the causative agent for more than half of all human infections (Liu, 2012). The Foodborne Diseases Active Surveillance Network (Food Net) reported 19,056 infections, 4,200 hospitalizations, and 80 deaths caused by foodborne pathogen infections from 2006 to 2013. In this report, *S.* Typhimurium was one of the top serotypes among 6,520 *Salmonella* isolates (Crim et al., 2014).

S. Typhimurium has a broad-host range and can cause disease in cattle, pigs, horses, sheep, poultry, and rodents (Salyers and Whitt, 2002) and it can be regarded as ubiquitous due to its presence in water, soil, insects, food plants, food animals, animal feces, and raw foods (Food

and Administration, 2004). The natural microflora of animal's intestinal tract may contain *S.* Typhimurium without producing an illness; therefore, controlling *Salmonella* in food-production animals is a huge concern due to the high rate of food contamination (Olsen et al., 2001; Tauxe, 1991). As a result, the impact of *Salmonella* spp. on the population can be massive. As consumers become more conscious about the risks, control and elimination of *Salmonella* has become an important goal for the poultry industry, particularly at the preharvest phase (Santos, 2007). Control of *Salmonella* spp. is a concern from both health and economical aspects; therefore, it is crucial to prevent *Salmonella* colonization in chickens at farm levels to reduce the risk of poultry product contamination (Blankenship et al., 1993). In 2011, USDA-FSIS regulated their performance standard for *Salmonella* contamination of whole broiler chickens. Since chicken is generally purchased as cut-up parts, USDA-FSIS conducted a survey of raw chicken parts in 2012 and reported an estimated 24% prevalence of *Salmonella*. In 2013, USDA-FSIS announced its *Salmonella Action Plan* indicating that USDA-FSIS will conduct a risk assessment and improve performance standards for poultry parts during 2014, among other key activities (Crim et al., 2014). To reduce the transmission of *Salmonella* to humans through the food chain, it is crucial to determine potential sources of contamination within the poultry production system.

Feed additives such as prebiotics, probiotics and organic acids have been commonly used to control *Salmonella* infections in poultry, with the rate of success varying based on the additive used (Adhikari et al., 2017; Corrier et al., 1993; Van Immerseel et al., 2005). Inconsistent results from anti-*Salmonella* strategies have been reported in studies over the years, so new feed additives are needed to reduce *Salmonella* infections in chickens. The dietary effect of nitrocompounds such as 2-nitro-1-propanol (NP) and 2-nitroethanol have been tested for reducing *Salmonella* colonization in the internal organs of laying hens and supplementation with these nitrocompounds showed an immune response in the ileum with reduction of expression of IFN-Ɣ, IL-6, TLR-4 and IL-10 mRNA (Adhikari et al., 2017). Potential effects of various nitrocompounds (nitroethane, nitroethanol, NP and nitropropionic acid) were studied on ammonia volatilization in poultry manure. According to the results, use of nitrocompounds caused a reduction in ammonia volatilization in poultry manure by inhibiting growth of uric acid–utilizing microorganisms (Kim et al., 2006). A similar study was conducted to observe the effects of dietary nitrocompounds on bird performance, ammonia volatilization, and changes in

manure nitrogen. This study concluded that supplementation of nitroethanol or NP into broiler diets up to 33.3 mg/kg affects uric acid degradation and ammonia production in broiler manure while sustaining optimal growth performance (Mowrer et al., 2016). *In vitro* effects of incubating an experimental chlorate product, nitrate, or select short-chain nitrocompounds, alone or in combination, against experimentally inoculated *S.* Typhimurium and *E. coli* in porcine fecal suspensions were analyzed. In this experiment, NP and 2-nitroethanol showed bactericidal activity against *S.* Typhimurium and *E. coli* during 24 h of incubation (Anderson et al., 2007). The inhibitory activity of NP against *S.* Typhimurium, *E. coli* O157:H7 and *Enterococcus faecalis* was reported (Jung et al., 2004a). The same research group reported in a similar study that NP inhibited *Salmonella* colonization in the ceca of broiler chicks (Jung et al., 2004b).

In the light of these studies, we hypothesized that NP may reduce *S.* Typhimurium colonization in the ceca and other internal organs of broiler chicks, decrease fecal shedding of *S.* Typhimurium and produce an immune response in the ileum. The objectives of the study were 1) to evaluate the inhibitory effect of NP on *Salmonella* in ceca as well as other internal organs, 2) to reduce fecal shedding and 3) to promote an effective immune response in the ileum of broiler chicks.

MATERIAL AND METHODS

Salmonella strain and inoculum preparation

Nalidixic acid resistant *Salmonella* Typhimurium (ST^{NAR}) was used as the challenge organism. ST^{NAR} was stored at -80°C in Nutrient Broth (Acumedia, East Lansing, MI; NB) with 16% glycerol. ST^{NAR} was grown and maintained on brilliant green with sulphapyridine agar plates (Acumedia, East Lansing, MI; BGS) containing 200 ppm of NAL (Sigma Chemical Co., St. Louis, MO; BGS NAL) for 24 h at 37°C. Individual colonies were suspended into a sterile 0.85% saline solution. The absorbance was adjusted to 0.20 ± 0.01 OD_{540nm} using a spectrophotometer (Spect 20, Milton-Roy, Thermo Spectronics, Madison, WI). Culture solution was serially diluted and plated onto BGS-NAL plates for enumeration. Chicks were orally challenged with a 0.1 mL of approximately 2.7×10^7 cfu ST^{NAR}.

Chicks, housing and dietary treatments

A total of 240 one-day-old commercial broiler chicks were obtained from a local hatchery. The chicks were individually weighed upon arrival to maintain uniformity between treatments and were placed in battery cages in a light- and temperature-controlled room at

Poultry Research Center, University of Georgia. Chicks were allocated to one of four dietary treatments (10 birds/cage; 6 replicated cages per treatment): T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control), T3 = ST^{NAR} challenged + 100 ppm NP, T4 = ST^{NAR} challenged + 200 ppm NP. All chicks were fed a standard corn-soybean broiler starter diet with or without NP supplementation, based on the treatment group. The diet was formulated to provide 3,000 kg/kcal metabolizable energy (ME), 22% crude protein (CP), 0.9% Ca and 0.45% available P (Table 3.1). Chicks were provided free access to water and mash feed *ad libitum* throughout the experiment period. At day 4, all chicks broiler except in T1 (negative control) group was orally challenged with 0.1 mL of 10^7 cfu ST^{NAR}. Feed was withdrawn from all chicks 10 h before challenge and returned right after ST^{NAR} challenge. The experiment protocol was approved by the Institutional Animal Care and Use Committee of University of Georgia.

Sampling protocol and analyses

Feces, ceca, L/GB and spleen (bacteriological)

Fresh fecal samples were collected from each battery cage at 3, 6, 9 and 12 dpi to screen for fecal shedding. The individual fecal droppings were collected separately into 50 ml-centrifuged tubes, transported on ice and processed at the U.S. National Poultry Research Center, Athens, GA. The samples were individually weighed and diluted with buffered peptone water (BPW; 3X volume/weight). Afterwards, the samples were incubated (pre-enriched) overnight at 37°C before being streaked onto BGS-NAL for isolation of ST^{NAR}. The BGS-NAL plates were incubated overnight at 37°C for 24 h. The growth of ST^{NAR} were observed and recorded as positive or negative for the samples.

Two chicks from each cage were euthanized and ceca, L/GB and spleen were collected on 3, 7, 11 and 17 dpi**.** Ceca, L/GB and spleen samples were collected aseptically into sterile stomacher bags (VWR, Radnor, PA). The organs were sampled for the presence of ST^{NAR} by growth on BGS-NAL plates. All samples were macerated with a rubber mallet, individually weighed and diluted with BPW (3X volume/weight), and stomached (Stomacher 80, Techmar Company, Cincinnati, Ohio) for 60 s. L/GB and spleen samples were pre-enriched overnight at 37°C for 24 h before being streaked onto BGS-NAL plates which were incubated overnight at 37°C for 24 h for growth. The growth of ST^{NAR} was observed and recorded as positive or negative for the samples. Cecal samples were analyzed using a modification of the Blanchfield method (Blanchfield, et al., 1984). In brief, after stomaching for 60 s, two cotton-tipped swabs

were dipped and rotated in the cecal material for approximately 5 s. One BGS-NAL plate was surface-swabbed (plate A). The second swab was transferred into a sterile 9.9 mL BPW dilution tube. The tube was vortexed for approximately 10 s, and a third swab was used to surface-swab a second BGS-NAL plate (plate B). The contents of dilution tube were returned to the stomacher bag and all plates together with the cecal samples were incubated overnight at 37°C for 24 h. Negative samples were re-streaked from the overnight pre-enrichments onto a fresh BGS-NAL plate (plate C) and incubated overnight at 37°C for 24 h. Counts were approximated and converted to log10 cfu ST^{NAR} /g of cecal contents.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Ileum sections were aseptically excised, immediately frozen in liquid nitrogen and stored at -80°C until analyzed for inflammatory cytokines. Total RNA was extracted from100 mg of tissues using Qiazol lysis reagent (Qiazen, Valencia, CA) according to the manufacturer's instruction. The RNA concentration was measured at an optical density of 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). RNA samples were normalized to a concentration of 2 µg/µl, and purity was verified by evaluating the optical density ratio of 260 to 280 nm. The normalized RNA was reverse-transcribed using a High Capacity cDNA synthesis kit (Applied Biosystems, Life Technologies, CA, USA). The house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the immune cytokines. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using a Step One thermo cycler (Applied Biosystems, Foster City, CA). Primers for the chicken immune genes toll-like receptor (TLR-4), interleukins (IL-1ß, IL-6, and IL-10) and interferon (IFN)-Ɣ were designed according to National Center for Biotechnology Information (NCBI). Pairs of primers used in our study are shown in Table 3.5. Gene expression data were analyzed by the difference in cycle threshold (ΔΔCT) method (Livak and Schmittgen, 2001).

Statistical analyses

For L/GB, spleen and feces ST^{NAR} recovery, the prevalence was analyzed with Fisher's exact test. The mean of log10 viable ST^{NAR} counts obtained from the ceca was subjected to one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS, 2001). Significant differences between the means of different treatment groups were determined by Duncan's multiple-range test and significant differences were assessed at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of NP on Growth Performance

Bird weight and feed consumption was recorded at 0,7,14 and 21 days of age, and mortality recorded daily. The performance of birds was not significantly different among the treatments compared to the control (Table 3.2). When the cumulative results are compared within treatments, it can be seen that supplementation of NP did not affect the growth performance parameters of broiler chicks. At the end of the study, while the highest feed intake (FI) was recorded in T1 (negative control), the highest body weight gain (BWG) was seen in T4 compared to the rest of the treatments, but these differences were not significant. The FCR values for T1 (ST^{NAR} unchallenged = negative control), T2 (ST^{NAR} challenged = positive control), T3 (ST^{NAR} challenged + 100 ppm NP) and T4 (ST^{NAR} challenged + 200 ppm NP) were 1.28, 1.26, 1.27 and 1.32, respectively. It can be concluded that addition of 200 ppm of NP into the diet increased the feed conversion ratio (FCR), but this elevation was not significant.

In an earlier work evaluating the inhibitory effects of nitrocompounds on *Salmonella* Typhimurium colonization in the ceca of broiler chicks, there was no negative effect reported on growth performance when the chicks were fed up to 130 mg NP per bird (Jung et al., 2004b). Another study conducted to assess the effects of dietary nitrocompounds on bird performance, ammonia volatilization, and changes in manure nitrogen demonstrated. No significant differences in BWG, FI and FCR among the treatments indicating that the addition of up to 33.3 mg/kg of nitroethanol or NP has no adverse effects on broiler growth performance within 21 days (Mowrer et al., 2016). In the current study, supplementation of NP did not result in a difference in the growth performance parameters of broiler chicks among treatments, but further research is needed to determine the effective inoculum dose of NP without growth loss during full broiler growth period.

SE numbers and prevalence

Ceca and Feces

The ST^{NAR} numbers in ceca were counted and recorded as log10 cfu/g of cecal contents are shown in Figure 3.1 and 3.2. T1 (ST^{NAR} unchallenged = negative control) remained negative throughout the study. The ST^{NAR} numbers in the ceca from T3 (ST^{NAR} challenged + 100 ppm NP) were not different from T2 (ST^{NAR} challenged = positive control) on each sampling day. However, T4 showed a significant ($P < 0.05$) reduction, from 3.98 to 2.84 log10 cfu/g, in ST^{NAR}

number in ceca collected on day 11 compared to T2. All cecal ST^{NAR} counts in T2, T3 and T4 (ST^{NAR} challenged + 200 ppm NP) showed a trend throughout the study; peaking at day 11 and then declining at days 15 and 21.

Fecal samples were collected 3, 6, 9 and 12 dpi and assayed for recovery of ST^{NAR} (Table 3.3). At 3 dpi, there was a numerical decrease in fecal shedding in T4 compared to T2, but it was not statistically significant. At 6 dpi, T3 and T4 lowered the prevalence of ST^{NAR} in feces compared to T2, but only T4 had a significant effect ($P < 0.05$). At 9 and 12 dpi, the fecal samples from T2, T3 and T4 were 100% *Salmonella* positive, so there was no change among these treatments.

Salmonella generally infects chickens via the fecal–oral route with invasion starting from the intestinal tract at the distal ileum and ceca of the bird (Barrow et al., 2012). Oral ingestion of *Salmonella* gives rise to intestinal colonization (especially in the ceca) and shedding of the pathogen in excreted feces. The frequency and duration of intestinal colonization in poultry is affected by varied factors such as age, genetic line, immune status of the birds, by the strain and inoculum dose of *Salmonella* to which they are exposed. While young birds are more susceptible to the infection which may lead to early mortality, mature birds are more resistant and can host *Salmonella* in their intestinal tract without showing clinical signs (Brown et al., 1976). We compared our results from ceca samples with an earlier work focused on the bactericidal effect of nitrocompounds against *S.* Typhimurium *in vivo*. In this study, the inhibitory activity of NP was studied against *S.* Typhimurium in the ceca of 6 days of age broiler chicks. Two experiments were conducted with 6-day-old broiler chicks challenged via oral gavage with approximately 10^6 cfu (exact inoculum dose was not stated) of a novobiocin- and nalidixic acid–resistant *S.* Typhimurium. In experiment 1, chicks were allocated into three groups: 0 (control), 6.5, and 13 mg NP per bird. In experiment 2, chicks were allocated into four treatment groups: 0 (control), 13, 65, and 130 mg NP per bird. *S.* Typhimurium concentrations were reduced by up to 2 log units in the group treated with NP at the 13 mg per bird inoculum dose at both 24 and 48 h post-treatment relative to untreated controls. When compared with control treatments, *S.* Typhimurium concentrations were similarly reduced in all groups receiving NP regardless of dose level (Jung et al., 2004b). NP and 2-nitroethanol were supplemented into diets to reduce *S.* Enteritidis colonization in internal organs of mature laying hens. Inclusion of 100 ppm nitroethanol and 200 ppm NP into laying hen diets reduced cecal *S.* Enteritidis count (Adhikari et

al., 2017). In our study, we obtained a similar result compared to above-mentioned studies in which NP was used against *Salmonella* colonization the ceca of the birds. T4 (ST^{NAR} challenged + 200 ppm NP) showed a significant ($P < 0.05$) reduction, from 3.98 to 2.84 log10 cfu/g, in ST^{NAR} number in ceca collected on day 11 compared to T2.

In vitro studies have been conducted to reveal the inhibitory effect of nitrocompounds applied on fecal suspensions collected from different species. Additionally, the bactericidal activity of NP against select food-borne pathogens were tested *in vitro*. Fecal samples collected from a Holstein cow and were analyzed to reveal the inhibitory effect of NP against *S.* Typhimurium. After 24 h, concentrations of *S.* Typhimurium in fecal suspensions (already inoculated with 10^5 - 10^6 cfu/ml) containing 10 mM NP were 2.7 log10 cfu lower than concentrations in control incubations containing no NP. *S.* Typhimurium concentrations in fecal fluid containing 2.5 mM NP were 1.75 log10 cfu lower than concentrations in control incubations but this reduction was not significant (Jung et al., 2004a). *In vitro* effects of incubating an experimental chlorate product, nitrate, or select short-chain nitrocompounds, alone or in combination, against experimentally inoculated *S.* Typhimurium and indigenous *E. coli* in porcine fecal suspensions were tested. In this experiment, 2-nitro-1-propanol and 2-nitroethanol, but not necessarily nitroethane, exhibited bactericidal activity against *S.* Typhimurium and *E. coli* during 24 h of incubation in porcine fecal suspensions. When nitrocompounds are incubated with an added chlorate, the combined activity of the compounds is markedly enhanced. Coincubation of the fecal suspensions with nitrate also markedly enhanced the bactericidal effect of chlorate against these test bacteria (Anderson et al., 2007). The effect of the addition of nitroethane and nitroethanol at 21.8 mM in swine fecal slurries decreased the production of skatole, an odor pollutant in livestock waste, in swine fecal slurries at 24 h incubation (Beier et al., 2009). In the current study, our result was similar to previous studies in which NP was effective in reducing *Salmonella* presence in fecal samples. T4 (ST^{NAR} challenged + 200 ppm NP) lowered ($P < 0.05$) the prevalence of ST^{NAR} in feces compared to T2 (ST^{NAR} challenged = positive control) at 6 dpi.

L/GB and spleen

L/GB and spleen samples were assayed for the recovery of ST^{NR} at day 7,11,15 and 21 (Table 3.4). There were numerical decreases in ST^{NAR} prevalence in L/GB and spleen samples on varied days, but the difference among treatments was not significant compared to T2. *Salmonella* after ingestion by the chicken, encounters the acidic conditions of the proventriculus, the first barrier which is easily overcome by Salmonella which has an immediate adaptation to lower pH. *S.* Typhimurium can survive acidic conditions with a pH as low as 3 (Lee et al., 1994). *Salmonella* is able to colonize the small intestine and the underlying lymph tissue; however, the specific site of colonization along the tract based on bacteria serovars (Carter and Collins, 1974; Henderson et al., 1999). As the number of the organism increases, *Salmonella* is able to invade other body tissues after proliferation within the liver and spleen (Henderson et al., 1999). In the end, *Salmonella* is reintroduced via the gall-bladder into the small intestine (Carter and Collins, 1974). Supplementation of nitroethanol and NP to the diets of hens as an intervention strategy was used to reduce *Salmonella* prevalence in internal organs challenged with a 1 ml of approximately 1.9×10^8 cfu SE^{NR}. There was no difference in SE^{NR} reduction in L/GB samples after supplementation with either nitroethanol or NP (Adhikari et al., 2017). In the current study, although there were numerical decreases in ST^{NAR} prevalence in L/GB and spleen samples on various days, the differences among treatments were not significant compared to T2 (ST^{NAR} challenged = positive control).

Ileum immune gene expression

The pro- and anti-inflammatory cytokines interleukin (IL)-6, IL-1β and IL-10 and toll-like receptors (TLR)-4 gene expressions were analyzed in our study (Figure 3.3). Ileum samples from each treatment were collected at the end of the experiment. The house keeping gene, GAPDH, was used to normalize the immune cytokines. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed. Gene expression data were analyzed by difference in cycle threshold (ΔΔCT) method (Livak and Schmittgen, 2001).

Although mRNA expression of TLR-4, IL-6, IL-1β and IL-10 was detected in all treatment groups at the end of the study, there was no statistically significant difference in the expression of these genes between T1 (ST^{NAR} unchallenged = negative control), T2 (ST^{NAR} challenged = positive control) and T3 (ST^{NAR} challenged + 100 ppm NP). However, only the mRNA expression of IL-6 was upregulated ($P < 0.05$) in T4 (ST^{NAR} challenged + 200 ppm NP).

The primary function of the immune system is to identify and fight pathogens. In the presence of microorganisms in the gut, toll-like receptor (TLR), also known as pattern recognition receptors, may stimulate expression of proinflammatory cytokines such as IL-6 (Kaiser, 2010) as well as recognizing microbial-associated molecular patterns resulting in a chain of reaction triggering immune system (Aderem and Ulevitch, 2000). In addition, resistance to *Salmonella* has closely been associated with an upregulation of TLR-4 (Chaussé et al., 2011; Sadeyen et al., 2006) and abnormal expression of TLR-4 has been linked to susceptibility to *S.* Enteritidis infection in chickens (Gou et al., 2012). Components of the bacterial cell wall such as lipopolysaccharide stimulates TLR in host cells, which in turn triggers an inflammatory response in tissues, characterized by the production of cytokines (Young et al., 2002). IL-1β is released primarily by monocytes and macrophages during an infection, invasion, and/or inflammation (Copray et al., 2001). IL-6 is a multifunctional cytokine produced by different types of cells and has a role in acute-phase responses and immune regulation (Wigley and Kaiser, 2003). IL-6 activity has been found in various infectious diseases of chickens including salmonellosis and it has been proposed that induction of IL-6 may play a vital role in the response to different serovars of *Salmonella* in chickens (Kaiser et al., 2000). Invasion of chicken cells by *S.* Typhimurium leads to an 8-fold increase of IL-6 mRNA beginning a strong inflammatory and immune response, limiting the infections to the gut and preventing development of systemic disease (Wigley and Kaiser, 2003). It has been reported that *S.* Typhimurium challenge increased the mRNA expression of IL-6 mRNA level in the cecal tonsils of hens at 7 dpi (Bai et al., 2014). IL-10, a regulatory cytokine which has a role in inhibition of inflammatory response to *Salmonella* infection (Eckmann and Kagnoff, 2001), exhibits a negative effect on IFN-Ɣ expression by Th1 cells, promotes proliferation of Th2 cells (Rothwell et al., 2004), and deactivates macrophages (O'Farrell et al., 1998).

A study conducted to test the effects of probiotics on cytokine gene expression in chicken cecal tonsils following *Salmonella* infection demonstrated that although the IL-10 was expressed at all time points and in all treatment groups, there was no significant difference in the expression rate among the various treatments (Haghighi et al., 2008). Another study reported that oral administration of a combination of lactic acid bacteria reduced the *Salmonella* invasion and inflammation of broiler chicks by elevating the expression of IL-10 in cecal tonsils and the expression level of the IL-6 gene increased significantly in *Salmonella* (10^6 cfu/mL)-challenged

broiler chicks, but was downregulated by the probiotic treatment on 6 dpi (Chen et al., 2012). An increase in the expression of IL-6 was detected in the ileum of chickens challenged with *S.* Typhimurium (10^4 cfu/0.25ml) only and other groups treated with *Lactobacillus* on 1 dpi when compared to uninfected control group (Hu et al., 2015). The effect of supplementing nitrocompounds into the diets on the mRNA expression of TLR-4, IL-1β, IL-10 and IL-6 in the ileum of hens challenged with *S.* Enteritidis was tested (Adhikari et al., 2017). In this study, IL-10 mRNA expression was upregulated by adding 200 ppm of nitroethanol to the diets compared to unchallenged control treatment. However, IL-10 mRNA expression was not different from the unchallenged control treatment when the diets were supplemented with 100 and 200 ppm of NP, respectively. Addition of 100 ppm NP to the diets downregulated TLR-4 mRNA expression. Supplementation of nitroethanol and NP resulted in a downregulation of IL-6 expression in the ileum of hens challenged with *S.* Enteritidis. It was stated that NP has a potential to inhibit the expression of pro-inflammatory cytokines and interacts with the host either by modulating the gut microbiome or direct contact with *S.* Enteritidis (Adhikari et al., 2017). In the current study, it appears that NP supplementation as a potential intervention strategy against ST^{NAR} challenge was not effective to downregulate the expressions of TLR-4, IL-1β, IL-10 and IL-6 so it could not reduce the inflammation in the ileum caused by the infection. In addition, the inoculum dose (10^7 cfu/0.1 ml) of ST^{NAR} used in the experiment might be excessive or the supplementation levels of NP to the diets might be too low to adequately test the potential inhibitory activity of NP against ST^{NAR} infection.

CONCLUSION

NP supplementation of the diets did not have an adverse effect on growth performance for the 21 day grow-out period but did decrease the prevalence of ST^{NAR} in feces and reduced the colonization in ceca at specific sampling days during the study. NP did not downregulate the mRNA expression of cytokines having vital roles in the ileal response to ST^{NAR} infection. Further studies need be conducted to reveal the effective dose of NP to obtain a consistent or sustainable inhibition against bacterial infections in poultry.

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Table 3.1. Diet composition of broiler ration supplemented with different levels 2 nitro-1-propanol (NP)[1]

Item	**Diets**		
Ingredient (% of the diet)	**T1/T2**	**T3**	**T4**
Corn, Grain	62.27	62.26	62.25
Soybean Meal -48%	31.00	31.00	31.00
Limestone	0.65	0.65	0.65
Soybean Oil	1.04	1.04	1.04
Defluor. Phos.	1.74	1.74	1.74
DL-Methionine	0.33	0.33	0.33
Common Salt	0.30	0.30	0.30
Vitamin Premix [2]	0.25	0.25	0.25
L-Lysine HCl	0.32	0.32	0.32
Threonine	1.35	1.35	1.35
Mineral Premix [3]	0.08	0.08	0.08
Sand	0.67	0.67	0.67
NP	0.00	0.01	0.02
Calculated composition			
ME (kcal/kg)	3000	3000	3000
CP (%)	22	22	22
Ca (%)	0.9	0.9	0.9
Available P (%)	0.45	0.45	0.45
Met + Cys (%)	0.98	0.98	0.98
Met (%)	0.64	0.64	0.64
Lysine (%)	1.32	1.32	1.32

[1] T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control), T3 = ST^{NAR} challenged + 100 ppm NP, T4 = ST^{NAR} challenged + 200 ppm NP. [2] Supplied per kilogram of diet: vitamin A, 5511 IU; vitamin D3, 1102 ICU; Vitamin E, 11.02 IU; vitamin B12, 0.01 mg; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic Acid, 11.02 mg; Vitamin B6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 191.36 mg. [3] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg. [4] ME = Metabolizable energy; CP = Crude protein.

Table 3.2. 0-21 days growth performance values of broilers fed diets supplemented with different levels of 2 nitro-1-propanol (NP)[1]

	Diets				
Item[3]	**T1**	**T2**	**T3**	**T4**	***P- value***
BWG (g/bird)	558.34	576.53	574.53	613.24	0.7998
FI (g/bird)	4526	4419	4479	4360	0.9429
FCR (g:g)	1.28	1.26	1.27	1.32	0.3243

[1] T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control), T3 = ST^{NAR} challenged + 100 ppm NP, T4 = ST^{NAR} challenged + 200 ppm NP.

[2] Chicks were 12 per treatment group (n=12)

[3] BWG = Body weight gain; *FI = Feed intake*; *FCR = Feed conversion ratio*

Table 3.3 Presence and absence of ST^{NAR} in feces collected from broilers fed diets supplemented with different levels of 2 nitro-1-propanol (NP)[1].

	Incidence (%) of ST^{NAR} – positives[2]			
	Treatments			
	T1	**T2**	**T3**	**T4**
Day 3	0	83	100	50
Day 6	0	100	83	67
Day 9	0	100	100	100
Day 12	0	100	100	100

[1] T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control), T3 = ST^{NAR} challenged + 100 ppm NP, T4 = ST^{NAR} challenged + 200 ppm NP

[2] Chicks were 12 per treatment group (n=12)

Table 3.4 Presence and absence of ST^{NAR} in liver with gall bladder (L/GB) and spleen in broilers fed diets supplemented with different levels of 2 nitro-1-propanol (NP)[1].

								Incidence (%) of ST^{NAR} – positives[2]
Organs	**L/GB**				**Spleen**			
Days	**Day 7**	**Day 11**	**Day 15**	**Day 21**	**Day 7**	**Day 11**	**Day 15**	**Day 21**
Treatments								
T1	0	0	0	0	0	0	0	0
T2	58	100	92	42	17	58	42	58
T3	50	100	75	84	8	67	50	75
T4	58	100	92	50	25	58	67	50

[1] *T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (10^7 cfu/ml), T3 = ST^{NAR} challenged (10^7 cfu/ml) + 100 ppm NP, T4 = ST^{NAR} challenged (10^7 cfu/ml) + 200 ppm NP.*

[2] *Chicks were 12 per treatment group (n=12)*

Table 3.5 Chicken cytokines and toll-like receptor primer sequences

Gene[2]	**Primer sequence (5′–3′)**	**Gene bank accession no.**	**Fragment size, bp**	**Annealing temperature, °C**
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	K01458	116	56
TLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	190	56
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	AJ309540	233	59
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	NM001004414	103	56
IL-1ß	F: CACAGAGATGGCGTTCGTTC R: GCAGATTGTGAGCATTGGGC	NM204524	118	56

[2] *IL = interleukin; TLR = Toll-like receptor; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase*

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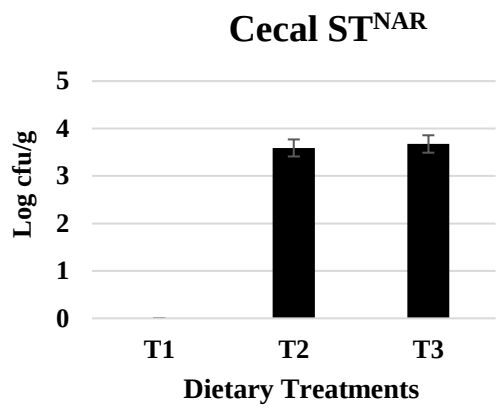

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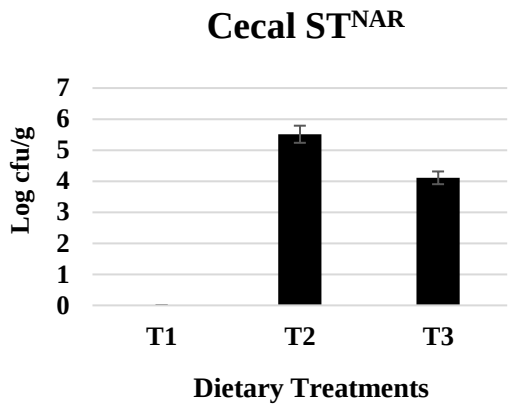

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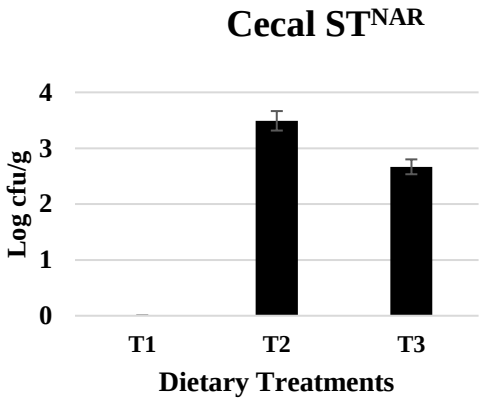

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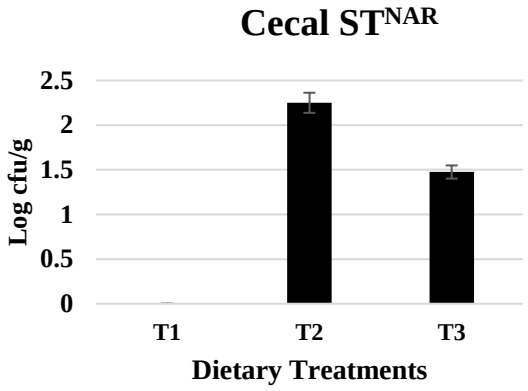
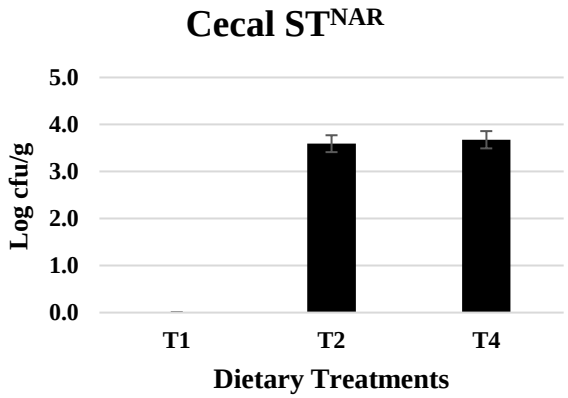


Figure 3.1 The comparison of T2 with T3 in terms of ST^{NAR} counts in ceca (n=12) collected on days 7, 11, 15 and 21. Broilers were fed diets supplemented with different levels of 2 nitro-1-propanol (NP). Chicks were challenged with Salmonella Typhimurium (ST^{NAR}). Error bars represent standard errors. Dietary treatments: T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control) and T3 = ST^{NAR} challenged + 100 ppm NP.

a) Day 7, b) Day 11, c) Day 15, d) Day 21.

a)



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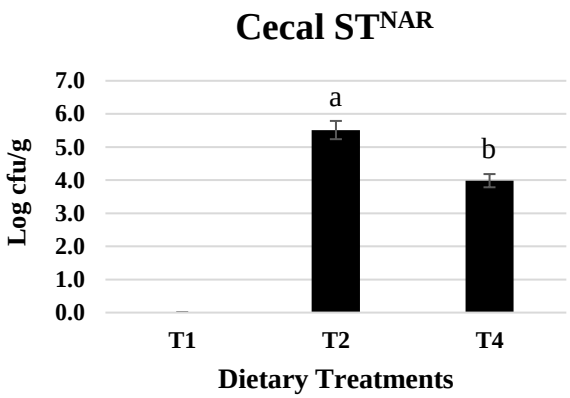

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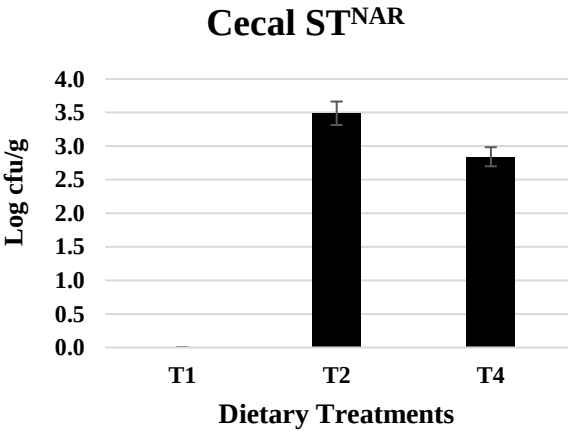

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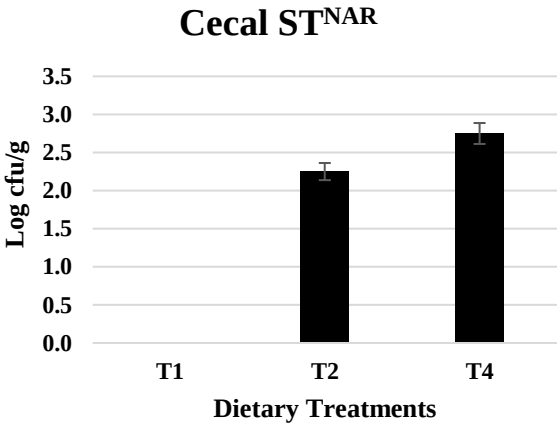
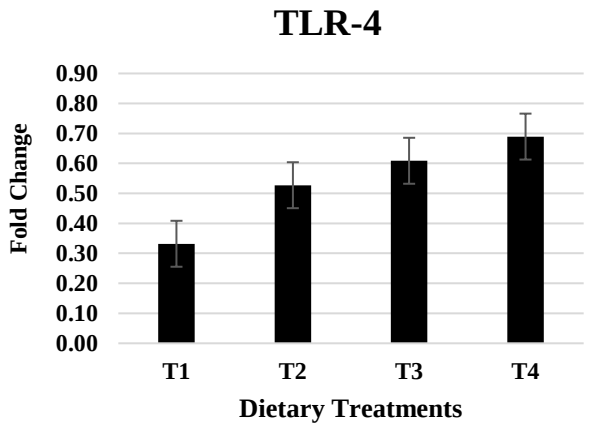


Figure 3.2 The comparison of T2 with T4 in terms of ST^{NAR} counts in ceca (n=12) collected on days 7, 11, 15 and 21. Broilers were fed diets supplemented with different levels of 2 nitro-1-propanol (NP). Chicks were challenged with Salmonella Typhimurium (ST^{NAR}). Error bars represent standard errors. Bars with different letters (a to b) differ significantly across the treatment groups ($P < 0.05$). Dietary treatments: T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control) and T4 = ST^{NAR} challenged + 200 ppm NP.

a) Day 7, b) Day 11, c) Day 15, d) Day 21.

a)



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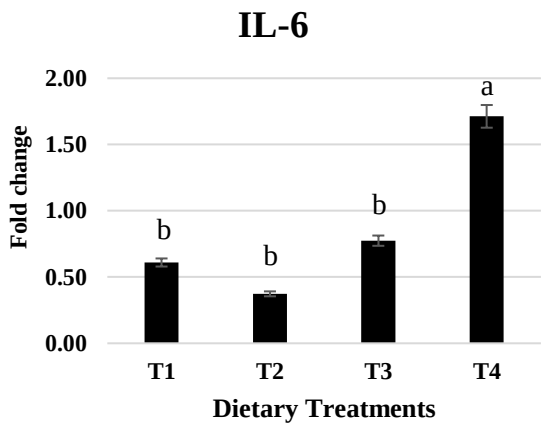

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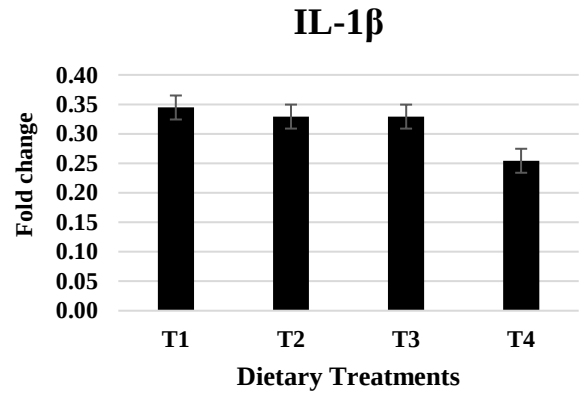

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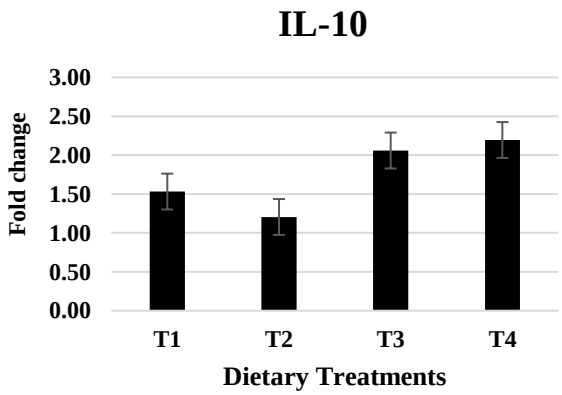

Figure 3.3 Ileal immune gene expressions of cytokines: a) Toll-like receptor (TLR)-4, b) Interleukin (IL)-6, c) IL-1β and d) IL-10. Chicks were fed diets supplemented with different levels of 2 nitro-1-propanol (NP) and challenged with Salmonella Typhimurium (ST^{NAR}). Error bars represent standard errors. Bars with different letters (a to b) differ significantly across the treatment groups (P < 0.05). Dietary treatments: T1 = ST^{NAR} unchallenged (negative control), T2 = ST^{NAR} challenged (positive control, T3 = ST^{NAR} challenged + 200 ppm NP and T4 = ST^{NAR} challenged + 200 ppm NP.

CHAPTER 4

CONCLUSION

Salmonellosis is a worldwide health problem with the majority of human salmonellosis associated with the consumption of contaminated poultry products such as meat and eggs. Research has been conducted to improve our understanding of *Salmonella* ecology and pathogenicity; however, *Salmonella* still causes new food safety challenges in the poultry industry and remains one of the major food-borne pathogens in the world. Therefore, controlling *Salmonella* in food animals is a huge concern due to high rate of contamination. Numerous feed additives have been used to reduce *Salmonella* infections in poultry, with the rate of success based on the additive used; however, alternative feed additives are still needed to create a sustainable inhibition or even elimination of *Salmonella* in the industry. In this study, we evaluated the effect of 2-nitro-1-propanol (NP) supplementation on the colonization of *Salmonella* in mature laying hens and broilers.

In the first study, challenging mature laying hens with two different inoculum doses of SE^{NR} served the purpose of revealing the inhibitory effect of NP as a potential feed additive. NP supplementation of the diets numerically reduced the counts of SE^{NR} in the ceca and altered the prevalence of SE^{NR} in other internal organs and feces. Also, it significantly downregulated the mRNA expressions of varied cytokines playing vital roles in the immune response of the ileum to *S.* Enteritidis infection in mature laying hens.

In the second study, NP supplementation of the diets against ST^{NAR} infection in broilers did not show adverse effects on growth performance within 21 days and significantly decreased the prevalence of ST^{NAR} in feces as well as reduction of colonization in the ceca. However, NP supplementation of the diets did not downregulate the mRNA expression of cytokines playing crucial roles in the ileum response to *S.* Typhimurium infection in broilers.

Further research is needed to determine the effective dose of NP to reveal its multifaceted effects against *Salmonella* infections so that a sustainable inhibition against bacterial infections can be achieved in the poultry industry.