DIETARY INTERVENTIONS TO REDUCE *SALMONELLA* ENTERITIDIS IN CECA AND INTERNAL ORGANS OF LAYING HENS: BACTERIOLOGY AND IMMUNOLOGY ASPECT

by

PRATIMA ACHARYA ADHIKARI

(Under the Direction of WOO KYUN KIM)

ABSTRACT

Control of *Salmonella* Enteritidis (SE) in mature laying hens is a major concern in the layer industry. We evaluated both OR and IC route of *Salmonella* by challenging White Leghorns using nalidixic acid resistant *Salmonella* Enteritidis (SE^{NAR}). After finding the OR route an easier and safer method to challenge hens, we carried out research incorporating prebiotics, probiotics, bacteriophages (BP) and nitrogen containing compounds into the diets.

Prebiotics, fructoligosaccharides (FOS) in the diet showed a trend towards decrease in the level of colonization of SE^{NAR}. Laying hens were challenged and fed two levels of FOS. Ceca, and internal organs were analyzed for SE^{NAR}. The results showed FOS lowered ceca SE^{NAR}. Fecal shedding was significantly ($P<0.05$) lower in the 1.0% of FOS supplemented groups compared to the SE^{NAR} challenge control. There was a significant upregulation ($P<0.05$) of mRNA toll-like receptor (TLR)-4 and interferon gamma (IFN-γ) levels but no changes in other cytokines, such as interleukin (IL)- 1ß, IL-6 or IL-10 mRNA levels. Immunohistochemistry (IHC) of ileum showed the numbers of immunoglobulin A (IgA) positive cells were higher in the

Salmonella challenged and 1.0% FOS supplementation, with the fewer numbers in challenged control plus 0.5% FOS.

Supplementation with BP, at 0.2% in a hens' diet showed greater efficacy in lower *Salmonella* in the internal organs. Immune cytokine mRNA expression levels of IFN-γ, interleukin IL-6 and IL-10, were significantly higher ($P<0.05$) in the ileum of SE^{NAR} challenged hens as well as challenged and BP treated hens when compared to the negative control.

Supplementation of 100 ppm NE and 200 ppm NP significantly reduced ($P<0.05$) ceca SE^{NAR} count. Cytokines mRNA levels of IFN-γ, IL-1ß, IL-6, TLR-4 and IL-10, were significantly upregulated ($P<0.05$) by SE^{NAR} challenge. Supplementation of probiotics showed a significant upregulation of mRNA levels of pro- and anti-inflammatory cytokines by SE^{NAR} challenge. Highest probiotic level resulted in a significant decrease in IFN-γ and elevation of IL-10 gene expression in the ileum. The research demonstrates that prebiotics, probiotics, BP and nitrocompounds can be included as the dietary strategies of the laying industry to reduce the SE^{NAR} infection on the farm and in the hens.

INDEX WORDS: *Salmonella* Enteritidis, prebiotics, probiotics, fructoligosaccharides, nitrocompounds, bacteriophage, oral and intraclaocal challenge, The University of Georgia

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PRATIMA ACHARYA ADHIKARI

B. V. Sc. and A. H., Institute of Agriculture and Animal Science, 2010

M. S., University of Manitoba, 2013

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PRATIMA ACHARYA ADHIKARI

Major Professor: Woo K. Kim
Committee: Nelson A. Cox
Robert M. Gogal
Casey W. Ritz
Douglas E. Cosby

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2017

DEDICATION

I would like to dedicate this dissertation to my beloved family. The accomplishment would be incomplete without support and love of my husband, Roshan Adhikari, my mom Sita, dad Somraj, sisters Sushma, Garima and Ashmita. Your love, understanding, and motivation cannot be described in these words. I am indebted to the encouragements and sacrifices of my mom, without which I would not have been able to achieve this honor. Love you so much mom.

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1. INTRODUCTION

The prevalence of *Salmonella enterica* is of both public health and economic concerns. Due to the threat of bacterial resistance against antibiotics, use of in-feed antibiotics at both therapeutic and sub-therapeutic levels is being limited by consumer demand and government regulation. Complete withdrawal of antibiotics as a growth promotants (AGP) has led to a demand for newer alternatives, such as prebiotics, probiotics, bacteriophages (BP), organic acids or nitrocompounds. Prebiotics and probiotics have become potential feed additives to improve gut health, immune system and microbiota by using various mechanisms of action, while enhancing growth performance in chickens. Recent studies on BP have showed it as one of the best methods for eliminating gut pathogens and producing a healthy gut.

In this study, we report on *Salmonella* recovery from the internal organs, ceca and feces of laying hens as well as the immune response after challenge with a nalidixic acid resistant *Salmonella* Enteritidis (SE^{NAR}). Bacterial colonization and pro- and anti-inflammatory cytokines signalling post *Salmonella* challenge and feed were studied. Mature laying hens are difficult to challenge with *Salmonella* as they have higher immunity and a mature gut microflora which works to prevent colonization of the gut. In this study, we initially evaluated the appropriate route and dose of the *Salmonella* challenge and thereafter, we assessed the different dietary strategies that might reduce the *Salmonella* colonization/shedding.

We hypothesized that there would be an effect from the dietary alternatives reducing the SE^{NAR} levels in the gut and organs as well as the prevalence in laying hens. We also hypothesize

that mRNA expressions of pro- and anti-inflammatory cytokines in the ileum would occur when hens are challenged with SE^{NAR} and supplemented with different forms of dietary alternatives.

2. LITERATURE REVIEW

***Salmonella* history and taxonomy**

Salmonella was isolated in 1885 by Salmon and Smith, initially diagnosed as hog cholera and classified as "Hog-cholerabacillus" then later as *Salmonella* Cholaerasuis. *Salmonella* spp. are gram negative, rod-shaped, facultative anaerobic bacteria which are motile by means of peritrichous flagella (Bell, 2004). *Salmonella* spp. grow at an optimal temperature of 35 - 40°C, with a growth range of 2 - 54°C depending on the serotype. Some biochemical characteristics of *Salmonella* that differentiates it from other bacteria of the same family are its fermentation of glucose with gas production, utilization of thiosulfate with hydrogen sulfide production, and inability to ferment lactose (Brenner et al. 2005).

The genus *Salmonella* is in the family Enterobacteriaceae, and is comprised of two species (*S. enterica, S. bongori*), 6 subspecies *enterica, salamae, arizonae, diarizonae, houtenae and indica*. Most of the serovars belong to the subspecies *S. enterica* subsp. *enterica*. and are human pathogens with the ability to cause a variety of diseases, such as enteritis and other systemic disease. Typhoidal *Salmonella* strains are human host-restricted organisms that cause typhoid fever (*S.* Typhus) and paratyphoid fever (*S.* Paratyphus), referred to as enteric fever. Non typhoidal *Salmonella* (NTS) strains may act as host specific such as *S.* gallinarium or *S.* pullorum (both of which are specific for poultry) or non-host specific which are capable of infecting or colonizing a broad range of insects, avian and vertebrate animals (Feasey et al., 2012). NTS such as Enteritidis cause mild to moderate gastroenteritis unlike typhoidal *Salmonella* which cause severe typhoid or paratyphoid fever which are often fatal There are 3 major antigenic combinations of *Salmonella* that has produced greater than 2,600 serovars (CDC, 2014). Each

Salmonella serovar has a unique combination of surface antigens (O: somatic or outer membrane antigens, H: flagellar antigens, Vi: capsular antigens) (Bell, 2004).

Epidemiology of Non-Typhoidal *Salmonella* (NTS)

Salmonella is a leading food-borne pathogen which causes the disease most common known as salmonellosis and is among the most commonly isolated bacterial infections. Further, *Salmonella enterica* subsp. *enterica* is one of the most common causative agents of food-borne illnesses in the world. *Salmonella* has been linked to approximately 1-1.2 million illness annually in the United States of which more than 40,000 cases require hospitalization with approximately 400 resulting in death (Scallan et al., 2011). In US alone, the healthcare costs related to salmonellosis is approximately $15.6 billion annually (Flynn, 2014).

Among the several serovars, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S*. Enteritidis; SE) is one of the serotypes most commonly reported as being associated with the consumption of infected eggs and poultry or meat products (Chai et al., 2012). Contamination of chickens with this serovar is asymptomatic in adult chickens, but poses a risk to human health. *Salmonella* Kentucky is most frequently recovered from carcasses and SE and *Salmonella* Typhimurium (ST) are the most commonly isolated serovars from human illness (Jones et al., 2008). Management practices at the farm levels, especially with laying hens, aim to minimize the risk of salmonellosis in humans.

Pathogenicity

Salmonella must overcome barriers such as low pH, bile salts, reduced oxygen concentration and resident microflora to colonize the lower intestinal tract. To possibly

overcome the stomach's acidic PH, *Salmonella* must induce an acidic tolerance response. After *Salmonella* is ingested, it is transported to small intestine where the primary infection occurs at specialized micro-fold cells (M cell), located in Peyers patches of the intestine. This invasion is mediated by a type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island (SPI) (Foley et al., 2013). After *Salmonella* gains access to the M cells and Peyers patch, it invades intestinal lymphoid tissues, destroying various phagocytes. Finally, *Salmonella* migrates through the lymphatics system ultimately reaching systemic circulation. This enables *Salmonella* access to the organs with high numbers of phagocytes and lymphocytes especially the spleen, liver and bone marrow (Griffin et al., 2011). However, an alternative mechanism for *Salmonella* invasion occurs when they are engulfed by dendritic cells (Mastroeni et al., 2003).

Cytokines and immunity

Cytokines are small secretory proteins released by many different cells, such as, T cells and macrophage, which mediate cell-to-cell interactions and communication (Zhang et al., 2007). Cytokines are classified by cell type and include lymphokine (made by lymphocytes), monokine (made by monocytes), chemokine (with chemotactic activities), interleukin (made by one leukocyte and acting on other leukocyte) (Zhang, et al., 2007). Others include interferons and growth factors.

Cytokines are an integral part of the immune response to *Salmonella* in avian species (Swaggerty et al., 2006). Pro-inflammatory cytokines are involved in the up-regulation of inflammatory reactions. Some of these include interleukin (IL)- 1ß, tumor necrosis factor (TNF) - α, IL-6, and IL-1. IL-1ß is released from monocytes and macrophages during inflammation, and is crucial to host defense responses to infection and injury (Lopez-Castejon et al., 2011).

Other cytokines related structurally and actively to IL-1ß include IL-6 and TNF- α. IL-6 is also a pro-inflammatory, multifunctional cytokine that is usually elevated after pathogen infection like *Salmonella* (Wigley et al., 2003). The expression of IL-6 limits *Salmonella* in avian gut by inducing immune responses. TNF- α is another inflammatory cytokine produced by macrophages, T cells and NK cells which causes inflammation. After an infection with *Eimeria*, release of TNF- α from chicken macrophages was observed (Zhang et al., 1995). IFN-γ is the pro-inflammatory cytokine produced by activated T-cells with a role in host defense for combating against *Salmonella* (Bao et al., 2000).

Protection against *Salmonella* is due to expression of IFN-γ, TNF-α, IL-12, and IL-18, whereas down-regulation of inflammatory responses to *Salmonella* is due to IL-4 and IL-10 (Eckmann et al., 2001). For instance, IFN-γ is produced by natural killer (NK) cells in response to IL-12 and IL-18 and mediates the upregulation of nitric oxide synthase (iNOS)-dependent macrophage antibacterial mechanisms (Mastroeni, et al., 2003; Penha Filho et al., 2012). IFN-γ also plays a major role in controlling *Salmonella* infection with elevated levels correlating with better rates of bacterial clearance from the chicken. Innate and adaptive immunity are rapidly initiated following oral infection, but these effector responses can also be hindered by bacterial evasion strategies. *Salmonella* encounters different conditions in the gastrointestinal tract including high temperature, low pH, reduced oxygen tension, bile salts, and competing microorganisms (Foster et al., 1995; Park et al., 2008). Initial detection of *Salmonella* is possible by early activation of CD4 T-cell within the Peyer's patch after which rapid acquisition of Th1 cells occur. Thus, this stimulates the production of IFN-γ in large amount. Similarly, Th17 cells are found associated with *Salmonella* clearance. Innate B-cell responses to TLR ligands have

also been shown to be important for the development of Th1 responses *in vivo*. Overall, Th1, Th17 and B-cells help protecting against *Salmonella* infection (Griffin, et al., 2011).

The first line of defense against the microorganism is the innate immunity that results in various inflammatory and anti-microbial response (Broz et al., 2012). The initial cellular barrier for *Salmonella* as it passes through the GI system is the gut epithelium which includes a thick mucus layer formed by goblet cells. The mucosal immune system also consists of immunoglobulin A (IgA) and mucosa-associated lymphocytes and leukocytes (Sheela et al., 2003). Macrophages and neutrophils also show up in the early responses to *Salmonella* infection. Requirement of neutrophils to the gut mucosa is a part of innate immunity where such cells are crucial to prevent the dissemination of *Salmonella* to gut. Salmonella exists the epithelium cells and transits to phagocytes where it is susceptible to neutrophils killing (Broz et al. 2012). On the other hand, *Salmonella* phagocytosed by macrophages are subjected to intracellular killing mechanisms including production of lysozyme, toxic reactive oxygen and nitrogen intermediates and exposure to the bactericidal peptides (Srikanth and Cherayil, 2007).

The innate response to *Salmonella* infection is followed by adaptive immune response (the acquired immunity) and includes both humoral and cell mediated responses, which play a major role in the clearance of *Salmonella* (Sheela, et al., 2003). The largest portion of acquired immunity is conducted by cell-mediated helper cells like CD4 cells (Hughes et al., 2002), including CD3 and CD8. CD4 cells when activated, secrete specific cytokines mediated by their mechanism of activation. $CD4^+$ TH1 cells produce and secrete IFN-γ and TNF-α inducing a pro-inflammatory cell-mediated state whereas $CD4^+$ TH2 cells produce IL-4, IL-5, and IL-13 and induce B cell activation (Hughes, et al., 2002).

Salmonella **Enteritidis in laying hens**

Salmonella Enteritidis is the most common serovar frequently found in the eggs of laying hens. The bacteria persist in the laying houses and throughout the subsequent flocks (Carrique-Mas et al., 2009; Wales et al., 2007). Laying hens infected with SE show no clinical signs or symptoms. The bacteria will extensively colonize the intestinal tract, initially, followed by the invasion of various internal organs (Gast, 1994). The bacteria colonize the intestinal tract with the crop and ceca being the organs primarily infected. Intestinal colonization, organ invasion, and egg contamination are the major pathological results of SE contamination in laying hens.

Naturally, non-host specific *Salmonella* infection, like SE, in hens occurs primarily through the oral route (Revolledo et al., 2012). Laying hens orally challenged with doses of 10^4, 10^6, or 10^8 cfu of a phage type 13a strain of SE resulted in 30 to 90% of recovery in liver samples at 5 days post inoculation (dpi) whereas the recovery was only 0 to 40% at 20 dpi (Richard K. Gast et al., 2011). However, for all above 3 inoculums, the recovery decreased as the dpi increased. The dose concentration is directly related to the shedding of *Salmonella* in feces. Further, the duration of SE PT4 fecal excretion was directly related to the concentration of the orally delivered dose (Humphrey et al., 1991). When inoculums of 10^3, 10^6 and 10^8 were administered to the birds, the mean periods of excretion of SE in feces were 3, 16 and 37 days, respectively (Humphrey, et al., 1991). Most hens seem to stop shedding detectable levels of bacteria in their feces approximately three weeks after the primary infection (Shivaprasad et al., 1990; Gast et al., 2005).

Dose and routes of SE infection

Different routes of challenge to laying hens at various SE doses could colonize different reproductive organs as well as eggs. The number of SE cells provided to the hens can affect the frequency and occurrence of egg contamination (Gast et al., 2013a). When hens were challenged with different doses of 10^4, 10^6 or 10^8 cfu/mL of SE, the higher doses were able to increase yolk and albumin contamination. Contamination of reproductive organs should eventually lead to egg contamination. However, there have been variation in the rate of contamination of the egg parts. For instance, 9.6% of yolk and 3.6% of albumin were contaminated by an oral dose of 10^6 cfu/mL of SE (Timoney et al., 1989). In another study, a similar dose provided intravenously was able to colonize only 6.9% of yolk and 2.3% of albumin (Okamura et al., 2001). In another study by Gast et al. (2002), oral challenge with 10^9 cfu/mL SE was able to contaminate 4–7% of yolk and 0 – 2% of albumin. Generally, when challenging hens via the oral route a higher dose was needed compared to either an intravenous or cloacal route. In other words, intravenous, cloacal or intravaginal routes of inoculation need fewer (10^5 to 10^7) SE cells to colonize the egg parts (Gast, et al., 2002; Kinde et al., 2000; Miyamoto et al., 1997). However, the results can be very variable depending upon many known and unknown conditions such as breed of hens, environment, age and maturity of the birds, past infection with SE, stress and others.

Dietary intervention strategies to reduce and control *Salmonella* in chickens

Subtheraputic antibiotics have been used as growth promoters (AGP) in healthy poultry as growth promotants for many years as well as in controlling intestinal pathogens such as E. coli and *Clostridium pefringens* for many years (Verstegen et al., 2002). AGP improves animal health and performance by suppressing clinical and sub-clinical diseases However, due to the growing

concerns regarding the antibiotics resistance strains of *Salmonella* and other pathogenic bacteria and the lack of development of new antibiotics in near future, various feed alternatives have become more widely used (Seal et al., 2013). The use of such dietary interventions strategies help eliminate the problem of antibiotics resistance due to misuse and/or overuse of antibiotics to control infections such as *Salmonella*. Prebiotics, probiotics, bacteriophage (BP), nitrocompounds are a few currently being looked at for use.

Prebiotics

Prebiotics are non-digestible feed ingredients, which beneficially affect the host by selectively stimulating the activity of one or a limited number of bacteria in the colon (Gibson et al., 1995). Prebiotics require characteristics such as: 1) not being absorbed in the upper gastrointestinal tract (GIT), 2) resistance to acidic pH, 3) stimulating growth of beneficial bacteria, and 4) enhancing the host defense system (Patterson et al., 2003). Commonly used prebiotics include various types of oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), mannanoligosachharides (MOS) and inulin. Prebiotics are not digested or absorbed in the upper GIT but, instead provide a food source for host beneficial bacteria, including *Lactobacillus* (LAB) and *Bifidobacteria* in the lower GIT. This allows for an expansion of beneficial bacteria in the GIT, minimizing sites for the attachment by gut pathogens. Thus, prebiotics affect gut microbial population by providing feed to beneficial bacteria reducing gut colonization of various pathogens, including *Salmonella*. GOS were shown to increase certain beneficial bacteria including LAB, *Bifidobacteria* or fermentation products of the two (Macfarlane et al., 2008). MOS are commonly derived from yeast and the outer cell of yeast. MOS modulate the immune system and eliminate pathogens from the intestinal tract (Fernandez et al., 2002).

Mechanism of action of prebiotics

There are several mechanisms in which prebiotics exhibit beneficial effects on the host, broadly classified into antimicrobial, immunological, epithelial, and microbial modulation from beneficial bacteria. The principle mechanism of action of prebiotics is via immunomodulation by selective growth of lactic acid-producing bacteria. Prominent mechanisms for MOS to act is the ability to bind with mannose-specific lectin of gram-negative pathogens, which express Type-1 fimbriae (*E. coli,* for example) facilitating their excretion from the intestine (Thomas et al., 2004). The increased concentrations of antimicrobials, such as short chain fatty acids (SCFA) (acetate, propionate, butyrate, etc.) modify the bacterial ecosystem by lowering pH that becomes intolerant to pathogens. Since pH within the cecum is low, prebiotics were shown to inhibit pathogen growth and stimulate growth of beneficial bacteria, like *Bifidobacterium* and LAB. The process is the most effective in cecum (Cummings et al., 2001). The overall gut integrity is improved from production of SCFA (Alloui Mohamed et al., 2013). Stimulation of the immune system includes increasing antibody production, such as secretory IgA, IgG and phagocytic cells activation (Macfarlane, et al., 2008). Prebiotics beneficially interact with host's physiology by selectively stimulating favorable microbiota in the intestinal system (Macfarlane, et al., 2008). Microbial flora, mostly consisting of LAB and *Bifidobacterium* sps., support the animal's defense system against pathogens invasion by stimulating GIT immune response (Mead, 2000). According to Seifert et al. (2007), prebiotics like inulin and oligofructans modulate the immune system directly. However, it is unclear whether prebiotics directly affect the pathogen or host in a microbiota-independent manner. Oligosaccharides, like beta-glucans stimulate performance by enhancing phagocytosis and proliferating monocytes and macrophages (Novak et al., 2008). Prebiotics compete for sugar receptors, thus preventing adhesion of pathogens including

Salmonella and *E. coli* (Iji et al., 1998). MOS have receptor properties for fimbriae of *E. Coli* and *Salmonella* leading to elimination of such pathogens with the digesta flow rather than binding to the mucosal receptor (Fernandez, et al., 2002).

Effect of prebiotics in chickens (growth performance, immune response and morphology)

Some major prebiotics shown to be beneficial to performances and gut health are shown in Table 1. Supplementation of MOS and FOS in broilers was found to be associated with improved body weight gain (BWG), feed conversion ratio (FCR) and carcass weight (Baurhoo et al., 2007; Sims et al., 2004; Xu et al., 2003). Production of SCFA is the reason for better growth performance as it increases the partition of nutrients into other body tissues (Ajuwon, 2015; Lu et al., 2012). Performance, egg cholesterol and gut microflora were improved by adding inulin to the diet of laying hens (Shang et al., 2010). Improvements in egg shell and bone quality and increased overall mineral metabolism due to inulin or oligofructose was also observed (Swiatkiewicz et al., 2012).

MOS, FOS and inulin modulated the immune responses in the gut-associated lymphoid tissue (GALT) of chickens like cecal tonsil, enhanced antibody titers of plasma IgM and IgG, cecum IgA levels, mucin mRNA expression and enhanced intestinal immune functions (Huang et al., 2015; Janardhana et al., 2009b). Prebiotic treated group were similar to an AGP treated group and improved GALT immunity in chickens (Janardhana et al., 2009a). Prebiotic-mediated immunological changes may in part be due to direct interaction between prebiotics and gut immune cells as well as due to an indirect action of prebiotics via preferential colonization of beneficial microbes and microbial products that interact with immune cells (Janardhana, et al., 2009b). In a study by Huang, et al. (2015), dietary inulin supplemented at 5-10 g/kg had better effects on feed intake (FI), and intestinal proportion of T $CD4^+$ lymphocyte and $CD4^+/CD8^+$ ratio

in ileum tissue, IgA in ceca content and IL-6 and IFN-g decreased in a starter phase (0-21 d), with no any beneficial effect on d 42 broiler chicks. It is presumed that increased villi height is associated with the increased absorption of feed due to increased surface area transporting more feed nutrients (Amat et al., 1996). Feeding MOS and lignin in poultry has resulted in low pH, high production of SCFA like butyric acids and healthy gut particularly, increased villi height (Baurhoo, et al., 2007). Morphological developments of intestine as well as balanced microbial community were observed in MOS-fed broilers (Baurhoo et al., 2009).

Abundance of LAB and *Bifidobacteria* in chicken gut has been associated with the prebiotics supplementation, mainly MOS, FOS and inulin type fructans in poultry (Baurhoo, et al., 2007; Geier et al., 2009; Kim et al., 2011). Length of time for adaptation and the exposure of GIT microbes to the supplemented FOS play major role in producing positive effect due to FOS. When FOS was added for a longer duration, it produced better results with villi height and crypt depth of intestine (Hanning et al., 2012).

Roles of prebiotics in reduction of pathogens

Studies have documented increased *Bifidobacteria* and LAB counts and decreased *Salmonella, E. coli and Clostridium perfringens* numbers in broilers fed MOS, FOS, fructan and lignin supplemented diets (Baurhoo, et al., 2007; Cao et al., 2005; Fernandez, et al., 2002; Macfarlane, et al., 2008; Spring et al., 2000; Zhao et al., 2013) (Table 1). The population of *Clostridium* and *E. coli* decreased with 0.25% FOS and 0.05% MOS supplementation whereas LAB diversity increased in the ileum of broilers by these two prebiotics (Kim, et al., 2011). Feeding lignin or MOS increased cecal population of LAB and *Bifidobacteria* whereas *E. coli* was reduced in the ceca of broilers (Baurhoo, et al., 2007). A possible explanation may be competitive exclusion (CE) where LAB and *Bifidobacteria* competed against *E. coli* for the

binding sites. On the other hand, bacteriocin produced by LAB and organic acids produced by *Bifidobacteria* might suppress the colonization of pathogenic bacteria. Increase in the lactic acid production resulted in elimination of pathogens like *Clostridium* from ileum and ceca, and the growth performance was better in chickens fed both prebiotics and probiotics (Abudabos et al., 2015). The increase in intestinal microbial diversity is believed to have positive effects on gut and overall host health (Janczyk et al., 2009). Due to the low pH created by SCFAs, pathogens like *Salmonella* and *Campylobacter* are reduced from the gut. Fermentation products such as SCFA increased after prebiotic supplementation because of oligosaccharide fermentation by resident microbiota (Macfarlane, et al., 2008). Thus, production of SCFA and reduction of gut pH are key mechanisms of prebiotics in order to limit pathogen colonization and maintain optimal growth performance and health in poultry.

Probiotics

Probiotics are either single or mixed culture of live microorganisms which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). According to Food and Agriculture/World's Health Organization (FAO/WHO), probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). The characteristics of good probiotics are: 1) they should be a strain capable of exerting beneficial effects on the host animal, 2) they should be non-pathogenic and non-toxic, 3) they should be present as viable cells, 4) they should be capable of surviving and metabolizing in the gut environment and 5) they should be stable and capable of remaining viable for periods under storage and field conditions (Fuller, 1989). Probiotics are also called 'direct fed microbials'. Commonly used probiotics in animals are: LAB (*L. bulgaricus, L. plantarum, L. acidophilus, L. helveticus, L. lactis, L. salivarius, L. casei, Bacillus subtilis*),

Enterococcus (*E. faecalis, E. faecium*), *Bifidobacterium* spp., *Steptococcus, Enterococcus, Lactococcus, E. coli* and fungi and yeast (*Aspergillus oryzae, Saccharomyces cerevisiae*) (Huang et al., 2004). LAB and *Bifidobacterium* species have been used most extensively in humans as well. *Bacillus, Enterococcus*, and *Saccharomyces* yeast have been the most commonly used organisms in livestock (Ferreira et al., 2011). Multiple strains may be more beneficial than a single strain as they act on different sites and provide different modes of action that create synergistic effects (Klose et al., 2006; Sanders et al., 1999; Timmerman et al., 2004).

Mechanism of action of probiotics

Mechanisms of action of probiotics mostly depend on the particular strains of bacteria or microorganisms used. The capacity of probiotics to modulate the intestinal bacteria is the principle mechanism. SCFA production, intermediary metabolites with antimicrobial activity, interaction with receptor and stimulation of immune function have been found common in probiotics feeding (Sherman et al., 2009). The most common MOA of probiotics is competitive exclusion (CE). CE was originated on the finding that the newly hatched chicken could be protected against *Salmonella* colonization of the gut when they were provided with a suspension of gut content prepared from healthy adult chickens (Nurmi et al., 1973). CE refers to the physical blocking of opportunistic pathogen colonization and altering the environmental niches within the intestinal tract like intestinal villus and crypts leading to better immune system (Duggan et al., 2002). It involves the addition of a non-pathogenic culture either single or multiple strains in order to reduce the pathogenic bacteria in the GI tract (Fuller, 1989). Simply, the non-pathogenic bacteria compete with the pathogenic bacteria for energy and nutrients in the gut. CE due to probiotics includes competition for physical attachment sites, enhancement of host immune system, production of antimicrobial compound like SCFAs and bacteriocins or

colicins from metabolic reactions (Callaway et al., 2008; Stahl et al., 2004). A front line of defense against the adverse effect of pathogens is provided by probiotics showing its antimicrobial effect. For example, lactic acid producing probiotics show antimicrobial effects by reducing the pH of the gut (Corr et al., 2007; Fayol-Messaoudi et al., 2005). On the other hand, some strains of LAB that are used as probiotics inhibit the virulence factor expression of pathogens and directly reduce their invasiveness (Carey et al., 2008; Lavermicocca et al., 2008). By competing for the common niche in the gut, probiotics exclude the sites for pathogen replication (Wu et al., 2008). It has been shown that lactic acid producing bacteria produces lactic acid, which is used by anaerobic butyrate producing bacteria for producing large amount of butyric acids, and this is called cross feeding (Duncan et al., 2004).

Effects of probiotics in chickens (Growth performance, immune response and intestinal morphology

The specific action of probiotics to modulate the immune function depends on the strain of probiotics (Huang, et al., 2004). Through the interaction of host and the probiotic cultures, enhancement of both natural and specific antibodies, interferon or cytokines as well as activation or suppression of T-cells that eventually lead to the cytokine expression have been observed in many studies (Castellazzi et al., 2007; Haghighi et al., 2008; Haghighi et al., 2005). Probiotic strains differentially modulate pro- and anti-inflammatory cytokines in order to balance pro-and anti-inflammatory responses (Foligne et al., 2010). Pro-inflammatory cytokines like TNFα, IL-1β and IL-6 released from monocytes and macrophages are augmented by LAB and *Bifidobacteria* (Helwig et al., 2006; Miettinen et al., 1998). Anti-inflammatory cytokine like IL-10 is also released from cells like dendritic cells and monocytes in chickens due to LAB or *Bifidobacteria* feeding (Braat et al., 2004; Smits et al., 2005). Moreover, production of

antimicrobial peptides and cytokines such as IL-12, IFN-γ, IL-10, and TNF-α from the intestinal epithelium has been found in LAB-fed broilers (Arvola et al., 1999).

The major effects observed in poultry due to probiotics including yeast cultures supplementation are in growth performance, meat quality, immune response, intestinal morphology, and intestinal microbiota (Table 2) (Bai et al., 2013; Gao et al., 2008; Samanya et al., 2002). In poultry, probiotics feeding has been shown to maintain balanced flora mainly by CE (Kizerwetter-Swida et al., 2009), improve feed consumption / digestion and gut health (Awad et al., 2009), and stimulate the immune system (Brisbin et al., 2008). Probiotics may potentially stimulate growth through increased SCFA production in poultry and through selective regulation of insulin signaling in different tissues (Ichikawa et al., 2002). SCFA like acetate, propionate and butyrate are used as energy source in tissues. Particularly in chickens, butyrate has shown beneficial effects by selectively partitioning the nutrients away from liver and adipose tissues towards muscles through upregulation of insulin receptors in muscle (Matis et al., 2015). SCFA production due to probiotics helps to promote intestinal health and integrity by directly stimulating epithelial cell proliferation and acts as the epigenetic regulators of the gene expression of multiple genes that improve growth and overall health of poultry (Kang et al., 2014; Meimandipour et al., 2010; Wu et al., 2009). Carcass and meat quality are also improved by supplementation with *Bacillus subtilis*; *Bacillus subtilis* and *Bacillus licheniformis*; and *Saccharomyces cerevisiae* in broilers (Pelicano et al., 2003). In another study, both meat quality and growth performance were improved by diet supplemented with *Bacillus licheniformis* (Liu et al., 2012).

Growth performance was better with 7.5 g/kg of yeast culture, whereas immune modulation by production of mucosal IgA was better with yeast culture supplemented diets at

level of 2.5 g/kg among the various levels provided (0, 2.5, 5.0 and 7.5 g/kg) (Gao, et al., 2008). Similarly, probiotics containing LAB and *Saccharomyces cerevisae* supplemented at 0.2% enhanced growth performance as well as T cell function in broilers (Bai, et al., 2013). Probiotics supplementation increased production of natural antibodies like intestinal IgA, serum IgG and IgM, all indicators of enhanced immunity (Haghighi et al., 2006). Chickens fed dietary *B. subtilis* for 28 days tended to display greater growth performance as well as pronounced intestinal morphology changes, including prominent villus height, extended cell area and consistent cell mitosis compared to the controls feed (Samanya, et al., 2002).

Production of cytokines leads to the overall immune modulation in the chicken. LAB has shown the modulating effects on the immune system of both layer- and meat-type chickens. The ability of LAB to modulate chicken cytokines, toll-like receptors and chemokine gene expression has been demonstrated (Brisbin et al., 2011; Haghighi, et al., 2008). Increase in the antibody secretion due to increase in B-lymphocytes (humoral immunity) is a potential mechanism by LAB in boosting the immunity in broiler chicks (Apata, 2008). The increase in the population of white blood cells may be attributed to the presence of LAB in the diet stimulating the production of lymphocytes, particularly the B-cells that are responsible for forming antibodies.

Role of probiotics in reduction of pathogens

Pathogens like *Salmonella*, *Campylobacter*, *Clostridium* and *E. Coli* are displaced or reduced by probiotics bacteria supplementation in chickens (Table 2). Enhancement of gut barrier function through modulation of the cytoskeleton and epithelial tight junctions in the intestinal mucosa is one of the mechanisms of probiotics in preventing pathogen infection (Ng et al., 2009). Probiotics have been shown to inhibit pathogens both *in vitro* and *in vivo* (Thomke and Elwinger, 1998). Supplementation of probiotics-in feed helps in reducing *Salmonella*

colonization in ceca and other internal organs either by the mechanism of CE (Nurmi, et al., 1973) or reduction of the colonization of opportunistic bacteria in the GI tract (Callaway, et al., 2008; Patterson, et al., 2003; Vicente et al., 2008). However, the idea behind using probiotic cultures as CE in chickens was that the chickens should be *Salmonella* free and the CE cultures should be given at the earliest period of age (Mead, 2000). LAB culture has shown accelerated development of healthy and beneficial microflora in broiler chickens, providing increased resistance against *Salmonella sp.* infections (Higgins et al., 2010; Vicente, et al., 2008). The mucosal flora is an important component to limit *Salmonella* colonization, and microbial attachment to the mucosal surface is the key to *Salmonella* exclusion (Mead, 2000). Innate and adaptive responses in broilers infected with *Eimeria* and treated with *Lactobacillus*-based probiotic were also observed where surface markers of immune responses like cluster of differentiations, CD3, CD4, CD8, and αβ T-cell receptor (TCR), were increased in pronounced numbers in feces, sera and intestinal washes (Dalloul et al., 2003).

Oral administration of *Klebsiella pneumoniae, Citrobacter diversus*, and *E. coli* significantly reduced *Campylobacter jejuni* colonization of chickens (Stern et al., 2001). Downregulation of flagellar genes including flaA by LAB supplementation was able to reduce pathogenesis of the *Campylobacter* in chicken (Ding et al., 2005). Similarly, a study showed that probiotics were able to enhance the cell-mediated immunity and the shedding of fecal oocysts of *Eimeria acervulina* (Dalloul et al., 2005). Mortality due to Necrotic Enteritis was reduced from 60 to 30% due to lactic acid bacteria added in feed (Hofacre et al., 2003). Dietary supplementation of *Bacillus subtilis* reduced FCR as well as reduced intestinal lesions in broilers challenged with *Clostridium* and *Eimeria* (Jayaraman et al., 2013). A study with *Bacillus* in *Eimeria maxima* infected broiler chickens found that *Bacillus subtilis* reduced the clinical signs

of experimental avian coccidiosis and increased parameter of innate immunity like nitric oxide, in broiler chickens (Lee et al., 2010b).

Although probiotics have great potential to improve growth performance and immune function, and prevent pathogen colonization in poultry, the positive effects of probiotics supplementation are not always warrant. The reason behind the variability due to probiotics may include physiological state of bird, actual microbiota already present in the gut, dose and nature of strains used for probiotics culture, probiotics species, method of preparation of probiotic strains, route of administration and timing of application relative to any pathogen challenge (Ajuwon, 2015; Brisbin, et al., 2011; Huyghebaert et al., 2011).

Bacteriophage

Mechanism of action and studies in chicken

Bacteriophage (BP) are small viruses that infect and replicate within bacteria (Deresinski, 2009). Such viruses can lyse the specific bacterial cells during their rapid replication. Like all viruses, phages are obligate parasites. Bacteriophages were discovered nearly a century ago and were used for more than 60 years for bacterial control in the pre-antibiotics period. They were first described by the British pathologist Frederick William Twort in his study of *Micrococcus* in 1915. Generally, there are 3 basic steps in lifecycle of BP; adsorption, infection and release. The adsorption stage begins as BP attaches itself to the host bacterium and there is a collision between the bacterium and BP. Lipopolysaccharides, teichoic acids or flagella are recognized by the BP to attach to the host. There occurs an injection of DNA material from BP into the bacterium cells and subsequently multiple copies of BP form inside the bacterium. The bacterial cell wall lyses and releases large numbers of BP into the environment (Thiel, 2004).

Due to the host specificity, BP are often of limited use in poultry and other livestock as a pre-harvest control measure of infection. BP has been used as a pre-harvest control measure in both broilers and layers. BP was used against *Campylobacter* infection to reduce the contamination on broiler skin and it reduced the numbers of bacteria up to 2.3 logs cfu/cm^2 on the skin (Atterbury et al., 2003). In another study by Atterbury et al. (2007a), *SE* colonization in the ceca was reduced by ≥ 4.2 $\log_{10}$ cfu whereas ST was reduced Typhimurium by ≥ 2.19 $\log_{10}$ cfu. A number of previous studies have demonstrated a role for BP in reducing *Salmonella* in food, meat and poultry products (Bielke et al., 2007; Bigwood et al., 2008; Fiorentin et al., 2005; Goode et al., 2003).

Nitrocompounds

Mechanism of action and studies in chicken

Nitrocompounds such as 2 nitroethanol (NE) and 2 nitropropanol (NP) have been used as alternative methods to reduce antibiotic resistance both *in vitro* and *in vivo*. Several of the nitrocompounds when supplemented at 50 to 100 mM have been shown to inhibit bacterial degradation of uric acid by avian gut microbes (Kim et al., 2009). Early work by Angermaier et al. (1983) demonstrated the inhibition of electron transfer in a reconstituted clostridial ferredoxin-hydrogenase system by NE and thus it is possible that a similar mechanism may be involved with structurally related nitrocompounds. Broad-spectrum antibacterial effect of NP against *Salmonella, E. coli and E. feacalis in vitro* was observed (Jung et al., 2004). A bactericidal effect was observed against foodborne pathogens like *Salmonella* and *Campylobacter* (Horrocks et al., 2007).

A few nitrocompounds have been effectively tested as an anti-methanogenic intervention in ruminal contents. In chicken, ceca methane was completely depleted when treated with nitrocompounds (Saengkerdsub et al., 2006). Ammonia concentrations and volatilization

decreased with the addition of short chain nitrocompounds like NE, NP and nitropropanoic acid on *in vitro* incubations of ruminal fluid (Anderson et al., 2003; Kim et al., 2006). Earlier studies have shown that oral administration of NP resulted in significant reductions of *Salmonella* Typhimurium and naturally occurring *Campylobacter* concentrations, thus demonstrating that this compound may have an application in reducing foodborne pathogens in animals (Jung and others 2003, 2004). Besides NP, NE and nitroethane showed inhibition against *Listeria* monocytogens and *L*. innocua *in vitro* (Dimitrijevic et al., 2006). By reducing the chlorate activity in swine gut contents, reduction of *Salmonella* and *E.* coli was observed after nitrocompounds were used as supplements in the diet (Anderson et al., 2004b). Whether the nitrocompounds can be developed for use as feed additives to control *Campylobacter, Listeria*, and *Salmonella* will undoubtedly depend on further studies examining their potential, toxicity and metabolism.

Organic acids

Mechanism of action, studies in chicken

Organic acids are also called short chain or volatile fatty acids. Organic acids reduce the gastrointestinal pH. The use of organic acids in poultry may be CE, enhance nutrient utilization, and improve feed conversion. Over the years, organic acids have been used as antimicrobials to inhibit *Salmonella* in poultry products (Mani-López et al., 2012). Short chain fatty acids like butyric acid downregulates the expression of invasion genes in *Salmonella* spp. (Van Immerseel et al., 2006). Bacteria can use organic acids for carbon metabolism and energy source. The mechanism of antimicrobial activity of organic acids can be explained by the ability of these acids to pass across the cell membranes in undissociated form where they dissociate and acidify the cell cytoplasm and cause disruption (Borsoi et al., 2011; Van Immerseel, et al., 2006). Both bactericidal as well as bacteriostatic property of organic acids have been observed against

Salmonella and *E. coli*. A study reported that 25 mM of medium chain fatty acids like C6 to C10 but not 100 mM of SCFA were found effective against *Salmonella* (Van Immerseel et al., 2004). Organic acids either single or as a mixture have been found to be administered and combat against *Salmonella* in both broilers and laying hens (Adil et al., 2010; Borsoi, et al., 2011; C. et al., 1997; Sterzo et al., 2007).

References

Abudabos, A. M., H. A. Al-Batshan, and M. A. Murshed. 2015. Effects of prebiotics and probiotics on the performance and bacterial colonization of broiler chickens. S. Afr. J. Anim. Sci. 45:419-428.

Ajuwon, K. M. 2015. Toward a better understanding of mechanisms of probiotics and prebiotics action in poultry species. J. Appl. Poult. Res. 0:1-7.

Alloui Mohamed, N., W. Szczurek, and S. Świątkiewicz. 2013. The Usefulness of Prebiotics and Probiotics in Modern Poultry Nutrition: a Review. Ann. Anim. Sci. J. 13:17-32.

Amat, C., J. M. Planas, and M. Moreto. 1996. Kinetics of hexose uptake by the small and large intestine of the chicken. Am. J. physiol. 271:1085-1089.

Anderson, E. C., G. E. Carstens, R. K. Miller, T. R. Callaway, C. L. Schultz, T. S. Edrington, R. B. Harvey, and D. J. Nisbet. 2004. Effect of nitroethane administration on ruminal VFA production and specific activity of methane production. J. Anim. Feed Sci. 13:23-26.

Anderson, R. C., T. R. Callaway, J. A. Van Kessel, Y. S. Jung, T. S. Edrington, and D. J. Nisbet. 2003. Effect of select nitrocompounds on ruminal fermentation; an initial look at their potential to reduce economic and environmental costs associated with ruminal methanogenesis. Bioresour. Technol. 90:59-63.

Angermaier, L., and H. Simon. 1983. On the reduction of aliphatic and aromatic nitrocompounds by clostridia, the role of ferredoxin and its stabilization. Hoppe Seylers Z. Physiol. Chem. 364:961-975.

Apata, D. F. 2008. Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of *Lactobacillus bulgaricus*. J Sci Food Agr 88:1253-1258.

Arvola, T., K. Laiho, S. Torkkeli, H. Mykkanen, S. Salminen, L. Maunula, and E. Isolauri. 1999. Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. Pediatrics 104:64.

Atterbury, R. J., P. L. Connerton, C. E. Dodd, C. E. Rees, and I. F. Connerton. 2003. Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. Appl. Environ. Microbiol. 69(10):6302-6306.

Atterbury, R. J., M. A. P. Van Bergen, F. Ortiz, M. A. Lovell, J. A. Harris, A. De Boer, J. A. Wagenaar, V. M. Allen, and P. A. Barrow. 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl. Environ. Microbiol. 73:4543-4549.

Awad, W. A., K. Ghareeb, S. Abdel-Raheem, and J. Bohm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88:49-56.

Bai, S. P., Y. Huang, Y. H. Luo, L. L. Wang, X. M. Ding, J. P. Wang, Q. F. Zeng, and K. Y. Zhang. 2014. Alteration in lymphocytes responses, cytokine and chemokine profiles in laying hens infected with *Salmonella* Typhimurium. Vet. Immunol. Immunopathol. 160:235-243.

Bai, S. P., A. M. Wu, X. M. Ding, Y. Lei, J. Bai, K. Y. Zhang, and J. S. Chio. 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult. Sci. 92:663-670.

Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* Typhimurium infection. Immunol. 99:464-472.

Baurhoo, B., P. R. Ferket, and X. Zhao. 2009. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult. Sci. 88:2262-2272.

Baurhoo, B., A. Letellier, X. Zhao, and C. A. Ruiz-Feria. 2007. Cecal populations of lactobacilli and bifidobacteria and *Escherichia coli* populations after *in vivo Escherichia coli* challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poult. Sci. 86:2509-2516.

Brenner, D. J., N. R. Krieg, and J. T. Staley. 2005. Pages 321-325 in Bergey's Manual of Systemic Bacteriology, 2nd edition. Springer.

Bielke, L., S. Higgins, A. Donoghue, D. Donoghue, and B. M. Hargis. 2007. *Salmonella* host range of bacteriophages that infect multiple genera. Poult. Sci. 86:2536-2540.

Bigwood, T., J. A. Hudson, C. Billington, G. V. Carey-Smith, and J. A. Heinemann. 2008. Phage inactivation of foodborne pathogens on cooked and raw meat. Food Microbiol. 25:400-406.

Braat, H., J. van den Brande, E. van Tol, D. Hommes, M. Peppelenbosch, and S. van Deventer. 2004. *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am. J. Clinic. Nutr. 80:1618-1625.

Brisbin, J. T., J. Gong, S. Orouji, J. Esufali, A. I. Mallick, P. Parvizi, P. E. Shewen, and S. Sharif. 2011. Oral treatment of chickens with lactobacilli influences elicitation of immune responses. Clinic. Vacc. Immunol. 18:1447-1455.

Brisbin, J. T., H. Zhou, J. Gong, P. Sabour, M. R. Akbari, H. R. Haghighi, H. Yu, A. Clarke, A. J. Sarson, and S. Sharif. 2008. Gene expression profiling of chicken lymphoid cells after treatment with *Lactobacillus acidophilus* cellular components. Develop. Comp. Immunol. 32:563-574.

Broz, P., M. B. Ohlson, and D. M. Monack. 2012. Innate immune response to *Salmonella* Typhimurium, a model enteric pathogen. Gut Microbes. 3:62-70.

Callaway, T. R., T. S. Edrington, R. C. Anderson, R. B. Harvey, K. J. Genovese, C. N. Kennedy, D. W. Venn, and D. J. Nisbet. 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim. Health Res. Rev. 9:217-225.

Cao, B. H., Y. Karasawa, and Y. M. Guo. 2005. Effects of green tea polyphenols and fructo-oligosaccharides in semi-purified diets on broilers performance and caecal microflora and their metabolites. Asian Australas J. Anim. Sci. 18:85-89.

Carey, C. M., M. Kostrzynska, S. Ojha, and S. Thompson. 2008. The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic *Escherichia coli* O157:H7. J. Microbiol. Methods 73:125-132.

Carrique-Mas, J. J., M. Breslin, L. Snow, I. McLaren, A. R. Sayers, and R. H. Davies. 2009. Persistence and clearance of different *Salmonella* serovars in buildings housing laying hens. Epidemiol. Infect. 137:837-846.

Castellazzi, A. M., C. Valsecchi, L. Montagna, P. Malfa, G. Ciprandi, M. A. Avanzini, and G. L. Marseglia. 2007. *In vitro* activation of mononuclear cells by two probiotics: *Lactobacillus paracasei* I 1688, *Lactobacillus salivarius* I 1794, and their mixture (PSMIX). Immunol. Investigations 36:413-421.

serovar Typhimurium infection in mice. Bio. Med. Central Microbiol. 11:1-12.

Corr, S. C., Y. Li, C. U. Riedel, P. W. O'Toole, C. Hill, and C. G. M. Gahan. 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc. Nat. Acad. Sci. 104:7617-7621.

Cox, N. A., M. E. Berrang, and J. A. Cason. 2000. *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs--a review. Poult. Sci. 79:1571-1574.

Cummings, J. H., G. T. Macfarlane, and H. N. Englyst. 2001. Prebiotic digestion and fermentation. Am. J. Clinic. Nutr. 73:415-420.

Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr. 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poult. Sci. 82:62-66.

Dalloul, R. A., H. S. Lillehoj, N. M. Tamim, T. A. Shellem, and J. A. Doerr. 2005. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. Comp. Immunol. Microbiol. Infect. Dis. 28:351-361.

Deresinski, S. 2009. Bacteriophage therapy: exploiting smaller fleas. Clin. Infect. Dis. 48:1096-1101.

Dimitrijevic, M., R. C. Anderson, T. R. Callaway, Y. S. Jung, R. B. Harvey, S. C. Ricke, and D. J. Nisbet. 2006. Inhibitory effect of select nitrocompounds on growth and survivability of *Listeria monocytogenes in vitro*. J. Food Prot. 69:1061-1065.

Ding, W., H. Wang, and M. W. Griffiths. 2005. Probiotics down-regulate flaA sigma28 promoter in *Campylobacter jejuni*. J. Food Prot. 68:2295-2300.

Duggan, C., J. Gannon, and W. A. Walker. 2002. Protective nutrients and functional foods for the gastrointestinal tract. Am. J. Clinic. Nutr. 75:789-808.

Duncan, S. H., P. Louis, and H. J. Flint. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl. Environ. Microbiol. 70:5810-5817.

Eckmann, L., and M. F. Kagnoff. 2001. Cytokines in host defense against *Salmonella*. Microbes and Infect. 3:1191-1200.

FAO/WHO. 2001 Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria; Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Pages 1-34. FAO/WHO ed., Amerian Córdoba Park Hotel, Córdoba, Argentina.

Fayol-Messaoudi, D., C. N. Berger, M.-H. Coconnier-Polter, V. Liévin-Le Moal, and A. L. Servin. 2005. pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella* enterica Serovar Typhimurium. Appl. Environ. Microbiol. 71:6008-6013.

Feasey, N. A., G. Dougan, R. A. Kingsley, R. S. Heyderman, and M. A. Gordon. 2012. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. Lancet 379:2489-2499.

Fernandez, F., M. Hinton, and B. Van Gils. 2002. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella* Enteritidis colonization. Avian Pathol. 31:49-58.

Ferreira, C. L., S. Salminen, G. Lukasz, M. A. Brizuela, L. Sanchez, H. Carneiro, and M. Bonnet. 2011. Terminology concepts of probiotics and prebiotics and their role in human and animal health. Revista de Salud Animal 33:137-146.

Fiorentin, L., N. Vieira, and W. Barioni Júnior. 2005. Use of lytic bacteriophages to reduce *Salmonella* Enteritidis in experimentally contaminated chicken cuts. Revista Brasileira de Ciência Avícola 7:255-260.

Flynn, D. 2014. USDA: US Foodborne Illnesses Cost More Than $15.6 Billion Annually Food Safety News.

Foligne, B., J. Dewulf, J. Breton, O. Claisse, A. Lonvaud-Funel, and B. Pot. 2010. Probiotic properties of non-conventional lactic acid bacteria: immunomodulation by Oenococcus oeni. Internat. J. Food Microbiol. 140:136-145.

Foster, J. W., and M. P. Spector. 1995. How *Salmonella* survive against the odds. Ann. Rev. Microbiol. 49:145-174.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gao, J., H. J. Zhang, S. H. Yu, S. G. Wu, I. Yoon, J. Quigley, Y. P. Gao, and G. H. Qi. 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. Poult. Sci. 87:1377-1384.

Gast, R. K. 1994. Understanding *Salmonella* Enteritidis in laying chickens: the contributions of experimental infections. Int. J. Food Microbiol. 21:107-116.

Gast, R. K., J. Guard-Petter, and P. S. Holt. 2002. Characteristics of *Salmonella* Enteritidis contamination in eggs after oral, aerosol, and intravenous inoculation of laying hens. Avian Dis. 46:629-635.

Gast, R. K., R. Guraya, and J. Guard. 2013. *Salmonella* Enteritidis deposition in eggs after experimental infection of laying hens with different oral doses. J. Food Prot. 76:108-113.

Gast, R. K., R. Guraya, J. Guard, and P. S. Holt. 2011. Frequency and magnitude of internal organ colonization following exposure of laying hens to different oral doses of *Salmonella* Enteritidis. Int. J. Poult. Sci. 10:325-331.

Gast, R. K., R. Guraya, J. Guard-Bouldin, P. S. Holt, and R. W. Moore. 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella* Enteritidis or *Salmonella* Heidelberg. Avian Dis. 51:40-44.

Gast, R. K., and P. S. Holt. 1998. Persistence of *Salmonella* Enteritidis from one day of age until maturity in experimentally infected layer chickens. Poult .Sci. 77:1759-1762.

Gast, R. K., H. D. Stone, and P. S. Holt. 1993. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella* Enteritidis by laying hens. Avian Dis. 37:1085-1091.

Geier, M. S., V. A. Torok, G. E. Allison, K. Ophel-Keller, and R. J. Hughes. 2009. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens. J. Appl. Microbiol. 106:1540-1548.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Griffin, A. J., and S. J. McSorley. 2011. Development of protective immunity to *Salmonella*, a mucosal pathogen with a systemic agenda. Mucosal Immunol. 4:371-382.

Haghighi, H. R., M. F. Abdul-Careem, R. A. Dara, J. R. Chambers, and S. Sharif. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. Vet. Microbiol. 126:225-233.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, B. Sanei, P. Parvizi, H. Gisavi, J. R. Chambers, and S. Sharif. 2005. Modulation of antibody-mediated immune response by probiotics in chickens. Clinic. Diagnostic Lab. Immunol. 12:1387-1392.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, H. Zhou, B. Sanei, J. R. Chambers, and S. Sharif. 2006. Probiotics stimulate production of natural antibodies in chickens. Clinic. Vacc. Immunol. 13:975-980.

Hanning, I., A. Clement, C. Owens, S. H. Park, S. Pendleton, E. E. Scott, G. Almeida, F. Gonzalez Gil, and S. C. Ricke. 2012. Assessment of production performance in 2 breeds of broilers fed prebiotics as feed additives. Poult. Sci. 91:3295-3299.

Helwig, U., K. M. Lammers, F. Rizzello, P. Brigidi, V. Rohleder, E. Caramelli, P. Gionchetti, J. Schrezenmeir, U. R. Foelsch, S. Schreiber, and M. Campieri. 2006. Lactobacilli, *Bifidobacteria* and *E. coli* nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells. World J. Gastroenterol. 12:5978-5986.

Higgins, J. P., S. E. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez, J. L. Vicente, B. M. Hargis, and G. Tellez. 2010. Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella* Enteritidis in neonatal broilers. Poult. Sci. 89:243-247.

Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res. 12:60-64.

Horrocks, S. M., Y. S. Jung, J. K. Huwe, R. B. Harvey, S. C. Ricke, G. E. Carstens, T. R. Callaway, R. C. Anderson, N. Ramlachan, and D. J. Nisbet. 2007. Effects of short-chain nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli in vitro*. J. Food Sci. 72:50-55.

Huang, M. K., Y. J. Choi, R. Houde, J. W. Lee, B. Lee, and X. Zhao. 2004. Effects of lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. Poult. Sci. 83:788-795.

Huang, Q., Y. Wei, Y. Lv, Y. Wang, and T. Hu. 2015. Effect of dietary inulin supplements on growth performance and intestinal immunological parameters of broiler chickens. Livest. Sci. 180:172-176.

Hughes, E. A., and J. E. Galán. 2002. Immune Response to *Salmonella*: Location, Location, Location? Immunity 16:325-328.

Humphrey, T. J., H. Chart, A. Baskerville, and B. Rowe. 1991. The influence of age on the response of SPF hens to infection with *Salmonella* Enteritidis PT4. Epidemiol. Infect. 106:33-43.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182-188.

Ichikawa, H., R. Shineha, S. Satomi, and T. Sakata. 2002. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. Digest. Dis. Sci. 47:1141-1146.

Iji, P. A., and D. R. Tivey. 1998. Natural and synthetic oligosaccharides in broiler chicken diets. World's Poult. Sci. J. 54:129-143.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. Bean. 2009a. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139:1404-1409.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. D. Bean. 2009b. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139:1404-1409.

Janczyk, P., B. Halle, and W. B. Souffrant. 2009. Microbial community composition of the crop and ceca contents of laying hens fed diets supplemented with *Chlorella vulgaris.* Poult. Sci. 88:2324-2332.

Jayaraman, S., G. Thangavel, H. Kurian, R. Mani, R. Mukkalil, and H. Chirakkal. 2013. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. Poult. Sci. 92:370-374.

Jones, T. F., L. A. Ingram, P. R. Cieslak, D. J. Vugia, M. Tobin-D'Angelo, S. Hurd, C. Medus, A. Cronquist, and F. J. Angulo. 2008. Salmonellosis outcomes differ substantially by serotype. J. Infect. Dis. 198:109-114.

Jung, Y. S., R. C. Anderson, T. S. Edrington, K. J. Genovese, J. A. Byrd, T. R. Callaway, and D. J. Nisbet. 2004. Experimental use of 2 nitropropanol for reduction of *Salmonella* Typhimurium in the ceca of broiler chicks. J. Food Prot. 67:1945-1947.

Kang, S. J., Y. I. Park, B. So, and H. G. Kang. 2014. Sodium butyrate efficiently converts fully reprogrammed induced pluripotent stem cells from mouse partially reprogrammed cells. Cell Reprogramm. 16:345-354.

Kim, C., K. Shin, K. Woo, and I. Paik. 2009. Effect of dietary oligosaccharides on the performance, intestinal microflora and serum immunoglobulin contents in laying hens. Korean J. Poult. Sci. 36:125-131.

Kim, G.-B., Y. M. Seo, C. H. Kim, and I. K. Paik. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90:75-82.

Kim, W. K., R. C. Anderson, A. L. Ratliff, D. J. Nisbet, and S. C. Ricke. 2006. Growth inhibition by nitrocompounds of selected uric acid-utilizing microorganisms isolated from poultry manure. J. Environ. Sci. Health B 41:97-107.

Kinde, H., H. L. Shivaprasad, B. M. Daft, D. H. Read, A. Ardans, R. Breitmeyer, G. Rajashekara, K. V. Nagaraja, and I. A. Gardner. 2000. Pathologic and bacteriologic findings in 27-week-old commercial laying hens experimentally infected with *Salmonella* Enteritidis, phage type 4. Avian Dis. 44:239-248.

Kizerwetter-Swida, M., and M. Binek. 2009. Protective effect of potentially probiotic *Lactobacillus* strain on infection with pathogenic bacteria in chickens. Polish J. Vet. Sci. 12:15-20.

Klose, V., M. Mohnl, R. Plail, G. Schatzmayr, and A. P. Loibner. 2006. Development of a competitive exclusion product for poultry meeting the regulatory requirements for registration in the European Union. Mol. Nutr. Food Res. 50:563-5671.

Lavermicocca, P., F. Valerio, S. L. Lonigro, A. Di Leo, and A. Visconti. 2008. Antagonistic activity of potential probiotic lactobacilli against the ureolytic pathogen *Yersinia enterocolitica*. Current Microbiol. 56:175-181.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Li, L., C. L. Xu, C. Ji, Q. Ma, K. Hao, Z. Y. Jin, and K. Li. 2006. Effects of a dried *Bacillus subtilis* culture on egg quality. Poult. Sci. 85:364-368.

Liu, X., H. Yan, L. Lv, Q. Xu, C. Yin, K. Zhang, P. Wang, and J. Hu. 2012. Growth performance and meat quality of broiler chickens supplemented with *Bacillus licheniformis* in drinking water. Asian-Australasian J. Anim. Sci. 25:682-689.

Lu, H., S. Su, and K. M. Ajuwon. 2012. Butyrate supplementation to gestating sows and piglets induces muscle and adipose tissue oxidative genes and improves growth performance. J. Anim. Sci. 90 (4):430-432.

Macfarlane, G. T., H. Steed, and S. Macfarlane. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J. Appl. Microbiol. 104:305-344.

Mani-López, E., H. S. García, and A. López-Malo. 2012. Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. Food Res. Int. 45:713-721.

Mastroeni, Pietro, and N. Ménager. 2003. Development of acquired immunity to *Salmonella*. J. Med. Microbiol. 52:453-459.

Matis, G., A. Kulcsar, V. Turowski, H. Febel, Z. Neogrady, and K. Huber. 2015. Effects of oral butyrate application on insulin signaling in various tissues of chickens. Domes. Anim. Endocrinol. 50:26-31.

Mead, G. C. 2000. Prospects for 'competitive exclusion' treatment to control Salmonellae and other foodborne pathogens in poultry. Vet. J. 159:111-123.

Meimandipour, A., M. Shuhaimi, A. F. Soleimani, K. Azhar, M. Hair-Bejo, B. M. Kabeir, A. Javanmard, O. Muhammad Anas, and A. M. Yazid. 2010. Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of *Lactobacillus*. Poult. Sci. 89:470-476.

Miettinen, M., S. Matikainen, J. Vuopio-Varkila, J. Pirhonen, K. Varkila, M. Kurimoto, and I. Julkunen. 1998. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. Infect. Immunol. 66:6058-6062.

Miyamoto, T., E. Baba, T. Tanaka, K. Sasai, T. Fukata, and A. Arakawa. 1997. *Salmonella* Enteritidis contamination of eggs from hens inoculated by vaginal, cloacal, and intravenous routes. Avian Dis. 41:296-303.

Ng, S. C., A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: recent advances. Inflamm. Bowel Dis. 15:300-310.

Novak, M., and V. Vetvicka. 2008. Beta-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. J. Immunotoxicol. 5:47-57.

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

Okamura, M., T. Miyamoto, Y. Kamijima, H. Tani, K. Sasai, and E. Baba. 2001. Differences in abilities to colonize reproductive organs and to contaminate eggs in intravaginally inoculated hens and *in vitro* adherences to vaginal explants between *Salmonella* Enteritidis and other *Salmonella* serovars. Avian Dis. 45:962-971.

Park, S. Y., C. L. Woodward, L. F. Kubena, D. J. Nisbet, S. G. Birkhold, and S. C. Ricke. 2008. Environmental dissemination of foodborne *Salmonella* in preharvest poultry production: reservoirs, critical factors, and research strategies. Crit. Rev. Environ. Sci. Technol. 38:73-111.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.

Pelicano, E., P. d. Souza, H. d. Souza, A. Oba, E. Norkus, L. Kodawara, and T. d. Lima. 2003. Effect of different probiotics on broiler carcass and meat quality. Revista Brasileira de Ciência Avícola 5:207-214.

Penha Filho, R. A., B. S. Moura, A. M. de Almeida, H. J. Montassier, P. A. Barrow, and A. Berchieri Junior. 2012. Humoral and cellular immune response generated by different vaccine programs before and after *Salmonella* Enteritidis challenge in chickens. Vaccine 30:7637-7643.

Qaisrani, S. N., M. M. van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2015. Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. Poult. Sci. 94:2152-2164.

Saengkerdsub, S., W.-K. Kim, R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2006. Effects of nitrocompounds and feedstuffs on *in vitro* methane production in chicken cecal contents and rumen fluid. Anaerobe 12:85-92.

Samanya, M., and K. E. Yamauchi. 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus* subtilis var. natto. Comp. Biochem. and Physiol. A. Mol. and Integrat. Physiol. 133:95-104.

Sanders, M. E., and J. Huis in't Veld. 1999. Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie Van Leeuwenhoek 76:293-315.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. Emerg. Infect. Dis. 17:7-15.

Seifert, S., and B. Watzl. 2007. Inulin and oligofructose: review of experimental data on immune modulation. J. Nutr. 137:2563s-2567s.

Shang, H. M., T. M. Hu, Y. J. Lu, and H. X. Wu. 2010. Effects of inulin on performance, egg quality, gut microflora and serum and yolk cholesterol in laying hens. Br. Poult. Sci. 51:791-796.

Sheela, R. R., U. Babu, J. Mu, S. Elankumaran, D. A. Bautista, R. B. Raybourne, R. A. Heckert, and W. Song. 2003. Immune responses against *Salmonella* enterica serovar Enteritidis infection in virally immunosuppressed chickens. Clin. Diag. Lab. Immunol. 10:670-679.

Sherman, P. M., J. C. Ossa, and K. Johnson-Henry. 2009. Unraveling mechanisms of action of probiotics. Nutr. Clinic. Prac. 24:10-14.

Sims, M. D., K. A. Dawson, K. E. Newman, P. Spring, and D. M. Hoogell. 2004. Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. Poult. Sci. 83:1148-1154.

Smits, H. H., A. Engering, D. van der Kleij, E. C. de Jong, K. Schipper, T. M. van Capel, B. A. Zaat, M. Yazdanbakhsh, E. A. Wierenga, Y. van Kooyk, and M. L. Kapsenberg. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J. Allergy Clinic. Immunol. 115:1260-1267.

Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. Poult. Sci. 79:205-211.

Srikanth, C. V. and B. J. Cherayil. 2007. Intestinal Innate Immunity and the Pathogenesis of *Salmonella* Enteritis. Immunol. Res. 37:61-78.

Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. J. Genovese. 2004. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhea and edema disease in swine. Antimicrob. Agents Chemother. 48:3119-3121.

Stern, N. J., N. A. Cox, J. S. Bailey, M. E. Berrang, and M. T. Musgrove. 2001. Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. Poult. Sci. 80:156-160.

Swaggerty, C. L., P. Kaiser, L. Rothwell, I. Y. Pevzner, and M. H. Kogut. 2006. Heterophil cytokine mRNA profiles from genetically distinct lines of chickens with differential heterophil-mediated innate immune responses. Avian Pathol. 35:102-108.

Swiatkiewicz, S., and A. Arczewska-wlosek. 2012. Prebiotic fructans and organic acids as feed additives improving mineral availability. World's Poult. Sci. J. 68:269-279.

Thomas, W. E., L. M. Nilsson, M. Forero, E. V. Sokurenko, and V. Vogel. 2004. Shear-dependent 'stick-and-roll' adhesion of type 1 fimbriated *Escherichia coli*. Mol. Microbiol. 53:1545-1557.

Timmerman, H. M., C. J. Koning, L. Mulder, F. M. Rombouts, and A. C. Beynen. 2004. Monostrain, multistrain and multispecies probiotics--A comparison of functionality and efficacy. Internat. J. Food Microbiol. 96:219-233.

Timoney, J., H. Shivaprasad, R. Baker, and B. Rowe. 1989. Egg transmission after infection of hens with *Salmonella* enteritidis phage type 4. Vet. Rec. 125:600-601.

Van Immerseel, F., B. De, F. Boyen, L. Bohez, F. Pasmans, J. Volf, M. Sevcik, I. Rychlik, F. Haesebrouck, and R. Ducatelle. 2004. Medium-chain fatty acids decrease colonization and invasion through hilA suppression shortly after infection of chickens with *Salmonella* enterica serovar Enteritidis. Appl. Environ. Microbiol. 70:3582 - 3587.

Van Immerseel, F., J. B. Russell, M. D. Flythe, I. Gantois, L. Timbermont, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2006. The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. Avian Pathol. 35:182-188.

Verstegen, M. W., and B. A. Williams. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim. Biotechnol. 13:113-127.

Vicente, J. L., A. Torres-Rodriguez, S. E. Higgins, C. Pixley, G. Tellez, A. M. Donoghue, and B. M. Hargis. 2008. Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella* enterica serovar Enteritidis-infected broiler chicks. Avian Dis. 52:143-146.

Wales, A., M. Breslin, B. Carter, R. Sayers, and R. Davies. 2007. A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. Avian Pathol. 36:187-197.

Wigley, P., and P. Kaiser. 2003. Avian cytokines in health and disease. Revista Brasileira de Ciência Avícola 5:1-14.

Wu, L. Y., Y. J. Fang, R. B. Tan, and K. J. Shi. 2009. A comparison of cecal microflora and volatile fatty acid concentration in goslings fed diets supplemented with or without a dried *Bacillus subtilis* culture. J. Appl. Anim. Res. 36:231-234.

Wu, X., B. A. Vallance, L. Boyer, K. S. Bergstrom, J. Walker, K. Madsen, J. R. O'Kusky, A. M. Buchan, and K. Jacobson. 2008. *Saccharomyces boulardii* ameliorates citrobacter rodentium-induced colitis through actions on bacterial virulence factors. Am. J. Physiol. Gastroint. Liver Physiol. 294:G295-306.

Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Z. and, and M. Q. Wang. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82:1030-1036.

Yörük, M. A., M. Gül, A. Hayirli, and M. Macit. 2004. The effects of supplementation of humate and probiotic on egg production and quality parameters during the late laying period in hens. Poult. Sci. 83:84-88.

Zhao, P. Y., J. P. Wang, and I. H. Kim. 2013. Effect of dietary levan fructan supplementation on growth performance, meat quality, relative organ weight, cecal microflora, and excreta noxious gas emission in broilers. J. Anim. Sci 91:5287-5293.

Table 2.1. Role of prebiotics supplementation in growth performance, immune modulation and pathogen reduction.

Reference	Type of prebiotics	Major outcomes
Fernandez et al. (2002)	MOS	Reduced *Salmonella* infection
Baurhoo et al. (2007)	MOS and lignin	Increased *Lactobacillus* (LAB) and *Bifidobacteria*, decreased *E. coli*, low intestinal pH, increased villi height
Baurhoo et al. (2009)	MOS	Increased intestinal microbial community and development of intestinal morphology
Xu et al. (2003)	FOS	Improved body weight gain, feed conversion and carcass weight, increased LAB and *Bifidobacteria*
Sims et al. (2004)	MOS	Improved body weight gain
Macfarlane et al. (2008)	GOS	Increased growth of LAB, *Bifidobacteria*, and/or their fermentation products
Zhao et al. (2013)	Fructan, FOS	Increased cecal LAB and *Bifidobacteria*, decreased *E. coli* and *C. perfringes*
Janardhana et al. (2009)	FOS, MOS	Increased immunity in GALT, increased IgG and IgM
Huang et al. (2015)	Inulin	Increased mucin mRNA expression of jejunum, increased cecum IgA level, increased intestinal immune function at d 21 but did not affect at d 42
Kim et al. 2011	FOS and MOS	Increased LAB and *Bifidobacteria*

Geier et al. 2009	FOS, MOS and inulin	Increased LAB and *Bifidobacteria*
Hanning (2012)	FOS	Improved villi height and crypt depth
Cao et al. (2005)	FOS + tea polyphenols	Reduced mortality in 28-42 d old broilers, FOS selectively promoted favorable microbes and inhibited microflora metabolites except volatile fatty acids in the cecum

Table 2.2. Role of probiotics supplementation in growth performance, immune modulation and pathogen reduction.

Reference	Type of probiotics	Major outcomes
Vicente et al. (2008)	*Lactobacillus*	Increased lactic acid producing bacteria, decreased gut lesions score in broilers due to *Eimeria* and *Salmonella*
Lee et al. (2010)	*Bacillus* (direct fed microbials)	Improved gut morphology and immunity against *Eimeria*
Yörük et al. (2004)	Humate and probiotic	Increased egg production, decreased mortality
Pelicano et al. (2003)	*Bacillus subtilis*; *Bacillus subtilis* and *Bacillus licheniformis*; and *Saccharomyces cerevisiae*	Improved carcass and meat quality in broilers
Liu et al. (2012)	*Bacillus licheniformis*	Enhanced growth promotion and meat quality
Bai et al. (2013)	*Lactobacillus fermentum* and *Saccha romyces cerevisiae*	Stimulated intestinal T cell immune system
Gao et al. (2008)	*Yeast culture*	Improved immune function, growth performance and intestinal mucosal morphology
Haghighi et al. (2006)	*Lactobacillus*	Produced natural antibodies like intestinal IgA, serum IgG and IgM

Samanya and Yamauchi (2002)	*Bacillus sublitis*	Improved growth performance as well as intestinal morphology
Higgins et al. (2010)	*Lactobacillus* cultures	Developed normal microflora in chicken gut and reduced incidence of *Salmonella*
Dalloul et al. (2003)	*Lactobacillus*	Improved innate and adaptive response against *Eimeria*
Stern et al. (2001)	*Klebsiella pneumoniae, Citrobacter diversus*, and *E. coli*	Reduced number of *Campylobacter jejuni*
Dalloul et al. (2005)	*Lactobacillus* based probiotic	Reduced fecal oocyst shedding of *Eimeria acervulina*
Hofacre et al. (2003)	Lactic acid bacteria	Reduced mortality due to *Necrotic Enteritis*
Jayaraman et al. (2013)	*Bacillus subtilis*	Reduced FCR and intestinal lesions in broilers challenged with *Clostridium* and *Eimeria*

3. COLONIZATION OF MATURE LAYING HENS WITH *SALMONELLA* ENTERITIDIS BY ORAL OR INTRACLOACAL INOCULATION[1]

[1]P. A. Adhikari, Douglas E. Cosby, Nelson A. Cox and Woo K. Kim. Submitted to *Journal of Applied Poultry Research.*

ABSTRACT

Evidence of *Salmonella* Enteritidis (SE) in internal organs of White Leghorns once they are inoculated via the oral (OR) or intracloacal (IC) route has not been consistently demonstrated. The aim of the current study was to evaluate OR or IC inoculation route of a nalidixic acid (Nal) resistant SE (SE^{NAR}) on the SE colonization of ceca and the invasion of internal organs in mature White Leghorns. Five experiments were conducted, and hens were inoculated with 10^8 colony-forming units (cfu) of SE^{NAR}. Hens were euthanized at 7 and 14 day post-inoculation (dpi), and the ceca, spleen, liver with gall bladder (L/GB) and ovaries were collected for bacteriological analyses. The recovery of SE^{NAR} in ceca was 100 % at 7 dpi. Recovery from the ovaries was lower than the other organs for both routes of inoculation. The SE recovery of L/GB, spleen and ovaries at 7 dpi were not different between the two routes. By 14 dpi, all organs approached negative, and the recovery rate was similar between OR and IC. Fecal shedding was 100 % positive at 3 dpi and reduced to almost 0 % by 14 dpi. Mature hens were colonized by SE^{NAR} with either OR or IC inoculation when using a larger volume and a higher cfu/mL (0.1 mL OR in experiment 1 vs. 1.0 mL OR and IC in the rest). SE^{NAR} showed some translocation into other organs, to a greater extent with IC. The colonization did not consistently persist either in ceca or the internal organs at 14 dpi.

Key words: *Salmonella* Enteritidis, leghorn, intracloacal, oral, inoculation

INTRODUCTION

Salmonella enterica subsp. serovar Enteritidis (SE) is the world's leading cause of human salmonellosis (Braden, 2006). A major route of *Salmonella* Enteritidis (SE) infection is the fecal-oral route. SE can invade beyond the intestinal tract of chickens to colonize internal organs such as liver with gall bladder (L/GB) and spleen within a few hours of inoculation (He et al., 2010), and this can eventually lead to reproductive organ colonization (Gantois et al., 2009). Colonization of reproductive organs could be a result of the systemic spread of *Salmonella* from the intestine. However, *Salmonella* colonization in the ovary and oviduct does not always correlate with egg contamination (Gast et al., 2004).

The frequency of internal organ colonization declines steeply after the first few weeks following oral inoculation of mature chickens and testing at longer post inoculation intervals is generally less informative (Gast et al., 2007). Newly hatched chicks are the most susceptible to *Salmonella* colonization because they lack mature gut microflora or feed in the alimentary tract (Snoeyenbos et al., 1978). The low pH of the upper intestinal tract makes it necessary to use higher levels of *Salmonella* in order to colonize young chicks when challenged by OR route (Bailey et al., 2005). *Salmonella* cells introduced via IC are not subjected to the same level of acidity found in the proventriculus and the gizzard, and thus a lower dose may be able to colonize in the ceca of chicks (Cox et al., 1996).

The inoculation of adult hens with large oral doses can sometimes lead to fecal shedding which can last for several months (Gast et al., 2011b). Fecal shedding of *Salmonella* by infected hens can be a major contributor to overall environmental contamination levels. However, the magnitude of fecal shedding does not always correlate with detection of the pathogen by environment sampling (Gast et al., 2015). Research is still lacking on the effectiveness of the 2 routes of SE challenge, OR and IC in mature White Leghorn hens. Thus, the aim of this study

was to evaluate the OR versus IC of a SE^{NAR} in mature laying hens to consistently colonize the ceca and other internal organs.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Georgia (IACUC number: A201407-016).

Bacterial Culture and Inoculum Preparation

Challenge pathogen: SE^{NAR} was obtained as a frozen stock from the USDA National Poultry Research Center (Athens, GA). Frozen stock cultures of SE^{NAR} were maintained at -80°C in nutrient broth and 16 % glycerol (Sigma) until needed. The bacteria (SE^{NAR}) was revived from frozen cultures onto brilliant green agar plates with sulphapyridine (BGS; Acumedia, East Lansing, MI) containing 200 ppm of Nal (Sigma Chemical Co., St. Louis, MO). The plates were incubated for 24 h at 37°C to ensure log phase growth. Isolated SE^{NAR} colonies were transferred to 9 mL of sterile 0.85 % saline solution. The absorbance value was adjusted to an optical density of 0.20 ± 0.01 at 540_{nm} with a spectrophotometer (Spect-20, Milton-Roy, Thermo Spectronics, Madison, WI) which yields approximately 1.0 x 10^8 cfu/mL. Cultures were serially diluted in sterile saline for enumeration. Hens were OR gavaged with a 1 cc tuberculin syringe (Becton, Dickinson and Co., Franklin Lakes, NJ) and an animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY), whereas IC inoculation was performed using only a 1 cc tuberculin syringe.

Experimental hens, Housing and Experimental Infection

Experiment 1

Thirty two - Single Comb White Leghorn hens (38 wk old at the beginning of the experiment) were housed at the Poultry Research Center, University of Georgia in Athens, GA.

Hens were kept separately in wire-laying cages and fed a corn-soy layer diet (Table 1). Hens were provided with water (automatic nipple-type drinkers) and mash feed *ad libitum*. The feed was formulated to provide crude protein of 16 %, metabolizable energy of 2,900 kcal/kg, calcium of 4.4 % and available phosphorus of 0.5 %. Hens were housed under a 16h light:8h dark regimen. All hens were adapted to the diet for 1 week after which they were challenged with the bacteria. Feed was withdrawn for 10 h before challenge, and feeders were replaced immediately post challenged. Out of 32 hens, 8 hens were unchallenged (negative control), and 24 hens were challenged via OR with a 0.1 mL volume of culture containing 1.6×10^8 cfu/mL SE^{NAR}.

Experiments 2 and 3

Thirty six - Single Comb White Leghorns were used in experiments 2 and 3. Hens were 44 and 50 wk old respectively at the beginning of each experiment. Animal husbandry, diet, feeding and handling were the same as those in experiment 1. In both experiments, hens were randomly distributed into 3 groups: 1) OR with 10^8 cfu of SE^{NAR} (14 hens), 2) IC with 10^8 cfu of SE^{NAR} (14 hens), and 3) unchallenged (negative) group (8 hens). Hens were inoculated with 1.0 mL dose of 3.3×10^8 and 1.2×10^8 cfu/mL SE^{NAR} in experiments 2 and 3, respectively.

Experiments 4 and 5

Number of hens, animal handling, feeding, husbandry and the allocation of hens in experiments 4 and 5 were similar to those in experiments 2 and 3. Hens were 55 and 72 wk old respectively, at the beginning of experiments 4 and 5. The SE^{NAR} counts in the ceca were estimated by the method of Blanchfield et al. (Blanchfield et al., 1984). Hens were inoculated with 1.0 mL of 2.6×10^8 and 3.1×10^8 cfu/mL SE^{NAR} in experiments 4 and 5, respectively.

Sampling Protocol, Bacteriological Examination and SE^{NAR} Recovery

1. Ceca, Spleen, L/GB and Ovaries Samples (Experiments 1, 2, 3 and 4)

In experiment 1, all 32 hens were humanely euthanized at 14 dpi, and the ceca, ovaries, spleen and L/GB were removed aseptically for bacteriological analyses. In experiment 2, 7 hens from the OR and IC groups and 4 hens from unchallenged groups were euthanized and sampled at 7 dpi. Similarly, at 14 dpi, the remaining 18 hens were sampled. The sampling procedure in experiments 3, 4 and 5 were similar to that of experiment 2. In addition to determining the presence or absence of SE^{NAR} in the ceca, counts were performed in experiments 4 and 5 using the method of Blanchfield et al. (5). All tissue samples were placed into labeled sterile plastic sampling bags (Fisher Scientific, Pittsburgh, PA) and transported on ice to the U.S. National Poultry Research Center, USDA, Athens, GA.

Presence and Absence of SE^{NAR} (Spleen, L/GB and Ovaries)

Samples were individually weighed and diluted in buffered peptone water (BPW) three times their weight. The sample bags were stomached (Techmar Company, Cincinnati, Ohio) for 60 s and pre-enriched overnight at 37^{o}C. Pre-enriched samples were streaked for isolation onto BGS-NAl plates and incubated overnight at 37 ^{o}C. The growth of SE^{NAR} was observed and recorded.

Estimation of SE^{NAR} in Ceca (Experiments 4 and 5)

The number of SE^{NAR} per g of cecal material was estimated using three swab plating method as described by Blanchfield et al. (Blanchfield, et al., 1984). In brief, ceca were aseptically excised from the hen and placed in stomacher bags. The ceca were weighed and diluted in BPW three times their weight. After stomaching for 60 s, a cotton-tipped swab was dipped and rotated in the cecal material for 5 s. A BGS-Nal plate was surface-swabbed (plate A). A second swab was placed into stomacher bag, transferred and broken off into a 9.9 mL BPW

dilution tube. The dilution tube was vortexed for 10 s. A second BGS-Nal plate (plate B) was taken and inoculated by dipping a fresh swab into the dilution tube and spreading as above. All plates together with the cecal samples were incubated at 37 ^{o}C overnight. Negative samples were swab-plated from the overnight pre-enrichments onto a BGS-Nal plate (plate C) and incubated at 37 ^{o}C overnight.

The swab dipped and rotated took up approximately 0.15 g of cecal material and can deposit approximately 0.055 g onto plate A. The cecal materials contained on the fresh swab created an approximate 100-fold dilution when vortexed in 9.9 mL of BPW. A fresh swab dipped into the BPW suspension picked up approximately 0.36 g and deposits approximately 0.0005 g of cecal material on plate B. The total factor for the estimation count was 54 (18×3) on the A plate, and the B plate is diluted 100 times so the multiplier was 5400 for the B plate. A log count of 1.5 was assigned to the *Salmonella* detected from pre-enriched samples.

2. Fecal samples (Experiments 2 and 3)

In experiments 2 and 3, fecal shedding was measured by collecting feces from all hens at 3, 6, and 13 dpi. Aluminum foil sheets were placed under each cage, and sterile cotton swabs were used to collect feces into 50 mL centrifuge tubes. BPW was added, approximately 3 times the weight and vortexed. A 10 µl portion of each sample was streaked for isolation onto BGS-Nal plates. Plates and sample tubes were incubated for 24 h at 37 °C. Plates that were negative by direct plating were again streaked into BGS-Nal plates from the overnight pre-enriched samples. The plates were read as negative or positive.

Statistical Analysis

The enrichment data were expressed as positive/total chickens (%), and the percent recovery of SE^{NAR} was compared using Fisher's exact test. The significance was accepted at $P \leq$

0.05. Log_{10} cfu values of SE^{NAR} per g of ceca were expressed as means ± SEM and deemed significant if $P \leq 0.05$. Data were analyzed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

SE^{NAR} in Ceca and Internal Organs

Salmonella can colonize birds through fecal-oral transmission. Each body opening including oral, nasal, ocular or IC routes of infection may be prominent in the colonization of younger chicks (Cox, et al., 1996). Cox et al. (Cox et al., 1990) reported that 100 fold fewer cells when administered through IC route were able to colonize the young broiler chicks compared to number required to colonize via the OR route. The low pH of crop and upper gastrointestinal tract requires a higher number of cells to colonize by OR versus IC (Cox, et al., 1990). In our study, we did not observe a difference between the two routes in the colonization and recovery of SE^{NAR}. No sample was positive for SE^{NAR} by OR challenge in experiment 1. In experiment 1, the low volume of 10^8 cells of SE^{NAR} provided to the mature hens was not enough to colonize them (Data not shown). A previous study reported that concentrations as high as 10^7 were needed to colonize hens over 29 weeks of age (Gast et al., 2013b). However, the actual amount / volume of the specific dose (e.g. 10^7) was not clear. Larger doses have been associated with a higher frequency and greater persistence of both intestinal and internal organ colonization (Gast et al., 2001).

The frequencies of SE^{NAR} recovery in ceca and internal organs for experiments 2 and 3 are shown in Table 2. In experiment 2, SE^{NAR} was recovered 100 % from all ceca when challenged by OR and IC routes at 7 dpi. There was no difference between OR and IC routes at 7 dpi. At 14 dpi, the recovery in ceca was reduced to 14.3 versus 28.6 % by OR and IC challenge.

The frequencies of recovery were significantly greater in hens challenged by OR in spleen samples versus IC (85.7 vs. 57.1 %; $P < 0.0001$), and greater in hens challenged by IC for L/GB versus OR (42.8 vs. 85.7 %; $P = 0.0003$). The frequency of SE^{NAR} did not differ significantly ($P = 1.000$) between the two routes for ovaries (28.6 vs. 28.6 %). In experiment 3, SE^{NAR} was recovered from the ceca at 85.7 % for OR and 100 % for IC ($P < 0.0001$) at 7 dpi (Table 3). Frequency of SE^{NAR} recovery in the ovaries was greater in IC hens compared to OR (42.8 vs. 14.3 %; $P < 0.0001$). However, recoveries in spleen (42.8 vs. 57.1 % in OR and IC; $P = 0.0657$) and L/GB (57.1 vs. 71.4 % in OR and IC, $P = 0.0551$) were not significantly different with the two routes. At 14 dpi, recovery of SE from the ceca was reduced to 14.3 and 42.8 % in OR and IC, respectively ($P < 0.0001$). The frequencies of SE^{NAR} were reduced to 0 % in ovaries and spleen in both OR and IC challenged birds. By 14 dpi, SE recovery in L/GB was 14.3 % in both routes.

The frequencies of SE^{NAR} recovered in the ceca and internal organs for experiments 4 and 5 are shown in Table 3. In experiment 4, the recovery of SE^{NAR} from ovaries was lower than spleen or L/GB at 7 dpi, regardless of challenge routes. In experiment 4, the frequency of SE^{NAR} recovery in ovaries was similar for OR and IC at 7 dpi (14.3 vs. 14.3 %; $P = 1.000$). In experiment 5, there were more positive ovaries in IC hens compared to OR (14.3 vs. 28.6 %; $P = 0.0153$). Recovery of SE^{NAR} from the spleen at 7 dpi was 71.4 vs. 86 %; OR vs. IC ($P = 0.0153$) in experiment 4, and 57 vs. 71.4 %; OR vs. IC ($P = 0.055$) in experiment 5, respectively. The recovery from the L/GB was inconsistent with routes of challenge at 7 dpi. Recovery of SE^{NAR} from the L/GB at 7 dpi was 86 vs. 71.4 %; OR vs. IC ($P = 0.065$) in experiment 4, and 43 vs. 57 %; OR vs. IC ($P = 0.065$) in experiment 5, respectively. By 14 dpi, the frequency of L/GB was

reduced to 14.3 % in OR challenged hens in experiment 4, whereas there were 0 % recovery in experiment 5. All internal organs were negative by 14 dpi in experiments 4 and 5.

Cecal SE cfu/g was estimated in experiments 4 and 5, and the results are shown in Table 4. At 7 dpi in experiment 4, a greater SE^{NAR} count was observed in the ceca for IC route compared to OR route of challenge. There was a 1.4 $\log_{10}$ reduction from 1.8 at 7 dpi to 0.43 at 14 dpi in OR challenged hens and a 2.1 $\log_{10}$ reduction from 2.5 at 7 dpi to 0.43 at 14 dpi in IC challenged hens (experiment 4). There was a 3.0 $\log_{10}$ reduction from 3.2 at 7 dpi to 0.21 at 14 dpi in OR challenged hens and a 2.8 $\log_{10}$ reduction from 3.02 to 0.21, with IC challenged birds. Both OR and IC challenge in the present study led to 100 % colonization of ceca (1.8 to 3.2 in OR and 2.5 to 3.1 cfu/g of cecal material at 7 dpi). This level of cecal colonization was similar to previous studies where cecal SE recovery was more than 90 % at 7 dpi with SE compared to the rest (Gast, et al., 2013a; Gast et al., 1998). Our results showing a higher frequency of positive ceca when compared to rest of the organs are consistent with the previous studies (Gast, et al., 2013b; Kallapura et al., 2014).

The recovery rates in the ceca, spleen and L/GB of our hens were similar to the recovery rates observed in the previous study where 50 to 100 % recovery was reported from various internal organs at 7 dpi, regardless of routes (Cox, et al., 1990). The frequency and incidence of SE positives in the spleen in the present study were similar to another study by Gast et al. (Gast, et al., 2013b), which used a conventional housing system. The lower colonization rates in the organs like the spleen, L/GB and ovaries were similar to a study where recovery in the spleen and liver was lower than in the ceca (Fernandez-Rubio et al., 2009). After 5 dpi, the isolation of SE from the liver was higher when hens were given 10^8 rather than 10^4 or 10^6 cfu (Gast et al., 2011a). We did not sample before 7 dpi, therefore we have no information about the rate in

various tissues prior to 7 dpi. In our experiments, more positive results were obtained from the spleen and L/GB than for the ovaries. The deposition of *Salmonella* inside developing eggs results from the SE colonization and invasion of the reproductive tissues (ovaries or oviducts) but a high occurrence of bacteria in the reproductive tract does not always correlate to a high rate of eggs being infected (Barrow et al., 1991). This can also be related to fecal bacteria penetrating the shell and getting into eggshell membrane. During the 1st week post challenge, a high percentage of hens were colonized in the intestinal tract and visceral organs (Gast, et al., 2013b). Our studies have shown that the translocation of SE^{NAR} can occur with either route of inoculation (IC or OR).

A previous study with SE in broilers has shown an inverse relation between the colonization frequency in ceca and other internal organs like the liver and spleen (Kramer et al., 2001). The translocation pattern of SE^{NAR} to internal organs in our study was similar to a previous study by Bailey et al. (Bailey, et al., 2005) where SE was found in the lymphoid organs using either OR or IC challenge. After an oral exposure to SE, the spleens were colonized in laying hens without causing any clinical infections in them (Langkabel et al., 2014). The colonization frequencies decreased as the days after SE challenge increased. Oral inoculation of mature hens with 10^6 cfu of *Salmonella* was not associated with the recovery of the bacteria from viscera after 10 dpi (Cox et al., 1973). The reason behind the deceased SE at 14 dpi could possibly be due to an increased immunity in the hens towards *Salmonella*. However, we did not find any study that showed the relationship between the dpi and the immunity in hens of similar age group.

SE^{NAR} in Feces

Presence of SE^{NAR} in feces was measured in experiments 2 and 3 (Table 5). At 3 dpi, the recovery of SE^{NAR} in feces was 100 % when birds were challenged by either OR or IC route, whereas by 13 dpi, all fecal samples were negative in experiments 2 and 3. At 6 dpi, detection of SE^{NAR} in feces was greater when birds were challenged by OR route than IC route. In experiment 2, fecal shedding at 6 dpi was 92.8 and 85.7 % ($P = 0.1652$) and in experiment 3, it was 85.7 and 71.4 % ($P = 0.0153$), for OR and IC, respectively.

Fecal shedding may be used as an indication of intestinal colonization in mature laying hens (Sadler et al., 1969). It has been reported that the bacteria shed in the feces is a direct consequence of intestinal colonization (Gast, et al., 2011b). However, persistent fecal shedding has proven to be an inconsistent predictor of the likelihood of systemic infection and/or egg contamination by SE (Gast et al., 2000). Hens challenged with 10^8 cfu/mL of SE had 87.5 % positive feces after 7 dpi (Gast, et al., 2011b), and this is similar to our study where we had almost 100 % positive SE^{NAR} in feces and ceca at 7 dpi. The decrease in fecal shedding of SE^{NAR} can be correlated with the prevalence pattern of the bacteria in the intestinal tract especially the ceca (Freitas Neto et al., 2008). A study by Van Immerseel et al. reported that decreased cecal colonization correlates to decreased fecal shedding (Van Immerseel et al., 2005). The decline in the rate of fecal shedding is an indication that birds are capable of reducing the level of colonization (Holt et al., 2006). Gast et al. showed that fecal shedding declined by 14 dpi, while no SE was recovered in the feces/fecal droppings 3 weeks after inoculation (Gast, et al., 2011b). The age of the chicken plays an important role in SE colonizing the chicken. Hens used in our study were mature egg layers above 40 wks old, which have been more difficult to colonize with lower doses of SE.

Older birds are more difficult to colonize than young chicks due to their diversified microflora of the gut (Bailey et al., 1988) and mature immune system providing resistance to SE colonization (Crhanova et al., 2011). It has also been shown that 20-40 wk old laying hens are naturally more resistant to SE colonization (Humphrey et al., 1991). The persistence of SE^{NAR} in hens used in experiments 4 and 5 was lower at 14 dpi compared to experiments 2 and 3, perhaps due to the younger age of the hens used in the experiments 2 and 3.

We made inferences with regards to 14 dpi being the maximum limit for reliable colonization because the frequency of recovery from internal organs declines sharply in the first few weeks after oral inoculation (Gast, 2007).

CONCLUSIONS

Salmonella Enteritidis can colonize the intestinal tract of mature hens via either the OR or IC route. Once internalized the SE^{NAR} shows the ability to translocate (although somewhat limited) into other organs including the ovary, spleen and L/GB. However, the colonization does not consistently persist either in the ceca or other internal organs at 14 dpi in mature laying hens. There was no any *Salmonella* shedding observed in feces after 2 weeks of infection. The short-term persistence of colonization and translocation limit the ability to do research with mature hens over two weeks in duration. This limits any feeding trials only for 2 weeks or less to test the efficacy of treatments that use mature laying hens challenged with *Salmonella.*

References

Bailey, J. S., L. C. Blankenship, N. J. Stern, N. A. Cox, and F. McHan. 1988. Effect of anticoccidial and antimicrobial feed additives on prevention of *Salmonella* colonization of chicks treated with anaerobic cultures of chicken feces. Avian Dis. 32:324-329.

Bailey, J. S., N. A. Cox, D. E. Cosby, and L. J. Richardson. 2005. Movement and persistence of *Salmonella* in broiler chickens following oral or intracloacal inoculation. J. Food Prot. 68:2698-2701.

Barrow, P. A., and M. A. Lovell. 1991. Experimental infection of egg-laying hens with *Salmonella* Enteritidis phage type 4. Avian Pathol. 20:335-348.

Blanchfield, B., S. Stavric, T. Gleeson, and H. Pivnick. 1984. Minimum intestinal inoculum for Nurmi cultures and a new method for determining competitive exclusion of *Salmonella* from chicks. J. Food Prot. 47:542-545.

Braden, C. R. 2006. *Salmonella* enterica serotype Enteritidis and eggs: a national epidemic in the United States. Clinic. Infec. Dis. 43:512-517.

Cox, N. A., J. S. Bailey, and M. E. Berrang. 1996. Alternative routes for *Salmonella* intestinal tract colonization of chicks. J. Appl. Poult. Res. 5:282-288.

Cox, N. A., J. S. Bailey, L. C. Blankenship, R. J. Meinersmann, N. J. Stern, and F. McHan. 1990. Fifty percent colonization dose for *Salmonella* typhimurium administered orally and intracloacally to young broiler chicks. Poult. Sci. 69:1809-1812.

Cox, N. A., M. E. Berrang, and J. A. Cason. 2000. *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs--a review. Poult. Sci. 79:1571-1574.

Cox, N. A., B. H. Davis, A. B. Watts, and A. R. Colmer. 1973. *Salmonella* in the laying hen. 1. *Salmonella* recovery from viscera, feces and eggs following oral inoculation. Poult. Sci. 52:661-666.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella* enterica serovar Enteritidis infection. Infect. Immun. 79:2755-2763.

Fernandez-Rubio, C., C. Ordonez, J. Abad-Gonzalez, A. Garcia-Gallego, M. P. Honrubia, J. J. Mallo, and R. Balana-Fouce. 2009. Butyric acid-based feed additives help protect broiler chickens from *Salmonella* Enteritidis infection. Poult. Sci. 88:943-948.

Freitas Neto, O. C. d., A. L. Mesquita, J. B. d. Paiva, F. Zotesso, and A. Berchieri Júnior. 2008. Control of *Salmonella* enterica serovar Enteritidis in laying hens by inactivated *Salmonella* Enteritidis vaccines. Brazilian J. of Microbiol. 39:390-396.

Gantois, I., F. Ducatelle R Fau - Pasmans, F. Pasmans F Fau - Haesebrouck, R. Haesebrouck F Fau - Gast, T. J. Gast R Fau - Humphrey, F. Humphrey Tj Fau - Van Immerseel, and F. Van Immerseel. 2009. Mechanisms of egg contamination by *Salmonella* Enteritidis. Microbiol. Rev. 33:718-738.

Gast, R. K., J. Guard, and P. S. Holt. 2004. Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella* Heidelberg and *Salmonella* Enteritidis. Avian Dis. 48:863-869.

Gast, R. K., R. Guraya, and J. Guard. 2013a. *Salmonella* Enteritidis deposition in eggs after experimental infection of laying hens with different oral doses. J. Food Prot. 76:108-113.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2013b. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 92:468-473.

Gast, R. K., R. Guraya, J. Guard, and P. S. Holt. 2011a. Frequency and magnitude of internal organ colonization following exposure of laying hens to different oral doses of *Salmonella* Enteritidis. Int. J. Poult. Sci. 10:325-331.

Gast, R. K., R. Guraya, and P. S. Holt. 2011b. Frequency and persistence of fecal shedding following exposure of laying hens to different oral doses of *Salmonella* Enteritidis. Int. J. Poult. Sci 10:750-756.

Gast, R. K., R. Guraya, J. Guard-Bouldin, P. S. Holt, and R. W. Moore. 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella* Enteritidis or *Salmonella* Heidelberg. Avian Dis. 51:40-44.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2015. Persistence of fecal shedding of *Salmonella* Enteritidis by experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 94:1650-1656.

Gast, R. K., and P. S. Holt. 1998. Persistence of *Salmonella* Enteritidis from one day of age until maturity in experimentally infected layer chickens. Poult .Sci. 77:1759-1762.

Gast, R. K., and P. S. Holt. 2000. Deposition of phage type 4 and 13a *Salmonella* Enteritidis strains in the yolk and albumen of eggs laid by experimentally infected hens. Avian Dis. 44:706-710.

Gast, R. K., and P. S. Holt. 2001. Assessing the frequency and consequences of Salmonella Enteritidis deposition on the egg yolk membrane. Poult. Sci. 80:997-1002.

He, G. Z., W. Y. Tian, N. Qian, A. C. Cheng, and S. X. Deng. 2010. Quantitative studies of the distribution pattern for *Salmonella* Enteritidis in the internal organs of chicken after oral challenge by a real-time PCR. Vet. Res. Commun. 34:669-676.

Holt, P. S., L. E. Vaughn, R. W. Moore, and R. K. Gast. 2006. Comparison of *Salmonella* enterica serovar Enteritidis levels in crops of fed or fasted infected hens. Avian Dis. 50:425-429.

Horrocks, S. M., Y. S. Jung, J. K. Huwe, R. B. Harvey, S. C. Ricke, G. E. Carstens, T. R. Callaway, R. C. Anderson, N. Ramlachan, and D. J. Nisbet. 2007. Effects of short-chain nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli* in vitro. J. Food Sci. 72:M50-55.

Humphrey, T. J., H. Chart, A. Baskerville, and B. Rowe. 1991b. The influence of age on the response of SPF hens to infection with *Salmonella* Enteritidis PT4. Epidemiol. Infect. 106:33-43.

Kallapura, G., M. J. Morgan, N. R. Pumford, L. R. Bielke, A. D. Wolfenden, O. B. Faulkner, J. D. Latorre, A. Menconi, X. Hernandez-Velasco, V. A. Kuttappan, B. M. Hargis, and G. Tellez. 2014. Evaluation of the respiratory route as a viable portal of entry for *Salmonella* in poultry via intratracheal challenge of Salmonella Enteritidis and *Salmonella* Typhimurium. Poult. Sci. 93:340-346.

Kramer, J., A. H. Visscher, J. A. Wagenaar, A. G. Boonstra-Blom, and S. H. Jeurissen. 2001. Characterization of the innate and adaptive immunity to Salmonella Enteritidis PT1 infection in four broiler lines. Vet. Immunol. Immunopathol. 79:219-233.

Langkabel, N., A. Klose, H. Irsigler, D. Jaeger, L. Bräutigam, H. M. Hafez, and R. Fries. 2014. Comparison of methods for the detection of Salmonella in poultry. J. Appl. Poult. Res. 23:403-408.

Matis, G., A. Kulcsar, V. Turowski, H. Febel, Z. Neogrady, and K. Huber. 2015. Effects of oral butyrate application on insulin signaling in various tissues of chickens. Domes. Anim. Endocrinol. 50:26-31.

Mead, G. C. 2000. Prospects for 'competitive exclusion' treatment to control *Salmonellas* and other foodborne pathogens in poultry. Vet. J. 159:111-123.

Ng, S. C., A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: recent advances. Inflamm. Bowel Dis. 15:300-310.

Sadler, W. W., J. R. Brownell, and M. J. Fanelli. 1969. Influence of age and inoculum level on shed pattern of *Salmonella* typhimurium in chickens. Avian Dis. 13:793-803.

Schneitz, C. 1992. Research note: automated droplet application of a competitive exclusion preparation. Poult. Sci. 71:2125-2128.

Seifert, S., and B. Watzl. 2007. Inulin and oligofructose: review of experimental data on immune modulation. J. Nutr. 137:2563s-2567s.

Setta, A. M., P. A. Barrow, P. Kaiser, and M. A. Jones. 2012. Early immune dynamics following infection with *Salmonella enterica* serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. Comp. Immunol. Microbiol. Infect. Dis. 35:397-410.

Snoeyenbos, G., O. M. Weinack, and C. F. Smyser. 1978. Protecting chicks and poults from Salmonellae by oral administration of "normal" gut microflora. Avian Dis. 22:273-287.

Van Immerseel, F., F. Boyen, I. Gantois, L. Timbermont, L. Bohez, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2005. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poult. Sci.

Table 3.1. Diet composition and calculated composition of basal diet fed to laying hens.

Ingredient	Amount[1]
Corn, grain	62.62
Soybean meal, 48%	20.00
Limestone	9.62
Soybean oil	2.75
Defluor phosphate	2.15
L-Lysine	1.75
Vitamin premix[2]	0.50
Common salt	0.30
DL-Methionine	0.16
Mineral premix[3]	0.15
Calculated composition	
ME (Kcal/kg)	2.9
CP	16
Ca	4.4
Available P	0.5

[2]Vitamin premix provided the following (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; D-biotin, 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]Supplemeted 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; D-biotin, 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.

Table 3. 2. Recovery of *Salmonella* Enteritidis in ceca and internal organs (%) of hens challenged either orally (OR) or intracloacally (IC) at 7 and 14 dpi (Experiments 2 and 3).

		Ceca		Ovaries		Spleen		L/GB[1]	
Experiment	DPI	OR	IC	OR	IC	OR	IC	OR	IC
2	7	100 (7/7[a])	100 (7/7)	28.6 (2/7)	28.6 (2/7)	85.7 (6/7)	57.1 (4/7)	42.8 (3/7)	85.7 (6/7)
	P-Value	1.000		1.000		<0.0001		0.0003	
	14	14.3 (1/7)	28 (2/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)
	P-Value	1.000		1.000		1.000		1.000	
3	7	85.7 (6/7)	100 (7/7)	14.3 (1/7)	42.8 (3/7)	42.8 (3/7)	57.1 (4/7)	57.1 (4/7)	71.4 (5/7)
	P-Value	<0.0001		<0.0001		0.0657		0.0551	
	14	14.3 (1/7)	42.8 (3/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	14.3 (1/7)	14.3 (1/7)
	P-Value	<0.0001		1.000		1.000		1.000	

[a] Number positive/number sampled

[1]Liver with gall-bladder

N=36 hens

Table 3.3. Recovery of *Salmonella* Enteritidis in internal organs (%) of hens challenged either orally (OR) or intracloacally (IC) at 7 and 14 day post-infection (dpi) (experiments 4 and 5).

		Ovaries		Spleen		L/GB	
Experiment	DPI	OR	IC	OR	IC	OR	IC
4	7	14.3 (1/7[a])	14.3 (1/7)	71.4 (5/7)	86 (6/7)	86 (6/7)	71.4 (5/7)
	P-Value	1.000		0.0153		0.065	
	14	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	14.3 (1/7)	0 (0/7)
	P-Value	1.000		1.000		1.000	
5	7	14.3 (1/7)	28.6 (2/7)	57 (4/7)	71.4 (5/7)	43 (3/7)	57 (4/7)
	P-Value	0.0153		0.055		0.065	
	14	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)
	P-Value	1.000		1.000		1.000	

[a] Number positive/number sampled

[1]Liver with gall-bladder

N=36 hens

Table 3. 4. Cecal count of *Salmonella* Enteritidis in hens challenged oral (OR) and intracloacal (IC) routes, determined by swab-plate method at 7 and 14 days post-infection (dpi) (experiments 4 and 5)[1].

		Log_{10} cfu/g SE^{NAR} in cecal content	
Experiment	DPI	OR	IC
4	7	1.8 ± 0.335[a] (1.5-2.3)	2.5 ± 1.731[a] (1.5-6.7)
	14	0.43 ± 0.677[b] (0-1.5)	0.43 ± 0.677[b] (0-1.5)
P-Value		<0.0001	<0.0001
5	7	3.2 ± 1.691[a] (1.5-6.7)	3.1 ± 1.906[a] (1.5-6.7)
	14	0.21 ± 0.524[b] (0-1.5)	0.21 ± 0.524[b] (0-1.5)
P-Value		<0.0001	<0.0001

[1]The data for cecal count is the mean ± SEM. The count is as Log_{10} cfu/g.

[ab]Different letters in the same column denote means are significantly different ($P<0.05$).

N=10 hens

Table 3. 5. Recovery of *Salmonella* Enteritidis from feces (%) of hens challenged either orally (OR) or intracloacally (IC) at 3, 6 and 13 dpi (Experiments 2 and 3)

Experiment	DPI	OR	IC	P-value
2	3	100 (14/14[a])	100 (14/14)	1.000
	6	92.8 (13/14)	85.7 (12/14)	0.1652
	13	0 (0/7)	0 (0/7)	1.000
3	3	100 (14/14)	100 (14/14)	1.000
	6	85.7 (12/14)	71.4 (10/14)	0.0153
	13	0 (0/7)	0 (0/7)	1.000

[a] Number positive/number sampled

N=36 hens

4. THE EFFECT OF DIETARY FRUCTOOLIGOSACCHARIDE SUPPLEMENTATION ON INTERNAL ORGANS COLONIZATION, FECAL SHEDDING, ILEAL IMMUNE RESPONSE, ILEAL MORPHOLOGY AND IMMUNOHISTOCHEMISTRY IN LAYING HENS CHALLENGED WITH *SALMONELLA* ENTERITIDIS[2]

[2]P. A. Adhikari, D. E. Cosby, N. A. Cox, M. Franca, S. M. Williams, R. M. Gogal, C. W. Ritz and W. K. Kim. To be submitted to *Poultry Science Journal*.

ABSTRACT

Two experiments were conducted to evaluate the efficacy of fructoligosaccharides (FOS) in controlling the infection of *Salmonella* Enteritidis (SE) in White Leghorns. Thirty laying hens were challenged both orally (OR) and intracloacally (IC) with approximately 10^8 colony forming units (cfu) of nalidxic acid resistant SE (SE^{NAR}) and divided into 3 treatments: 1) SE^{NAR} challenged control 2) SE^{NAR} challenged + 0.5% FOS; Nutraflora®) and 3) SE^{NAR} challenged + 1.0% FOS. The recovery of SE^{NAR} in fecal shedding was measured at 3 and 6-day post-infection (dpi) and in ceca and internal organs the recovery was measured at 7 dpi. In experiment 1, there was a 1.0 $\log_{10}$ and a 1.4 $\log_{10}$ reduction in cecal SE^{NAR} by supplementation of FOS at 0.5 and 1.0%, respectively. In experiment 2, there was a 0.4 $\log_{10}$ and a 0.8 $\log_{10}$ reduction in cecal SE^{NAR} by supplementation of FOS at 0.5 and 1.0%, respectively. Fecal shedding was significantly lower (P<0.05) in 1.0% FOS supplemented groups compared to SE^{NAR} challenge control. There was no significant difference among three treatments on SE^{NAR} recovery in liver with gall bladder (L/GB) and ovaries. However, the frequency of positive SE^{NAR} in ovaries (10 to 40%) in SE^{NAR} challenge control was significantly lower (P<0.05) than L/GB (60 to 80%) in both experiments. There was a significant upregulation (P<0.05) of toll-like receptor (TLR)-4 in 1.0% FOS and interferon gamma (IFN- γ) in both 0.5 and 1.0% FOS but no changes in other cytokines such as interleukin (IL)- 1ß, IL-6 or IL-10 by supplementing FOS. Histology measurements of ileum villi height (VH) and crypt depth (CD) did not show any significant difference among the treatments. Immunohistochemistry analyses of ileal samples showed that the numbers of immunoglobulin A (IgA) positive cells were significantly highest (P<0.05) in 1.0% FOS than in *Salmonella* challenge control and 0.5% FOS. These results demonstrated that the cecal SE^{NAR} and feces SE^{NAR} were reduced by supplementing FOS. Due to both *Salmonella*

challenge and FOS supplementation, there were expression of immune cytokines as well as IgA expressing cells in lamina propria of the ileum.

Key Words: Fructoligosaccharide, laying hen, *Salmonella* Enteritidis, cytokine, histology, immunohistochemistry

INTRODUCTION

Foodborne diseases continue to be important health and economic issues in the United States with the higher incidence observed for *Salmonella* (CDC, 2014). *Salmonella enterica* serovar Enteritidis **(SE)** is a facultative intracellular food-borne pathogen that causes illness in chickens and humans (Babu et al., 2012). The major sources of human *Salmonella* infections are the contaminated meat and eggs from *Salmonella* carrier chickens (Kao et al., 2010). The misuse of antibiotics leading to the resistance in bacteria like *Salmonella*, has created demands for the several antimicrobial or inhibitory replacements (Cheng et al., 2014). Dietary interventions have been evaluated in some plant by-products including wheat-middling and alfalfa with fructoligosaccharides **(FOS)** to reduce the *Salmonella* colonization in molting hens (Dunkley et al., 2007; Seo et al., 2001). Typical studies have been conducted with SE challenge with prebiotics, probiotics and symbiotic supplementation in *Salmonella* free 1-day-old broilers as well as laying hens chicks (Murate et al., 2015). Studies reported the effect of FOS in chickens especially in broilers resulting in better gain and feed conversion (Bailey et al., 1991; Xu, et al., 2003). Dietary FOS supplementation has the potential to elevate the anti-*Salmonella* activity, which is mainly due to the shift of intestinal microbiota and the production of short chain fatty acids **(SCFA)** (Van Immerseel et al., 2009). Production of SCFA has been shown to modify the bacterial ecosystem in the ceca and inhibit the growth of enteric bacteria such as *Salmonella*, *Escherichia coli*, and *Clostridium perfringens* (Cummings et al., 2001; Cummings and Macfarlane, 2002). Dietary supplementation with FOS showed a four-fold reduction of *Salmonella* in chicken ceca (Bailey, et al., 1991) and had indirect benefits toward the immune system of chickens by promoting the growth of lactic acid producing bacteria (Xu et al., 2003). There has not been any study that evaluated bacteriological as well as immunological consequences due to the FOS supplementation in mature laying hens challenged with SE.

Therefore, hypothesis of the current study was that the FOS supplementation reduces SE in the ceca and internal organs as well as stimulates immunological effects. The objective of our study was to investigate the role of FOS supplementation on anti-*Salmonella* activity in feces, ceca, liver with gall bladder **(L/GB)** and ovary, immune response, intestinal morphology and immunohistochemistry parameters in the ileum of hens fed FOS.

MATERIALS AND METHODS

Salmonella strain, diluent and inoculum preparation

The chicken isolate of SE resistant to nalidixic acid **(SE^{NAR})** was used in the study at the USDA-Agricultural Research Service facility (Athens, GA). Tryptic soy broth (Acumedia, Neogen Corp., Lansing, MI) with 15% glycerol (Sigma-Aldrich., St Louis, MO) was used for the long-term preservation of SE^{NAR}. SE^{NAR} were grown on brilliant green agar with sulphapyridine (**BGS**; Acumedia, East Lansing, MI) containing 200 ppm of Nal (**BGS-Nal**; Sigma-Aldrich., St. Louis, MO). The agar plates were incubated for 24 h at 37°C. Isolated SE^{NAR} colonies were transferred to 9 mL of sterile 0.85 % saline solution. The absorbance value was adjusted to an optical density of 0.20 ± 0.01 at 540nm with a spectrophotometer (Spect-20, Milton-Roy, Thermo Spectronics, Madison, WI), which yields approximately 10^8 cfu/mL. Cultures were serially diluted in sterile saline for enumeration. Hens were individually gavaged orally (**OR**) with a 1 cc tuberculin syringe (Becton, Dickinson and Co., Franklin Lakes, NJ) and an animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY), whereas intracloacal (**IC**) inoculation was performed using only a 1 cc tuberculin syringe. Hens were challenged with the inoculum doses of 2.4×10^8 and 1.7×10^8 cfu/mL of SE^{NAR} in experiment 1 and 2, respectively.

Hens, husbandry and dietary treatments

Two experiments were conducted with 30 Single Combs White Leghorn hens (60 and 65 weeks old at the beginning of the first and second experiment, respectively). Hens were housed

individually in wire-laying cages and fed a corn-soy layer mash diet (Table 1). The diet was formulated to provide 2,600 kg/kcal ME, 16% CP, 4.4% Ca and 0.5% available P (NRC, 1994). All hens were allowed to acclimate to the basal diet for the first week after which they were randomly allocated to the respective treatment diets. Hens were housed under a controlled environment (27 ± 2°C) and a 16h light:8h dark regimen. Feed was withdrawn for 10 h before SE^{NAR} challenge, and feeders were replaced immediately post challenge. Hens were grouped to give 10 replicates per treatment. The treatments were as follows: 1) SE^{NAR} challenged control 2) SE^{NAR} challenged + 0.5% FOS; Nutraflora®) (GTC Nutrition, Bridgewater, NJ) and 3) SE^{NAR} challenged + 1.0% FOS. All experiment protocols were approved by the Institutional Animal Care and Use Committee of University of Georgia (AUP number = A2014 07-016).

Sampling protocol and processing

1. Fecal shedding (bacteriological)

Hens were monitored for fecal shedding on 3 and 6 dpi. Aluminum foil sheets were placed in the bottom of the cages overnight, and feces were collected on the next morning. Feces were collected in sterile 50 mL conical centrifuge tubes were used to collect feces and transported in an ice chest for bacteriological analysis. Briefly, feces were weighed, added with buffered peptone water (**BPW**) 3 times the sample weight was added and vortexed. A 10 µl portion of each sample was streaked for isolation onto BGS-Nal plates. Plates and sample tubes were incubated for 24 h at 37°C. Plates that were negative by direct plating were again streaked into BGS-Nal plates from the overnight pre-enriched samples. The plates were read as negative or positive.

2. Ceca, L/GB and ovaries (bacteriological)

All hens were humanely euthanized by electrocution on 7 dpi. Samples like ceca, ovaries and L/GB were collected aseptically for bacteriological analyses. All the samples were macerated by rubber mallet. Samples were individually weighed and diluted in BPW three times their weight. The sample bags were stomached (Techmar Company, Cincinnati, Ohio) for 60 s and pre-enriched for 24 h at 37°C. Pre-enriched samples for ovaries and L/GB were streaked for isolation onto BGS-Nal plates and incubated for 24 h at 37°C. The growth of SE^{NAR} was observed and recorded.

Cecal samples were analyzed using the modified 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 was 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-struck 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.

3. Ileum immune genes expression

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR

Section of the ileum were aseptically excised, immediately frozen in liquid nitrogen and preserved at -80°C for analysis of immune genes by quantitative real-time polymerase chain reaction **(qRT-PCR**). Total RNA was extracted using about 100 mg of tissues using QiAzol lysis reagents (Qiazen, Valencia, CA, USA) according to the manufacturer's instruction. The

samples were homogenized in a homogenizer (Biospec Products, Fisher Scientific, MA, USA) for 3 min. The RNAse-free water (Ambion, Applied BioSystems, Life Technologies, CA, USA) was used to dissolve the final pellet. The total RNA concentrations were determined at an optical density of 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). All RNA samples were normalized to a concentration of 2 µg/ µl, and the purity of RNA was verified by evaluating the optical density ratio of 260 nm to 280 nm. The normalized RNA was reversed transcribed using High Capacity cDNA synthesis kits (Applied BioSystems, Life Technologies, CA, USA). Individual transcripts were normalized to glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**). Primers for chicken immune genes such as 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) and given in Table 5. qRT-PCR was performed using a Step One thermo-cycler (Applied Biosystem, Foster City, CA).

4. Ileum histomorphology

Approximately 2 cm sections of the ileum were selected and fixed in 10% phosphate-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Briefly, tissues were routinely processed, and samples were embedded in paraffin and sectioned into 4.0 µm. Additionally, unstained sections were stained with standard hematoxylin-eosin solution. Additionally, unstained sections were mounted onto positively charged slides for further immunohistochemistry (**IHC**) analysis. The sections were observed for villi height (**VH)** and crypt depth (**CD**) at 100X magnification with a light microscope coupled with a camera (Leica DC500 camera, Leica Microsystems Inc., Buffalo Groove, IL, USA). A minimum of three readings per slide were made for both VH and CD and averaged into a single value for each. VH was measured as entire villus length from the tip to base. CD was measured from the villus-crypt

axis to the base of the specific crypt. Villus height to CD ratio and total mucosal thickness were also calculated for each ileum. Image J software was used to analyze and measure the length of the captured images.

5. Ileum immunohistochemistry (IHC)

Immunoglobulin A (**IgA**)-positive cells in the ileum were determined by IHC technique. Paraffin-embedded ileal sections were deparaffinized in xylene and hydrated in descending grades of alcohol. Antigen retrieval was executed in citrate buffer at pH 6.0 for 45 min with the use of a steamer. Blocking of peroxidase activity was performed with Bloxall (Vector Laboratories, Burlingame, CA) following manufacturer's instructions. After washing steps, sections were incubated with Protein Block solution (Dako, Carpinteria, CA) for 10 minutes to block nonspecific binding. A mouse monoclonal anti-chicken IgA (Southern Biotech, Birmingham, AL, USA) was diluted at 1:500 concentrations and used as a primary antibody. Tissue sections were incubated with the primary antibody at room temperature for 1 h. Slides incubated with phosphate-buffered saline were used as negative controls. Tissues were incubated with MACH3 mouse probe and polymer (Biocare Medical, Concord, CA) following manufacturer's recommendations. After washing steps, tissues were incubated with 3, 3' diaminobenzidine tetrahydrochlorine (DAB) (Vector Laboratories, Burlingame, CA) for 10 minutes at room temperature and counterstained with hematoxylin. Sections were examined by a bright field microscope. An Olympus DP25 camera was used to take photographs of three 100x fields of view per section. Counting of immunostained cells was performed with CellSens Standard software (Olympus, Center Valley, PA).

Statistical analyses

SE^{NAR} recovery from feces and internal organs was analyzed with Fisher's exact test for any *Salmonella* prevalence. The relative quantification analysis of qRT-PCR data was performed using the ΔΔCt method (Livak et al., 2001). The means of Log_{10} viable SE^{NAR} counts from the ceca, ileum immune gene expression, histomorpholgy and IHC data were subjected to one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS, 2009). Significant differences between the means of different treatments were determined by Duncan's multiple-range test, and significant differences were assessed at $P<0.05$.

RESULTS

Prevalence of SE^{NAR} in fecal samples

The results of fecal shedding from laying hens at 3 and 6 dpi are shown in Table 2. On 3 dpi, feces were 100% positive in SE^{NAR} in all treatments (Table 2). There was significantly lower ($P<0.05$) prevalence of SE^{NAR} in 1.0% FOS treated group at 6 dpi in both experiments compared to SE^{NAR} challenge control.

Cecal count of SE^{NAR}

The recovery of SE^{NAR} as log_{10} cfu/g of cecal contents in all 3 treatments is shown in Table 3. Supplementation of FOS at 1.0% significantly reduced ($P<0.05$) the cecal SE^{NAR} compared to control diet but 1.0% FOS was not different from 0.5% FOS in both experiments. In experiment 1, the mean log_{10} value of SE^{NAR} colonization was 4.2 in control that was reduced to log 3.2 in 0.5% FOS and to log 2.8 in 1.0% FOS. In experiment 2, the mean log_{10} value of SE^{NAR} colonization was log 3.7, 3.3 and 2.9 in control, 0.5 and 1.0% FOS, respectively. In experiment 1, there was 1.0 log_{10} and 1.4 log_{10} reduction in cecal SE^{NAR} by supplementation of FOS at 0.5 and 1.0%, respectively. In experiment 2, there was 0.4 log_{10} and 0.8 log_{10} reduction in cecal SE^{NAR} by supplementation of FOS at 0.5 and 1.0%, respectively.

Prevalence of SE^{NAR} in ovary and L/GB

The prevalence of SE^{NAR} in L/GB and ovary at 7 dpi is presented in Table 4. The SE recovery of ovary was significantly lower ($P<0.05$) compared to one of L/GB. Ovary was 40% positive in challenged control, while for 0.5% and 1.0%, it was 20 and 30% positive, respectively (experiment 1). In experiment 2, ovary was 20% positive in control, whereas it was 20 and 10% positive for 0.5% and 1.0% FOS. In L/GB, FOS supplementation at any level did not reduce the recovery.

Cytokine gene expression in the ileum

The SE^{NAR} challenge affected expression of ileal cytokine genes such as TLR-4 and IFN-γ ($P=0.0005$ and $P=0.003$; Figure 1). Supplementation of 1.0 % FOS significantly up-regulated the TLR-4 mRNA expression compared to both control and 0.5 % FOS (Figure 1b). Supplementation of 0.5 and 1.0 % FOS significantly up-regulated the IFN- γ mRNA expression compared to the SE challenged control group (Figure 1d). No significant differences were observed for rest of the cytokines including IL-1ß, IL-6 and IL-10 ($P=0.552$, $P=0.340$, and $P=0.786$, respectively).

Intestinal morphology and IgA count in the ileum

In both of the experiments, VH, CD or their ratio did not differ between the SE challenged control and FOS treatments (Table 6). The stain of IgA cells in lamina propria of ileum is shown in Figure 2. The number of IgA positive cells in the ileum sections is shown in Figure 3. IgA positive cells were detected in intestinal mucosa in all three treatment groups. 1.0% FOS treatment had significantly higher number of (20.5) IgA positive cells compared to 0.5% FOS treatment (15.5). SE challenged control and 0.5% FOS were not different from one another.

DISCUSSION

Salmonella in feces, ceca, L/GB and ovary

The FOS used in our study has shown to be effective in reducing *Salmonella* Enteritidis (SE) fecal shedding at 6 dpi. Since there is a high chance of egg contamination after the egg laying process, knowledge of SE fecal shedding pattern would help us to evaluate intervention approaches to reduce *Salmonella* contamination in eggs. A previous study reported that supplementation of 0.75% FOS reduced *Salmonella* prevalence in 12% and resulted in 0.75 log10 cfu reduction in *Salmonella* numbers when compared with control birds (Bailey, et al., 1991). Studies have shown that FOS alone or in combination with competitive exclusion cultures decrease organ colonization and recovery of SE from cecal contents of White Leghorn as well as broiler chicks (Bailey, et al., 1991; Fukata et al., 1999).

Invasion beyond intestine to internal organs like liver and spleen occurs within few hours of exposure to *Salmonella* infection (He, et al., 2010). Hens fed diets containing both alfalfa and FOS had significantly reduced fecal shedding as well as organs (liver and ovary) colonization of SE (Donalson et al., 2008). However, the above study was conducted with the complete feed withdrawal for 7 days unlike to only an overnight feed withdrawal in our study. Feed withdrawal in laying hens is one of the major stressors, and thus the incidence of SE is higher in such withdrawal periods. In our study, FOS was supplemented in feed and not in water it was previously reported that in-feed supplementation of FOS is more effective in reducing *Salmonella* numbers than via drinking water (Bailey, et al., 1991).

Immune gene expression changes by Salmonella and FOS

Salmonella infection has shown to upregulate inflammatory cytokines such as IL-1ß, IL-18 and IFN- γ (Babu, et al., 2012; Chappell et al., 2009; Yasuda et al., 2002). Interleukin-1ß is a pro-inflammatory cytokine mainly secreted from monocytes and macrophages (Corwin, 2000).

In our study, there was no difference between treatments in the expression of IL-1ß gene. Our finding on IL-1ß was similar to a study that reported IL-1ß was neither upregulated nor downregulated when chicks were challenged with *Salmonella* and supplemented with FOS (Janardhana, et al., 2009b). However, in one of the studies pro-inflammatory cytokines including IL-1ß were reduced by supplementing FOS-inulin diet in SE infected cells (Babu, et al., 2012).

Toll-like receptors can recognize the conserved pathogen-associated molecular patterns of the lipopolysaccharides (LPS) of gram-negative bacteria, and are involved in a chain reaction that stimulates the innate immune response (Aderem et al., 2000). Elevation of TLR-4 in 1.0 % FOS diet compared to the control and 0.5% FOS might be due to higher level of FOS showing higher inflammatory reaction in the ileum tissue. Orally administered prebiotics are non-inflammatory in basal conditions but are beneficial in experimental intestinal inflammation (Daddaoua et al., 2006). In a study, it has been reported that monocytes are activated by FOS and inulin possibly via TLR-4 ligation that results enhanced cytokines secretion (Daddaoua, et al., 2006). Also, such inflammation may provide knowledge about the small intestine being a main site for pathogen control of gut associated infections (Shang et al., 2015). In chickens, the TLR-4 is shown to be linked to the resistance to *Salmonella* infection (Leveque et al., 2003).

IL-6 serves as both pro- and anti-inflammatory cytokines and is also produced in monocytes and macrophages (Waititu et al., 2014). Increased chemokines and cytokine gene expression (IL-1ß, IL-6, IL-8, IL-18 and CCLi2) in heterophils, monocyte-derived macrophages, ceca and cecal tonsil are supposed to be associated with *Salmonella* resistance (Ferro et al., 2004; Setta et al., 2012). Higher expression of IL-6 may be associated with strong pro-inflammatory immune response. The current study did not show any difference in the IL-6 expressions between the treatments. This result agrees with other studies where positive effects of FOS or

prebiotics in IL-6 expression in the cecal tonsil, ileum and spleen of broilers were not observed (Janardhana, et al., 2009b; Yitbarek et al., 2015).

Interferon-γ is a pro-inflammatory cytokine that is responsible for increasing the expression of major histocompatibility complex (MHC) antigens and provides host defense against intracellular pathogens like *Salmonella* (Benbernou et al., 1994). Similarly, supplementing FOS also up-regulated ileal IL -1ß, -2, -10, -18, TLR-4, IFN-γ and splenic IL-18, IL -1ß expressions effects (Shang, et al., 2015). Similarly, a study that used dietary yeast cells found higher expression of IFN-γ in broilers (Shanmugasundaram et al., 2015). Interleukin-10 is a major anti-inflammatory cytokine, which can directly regulate both innate and adaptive T cell responses as well as suppresses inflammatory responses in tissues (Couper et al., 2008). The current study did not show any difference in IL-10 expression between treatments and our findings are like a previous study where FOS did not show any effects on IL-10 expression in the cecal tonsil (Janardhana, et al., 2009b). Our results contrasts with another study that found upregulation of IL-10 in cecal tonsil by supplementing a blend of yeast derived carbohydrates and probiotics (Yitbarek, et al., 2015).

Ileal morphology and IgA expression

Structure of the intestinal mucosa can provide the information about the health of the digestive tract (Bogusławska-Tryk, 2012). Stress factors in the digesta can lead to shortening of villi and deepening of crypts (Bogusławska-Tryk, 2012). Increasing the VH suggests an increased surface area capable of greater absorption of available nutrients (Caspary, 1992). The increase in CD or crypt to VH ratio indicates the greater need of cell proliferation to maintain the gut barrier function (Awad, et al., 2009). The reason behind not been able to see the differences in the hens used in our study might be due to the age and maturity of laying hens used on our

study; they were old enough not to have any effects at the gut integrity level. Similarly, Xu, et al. (2003) accessed the effects of three levels of dietary FOS added to a broiler basal diet at 2.0, 4.0 and 8.0 g/kg mixture and found no dietary effect detected for villi and microvilli or CD in the duodenum.

IgA is the major isotype of immunoglobulin secreted on the mucosal surface and protects the intestinal mucosal surfaces from invasion and colonization from pathogens (Macpherson et al., 2008). Intestinal immune system is considered to have the largest accumulation of antibodies in the body (Burkey et al., 2009). The IgA is the predominant in intestinal secretions and is synthesized by plasma cells in the lamina propria (Bos et al., 2001). Intestinal immune response plays an important defensive role for pathogens, particularly for those transmitted by fecal shedding (Bianco et al., 2014). There was an expression of IgA cells in all *Salmonella* infected groups, and this was similar to a previous study that reported increased IgA positive cells after *Salmonella* infection (Bobikova et al., 2015). The expression of IgA in 0.5% FOS treated hens compared to the challenge control in our study was similar to other studies that showed no variation in frequency of IgA positive cells in the intestine of broilers fed FOS (Janardhana, et al., 2009b; Kim et al., 2011a). Moreover, it has been reported that in white leghorns, there were high titers of IgA specific for SE in crop samples (Kim, et al., 2009; Seo et al., 2003), and there was a high correlation observed between crop and intestinal IgA level. This relates to the mucosal response and might be the protective mechanism against *Salmonella.*

CONCLUSION

FOS reduced fecal shedding and ceca SE^{NAR} numbers in mature hens. The SE^{NAR} challenge also significantly affected the immune responses of laying hens including the major cytokines such as TLR-4 and IFN- γ, as well as expression of IgA. However, there was no effect

observed for either VH or CD. The study shows that FOS can be used as one of the dietary interventions to reduce *Salmonella* infection in chickens thus helping the poultry industry.

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References

Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. Nature 406:782-787.

Awad, W. A., K. Ghareeb, S. Abdel-Raheem, and J. Bohm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88:49-56.

Babu, U. S., K. Sommers, L. M. Harrison, and K. V. Balan. 2012. Effects of fructooligosaccharide-inulin on *Salmonella*-killing and inflammatory gene expression in chicken macrophages. Vet. Immunol. Immunopathol. 149:92-96.

Bailey, J. S., L. C. Blankenship, and N. A. Cox. 1991. Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poult. Sci. 70:2433-2438.

Benbernou, N., and C. Nauciel. 1994. Influence of mouse genotype and bacterial virulence in the generation of interferon-gamma-producing cells during the early phase of *Salmonella* Typhimurium infection. Immunol. 83:245-249.

Bianco, C., V. Felice, S. Panarese, R. Marrocco, F. Ostanello, B. Brunetti, L. V. Muscatello, G. Leotti, T. Vila, F. Joisel, and G. Sarli. 2014. Quantitative immunohistochemical assessment of IgA, IgM, IgG and antigen-specific immunoglobulin secreting plasma cells in pig small intestinal lamina propria. Vet. Immunol. Immunopathol. 160:281-287.

Blanchfield, B., S. Stavric, T. Gleeson, and H. Pivnick. 1984. Minimum intestinal inoculum for Nurmi cultures and a new method for determining competitive exclusion of *Salmonella* from chicks. J. Food Prot. 47:542-545.

Bobikova, K., V. Revajova, V. Karaffova, M. Levkutova, and M. Levkut. 2015. IgA gene expression and quantification of cecal IgA+, IgM+, and CD4+ cells in chickens treated with EFAL41 and infected with *Salmonella* Enteritidis. Acta. Histochem. 117:629-634.

Bogusławska-Tryk, M., A. Piotrowska, and K. Burlikowska 2012. Dietary fructans and their potential beneficial influence on health and performance parametrs in broiler chickens. J. Cent. Euro. Agri. 13:272-291.

Bos, N., H. Jiang, and J. Cebra. 2001. T cell control of the gut IgA response against commensal bacteria. Gut 48:762-764.

Burkey, T. E., K. A. Skjolaas, and J. E. Minton. 2009. Board-invited review: porcine mucosal immunity of the gastrointestinal tract. J. Anim. Sci. 87:1493-1501.

Caspary, W. F. 1992. Physiology and pathophysiology of intestinal absorption. Am. J. Clin. Nutr. 55:299s-308s.

CDC. 2014. Incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. Sites, 2006–2013. Morb. Mortal. Wkly. Rep. 63:328-332.

Chappell, L., P. Kaiser, P. Barrow, M. A. Jones, C. Johnston, and P. Wigley. 2009. The immunobiology of avian systemic salmonellosis. Vet Immunol. Immunopathol. 128:53-59.

Cheng, G., H. Hao, S. Xie, X. Wang, M. Dai, L. Huang, and Z. Yuan. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Frontiers Microbiol. 5:217.

Corwin, E. J. 2000. Understanding cytokines. Part I: Physiology and mechanism of action. Biol. Res. Nurs. 2:30-40.

Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: The master regulator of immunity to infection. J. Immunol. 180:5771-5777.

Daddaoua, A., V. Puerta, P. Requena, A. Martinez-Ferez, E. Guadix, F. S. de Medina, A. Zarzuelo, M. D. Suarez, J. J. Boza, and O. Martinez-Augustin. 2006. Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis. J. Nutr. 136:672-676.

Donalson, L. M., J. L. McReynolds, W. K. Kim, V. I. Chalova, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* enteritidis infection, and intestinal shedding in laying hens. Poult. Sci. 87:1253-1262.

Dunkley, K. D., J. L. McReynolds, M. E. Hume, C. S. Dunkley, T. R. Callaway, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. Molting in *Salmonella* enteritidis-challenged laying hens fed alfalfa crumbles. I. *Salmonella* enteritidis colonization and virulence gene hilA response. Poult. Sci. 86:1633-1639.

Ferro, P. J., C. L. Swaggerty, P. Kaiser, I. Y. Pevzner, and M. H. Kogut. 2004. Heterophils isolated from chickens resistant to extra-intestinal *Salmonella* Enteritidis infection

express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol. and Infect. 132:1029-1037.

Fukata, T., K. Sasai, T. Miyamoto, and E. Baba. 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on *Salmonella* colonization of chicks. J. Food Prot. 62:229-233.

He, G. Z., W. Y. Tian, N. Qian, A. C. Cheng, and S. X. Deng. 2010. Quantitative studies of the distribution pattern for *Salmonella* Enteritidis in the internal organs of chicken after oral challenge by a real-time PCR. Vet. Res. Commun. 34:669-676.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. D. Bean. 2009. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139:1404-1409.

Kim, C., K. Shin, K. Woo, and I. Paik. 2009. Effect of dietary oligosaccharides on the performance, intestinal microflora and serum immunoglobulin contents in laying hens. Korean J. Poult. Sci. 36:125-131.

Kim, G.-B., Y. M. Seo, C. H. Kim, and I. K. Paik. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90:75-82.

Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredo-Osti, K. Morgan, and D. Malo. 2003. Allelic variation in TLR4 is linked to susceptibility to *Salmonella* enterica serovar Typhimurium infection in chickens. Infect. Immun. 71:1116-1124.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods (San Diego, Calif.) 25:402-408.

Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. Mucosal Immunol. 1:11-22.

Murate, L. S., F. G. Paião, A. M. d. Almeida, A. Berchieri Jr, and M. Shimokomaki. 2015. Efficacy of prebiotics, probiotics, and synbiotics on laying hens and broilers challenged with *Salmonella* Enteritidis. Poult. Sci. 52:52-56.

NRC. 1994. Nutrient Requirements of Poultry. Ninth Revised Edition. Natl. Acad. Press, washington, DC.

Rehman, H., C. Rosenkranz, J. Bohm, and J. Zentek. 2007. Dietary inulin affects the morphology but not the sodium-dependent glucose and glutamine transport in the jejunum of broilers. Poult. Sci. 86:118-122.

Samanya, M., and K. E. Yamauchi. 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus* subtilis var. natto. Comp. Biochem. and Physiol. A. Mol. and Integrat. Physiol. 133:95-104.

SAS. 2009. Statistical Analysis Systems users guide (9th ed.). SAS Institute Inc., Cary, N.C., USA.

Seo, K. H., P. S. Holt, and R. K. Gast. 2001. Comparison of *Salmonella* Enteritidis infection in hens molted via long-term feed withdrawal versus full-fed wheat middling. J. Food. Prot. 64:1917-1921.

Seo, K. H., P. S. Holt, L. E. Vaughn, R. K. Gast, and H. D. Stone. 2003. Detection of Salmonella enteritidis-specific immunoglobulin A antibodies in crop samples from chickens infected with *Salmonella* enteritidis. Poult. Sci. 82:67-70.

Setta, A. M., P. A. Barrow, P. Kaiser, and M. A. Jones. 2012. Early immune dynamics following infection with *Salmonella enterica* serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. Comp. Immunol. Microbiol. Infect. Dis. 35:397-410.

Shang, Y., A. Regassa, J. H. Kim, and W. K. Kim. 2015. The effect of dietary fructooligosaccharide supplementation on growth performance, intestinal morphology, and immune responses in broiler chickens challenged with *Salmonella* Enteritidis lipopolysaccharides. Poult. Sci. 94:2887-2897.

Shanmugasundaram, R., M. H. Kogut, R. J. Arsenault, C. L. Swaggerty, K. Cole, J. M. Reddish, and R. K. Selvaraj. 2015. Effect of *Salmonella* infection on cecal tonsil regulatory T cell properties in chickens. Poult. Sci. 94:1828-1835.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Z. and, and M. Q. Wang. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82:1030-1036.

Yasuda, M., S. Tanaka, H. Arakawa, Y. Taura, Y. Yokomizo, and S. Ekino. 2002. A comparative study of gut-associated lymphoid tissue in calf and chicken. Anat. Rec. 266:207-217.

Yitbarek, A., H. Echeverry, P. Munyaka, and J. C. Rodriguez-Lecompte. 2015. Innate immune response of pullets fed diets supplemented with prebiotics and synbiotics. Poult. Sci.

Table 4. 1. Diet formulation and calculated composition of FOS diet fed to laying hens[1] (Experiment 1 and 2)

Item	Diet		
	Basal	0.5% FOS	1.0% FOS
Ingredient (% of the diet)			
Corn, Grain	59.53	59.53	59.53
Soybean Meal -48%	23.13	23.13	23.13
Limestone	9.62	9.62	9.62
Soybean OiL	3.00	3.00	3.00
Defluor. Phos.	2.13	2.13	2.13
Vitamin Premix[1]	0.50	0.50	0.50
DL-Methionine	0.34	0.34	0.34
Common Salt	0.30	0.30	0.30
L-Lysine HCl	0.30	0.30	0.30
Mineral Premix[2]	0.15	0.15	0.15
FOS[3]	0.00	0.50	1.00
Sand	1.00	0.50	0.00
Calculated composition			
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]Diets are in as-fed basis.

[2]Supplemeted 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; D-biotin, 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.

[3]Fructoligosachharides: NutraFlora® (GTC Nutrition, Bridgewater, NJ)

Table 4. 2. Presence of *Salmonella* Enteritidis in fecal shedding analyzed at 3 and 6 days post infection (dpi) from laying hens fed fructoligosaccharides (FOS) diets (Experiment 1 and 2)

Experiments	Dpi	Treatments		
		Control	0.5% FOS	1.0% FOS
1	3	10/10[1] (100%)	10/10 (100%)	10/10 (100%)
	6	9/10[a] (90%)	7/10[a] (70%)	6/10[b] (60%)
2	3	10/10 (100%)	10/10 (100%)	10/10 (100%)
	6	8/10[a] (80%)	7/10[a] (70%)	6/10[b] (60%)

[1]Pos/Tot: number of SE-positive hens out of a total of 10 observations.

[a,b]Means within a row with no common superscripts differ significantly ($P<0.05$).

N = 10

Table 4. 3. The viable number (log10) of *Salmonella* Enteritidis (SE) in the cecal contents analyzed at 7 day post-infection (dpi) fed fructoligosaccharides (FOS) diets (Experiment 1 and 2)[1].

		Treatments		
Experiment	Count	Control	0.5% FOS	1.0% FOS
1	Log_{10} cfu/g	4.2 ± 1.674^{a} (2.7-6.7)	3.2 ± 1.463^{ab} (1.5-5.7)	2.8 ± 1.088^{b} (1.5-2.7)
2	Log_{10} cfu/g	3.7 ± 1.781^{a} (1.5-6.7)	3.3 ± 1.636^{ab} (1.5-5.7)	2.9 ± 1.280^{b} (1.5-5.7)

[1]The mean ± SEM count per gram from 10 hens.

[a,b] Means within a row with no common superscripts differ significantly ($P<0.05$).

Values in the parenthesis represent the range of viable log_{10} counts of SE^{NAR}.

N=10

Table 4. 4. Effects of FOS on *Salmonella* Enteritidis colonization on the liver gall bladder (L/GB) and ovaries on 7 day post-infection (Experiment 1 and 2).

Experiment	Organs	Treatments		
		Control	0.5% FOS	1.0% FOS
1	L/GB	8/10 (80%)[a]	8/10 (80%)[a]	8/10 (80%)[a]
	Ovaries	4/10 (40%)[b]	2/10 (20%)[b]	3/10 (30%)[b]
2	L/GB	6/10 (60%)[a]	6/10 (60%)[a]	6/10 (60%)[a]
	Ovaries	2/10 (20%)[b]	2/10 (20%)[b]	1/10 (10%)[b]

[a,b] Means within a column with no common superscripts differ significantly ($P<0.05$).

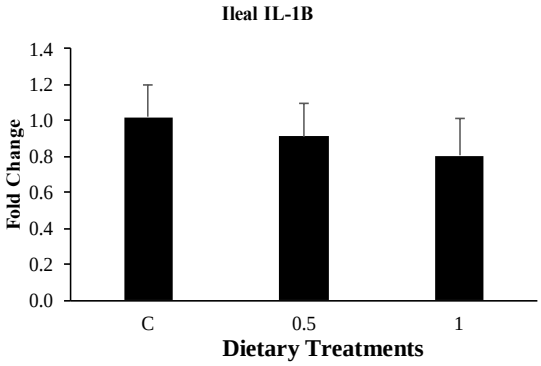
N=10

Table 4. 5. Chicken cytokines and toll-like receptor primer sequences in FOS

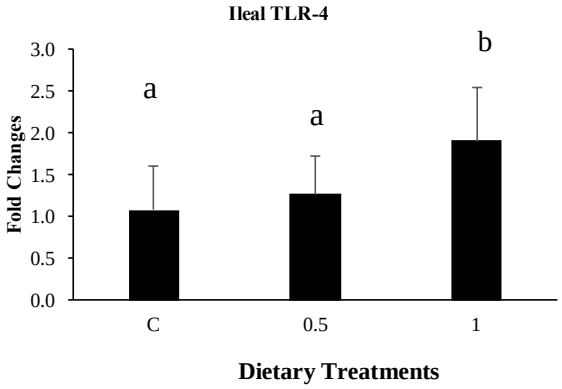
Gene[2]	Primer sequence[3] (5′–3′)	Gene bank accession no.	Fragment size, bp	Annealing temperature, °C
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	K01458	116	56
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	190	56
IL6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	AJ309540	233	59
IL10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	NM001004414	103	56
IL-1β	F: CACAGAGATGGCGTTCGTTC R: GCAGATTGTGAGCATTGGGC	NM204524	118	56
IFN- γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	NM205149	159	58

[2]IL = interleukin; IFN = interferon; TLR = Toll-like receptor.

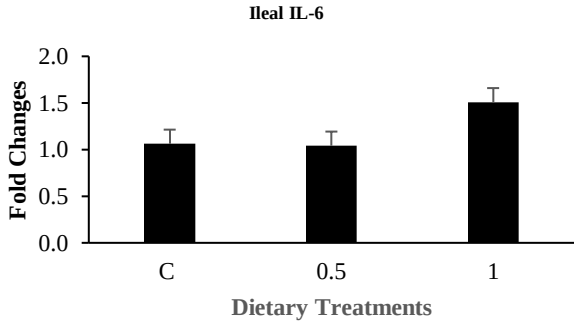
a)


Ileal IL-1B
Fold Change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
0.5
1
Dietary Treatments

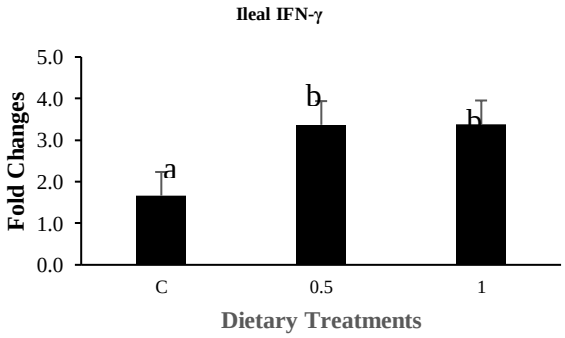
b)


Ileal TLR-4
Fold Changes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
C
0.5
1
Dietary Treatments

c)


Ileal IL-6
Fold Changes
2.0
1.5
1.0
0.5
0.0
C
0.5
1
Dietary Treatments

d)


Ileal IFN-γ
Fold Changes
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
C
0.5
1
Dietary Treatments

e)

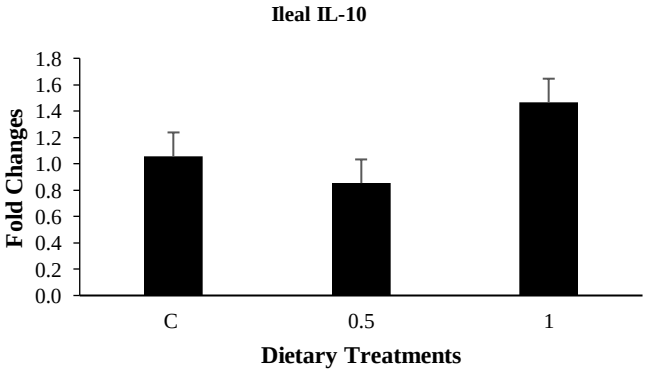

Ileal IL-10
Fold Changes
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
0.5
1
Dietary Treatments

Figure 4.1 Ileal immune gene expression of a) Interleukin (IL) – 1B, b) toll-like receptor (TLR) -4, c) IL-6, d) interferon (IFN) – γ, and e) IL- 10, under Salmonella Enteritidis challenge condition (N = 10/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 3 treatment groups (P<0.05). C= SENAR challenged control, 0.5 = SE^{NAR} challenged + 0.5% FOS (NutraFlora®) and 1.0= SE^{NAR} challenged + 1.0% FOS.

Table 4. 6. Effect of fructooligosaccharide (FOS) on the ileal morphology of laying hens[1]

Exp.	Site	Treatments[2]			SEM	*P*-value
		Control	0.5% FOS	1.0% FOS		
1	VH (μm)	615.9	615.4	595.4	41.68	0.924
	CD (μm)	101.1	75.6	95.6	9.88	0.181
	VH:CD	6.6	8.5	6.9	0.89	0.297
	Total mucosa thickness[3] (μm)	1027.7	992.8	1085.6	56.48	0.512
2	VH (μm)	750.6	742.7	669.7	40.52	0.343
	CD (μm)	75.5	90.5	88.9	84.90	0.318
	VH:CD	8.8	8.2	7.2	0.69	0.143
	Total mucosa thickness (μm)	1179.9	1148.5	1110.5	46.52	0.422

[1]Means of the three measurements of each villus height, crypt depth and total mucosa thickness of a hen, 10 hens per treatment.

[2] SE^{NAR} challenged control, SE^{NAR} challenged + 0.5% FOS; Nutraflora®) and SE^{NAR} challenged + 1.0% FOS.

[3]Total thickness of villus, crypt and muscularis mucosa.

N=3

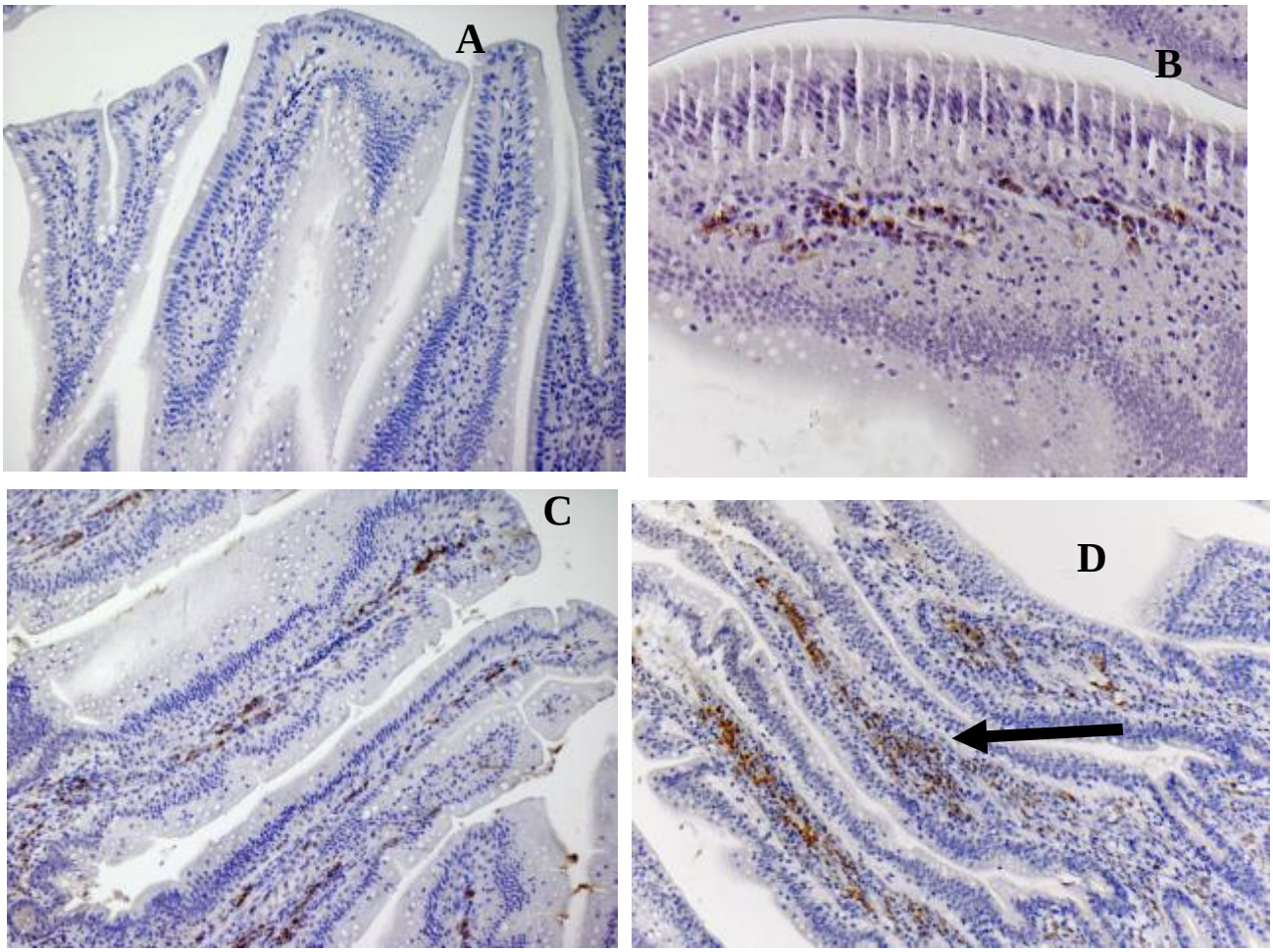


Figure 4.2 Immunohistochemical staining of IgA+ cells in the ileum of white leghorns. A) negative control, B) IgA positive cells in the lamina propria of Salmonella challenged group, C) IgA positive cells in the lamina propria of 0.5% FOS and D) IgA positive cells (arrows) in the lamina propria and crypt of 1.0% FOS (20x).

b

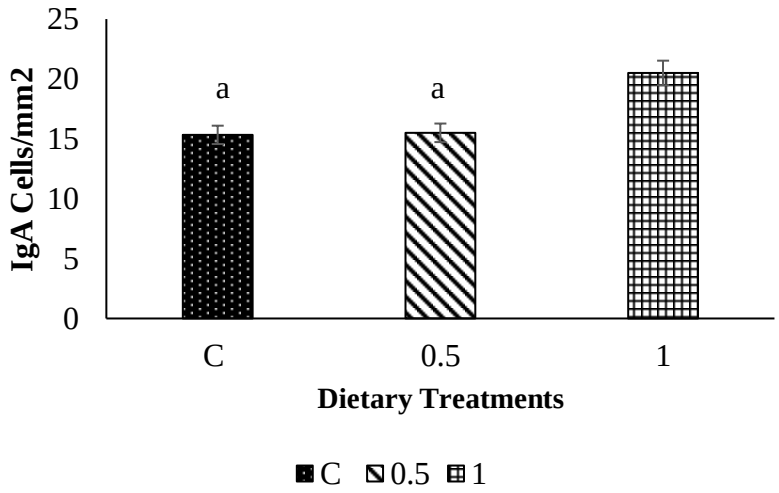


Figure 4.3 Immunoglobulin A (IgA) positive cells in the lamina propria of ileum section of hens. Hens *fed either C = SE^{NAR} challenged control, 0.5 = SE^{NAR} challenged + 0.5% FOS NutraFlora® or 1.0 = SE^{NAR} challenged + 1.0% FOS. Values are the mean ± SEM. The number of host cells showing positive staining on three randomly selected microscopic areas of each hen was counted.*

5. EFFECT OF DIETARY BACTERIOPHAGE SUPPLEMENTATION ON INTERNAL ORGANS, FECAL EXCRETION AND ILEAL IMMUNE RESPONSE IN LAYING HENS CHALLENGED BY *SALMONELLA* ENTERITIDIS[3]

[3]P. A. Adhikari, Douglas E. Cosby, Nelson A. Cox, J. H. Lee and Woo. K. Kim. Submitted to *Poultry Science Journal*.

ABSTRACT

A study was conducted to evaluate the role of bacteriophage (BP) against *Salmonella enterica* serovar Enteritidis (SE) on internal organs colonization and ileum immune response in laying hens. Hens were challenged both orally and intracloacally with 10^8 cfu/mL cells of nalidixic acid resistant *Salmonella* Enteritidis (SE^{NAR}). Thirty-two Single Comb White Leghorns were randomly allocated to four dietary treatments: 1) unchallenged control (negative control; T1), 2) SE^{NAR} challenged control (positive control; T2), 3) SE^{NAR} challenged + 0.1% BP (T3) and 4) SE^{NAR} challenged + 0.2% BP (T4). The number of SE^{NAR} in the ceca was significantly reduced by 0.2% BP supplementation (P<0.05) at 7 days-post infection (dpi). The respective number of SE^{NAR} was reduced from 2.9 log cfu/gm in T2 and T3 to 2.0 log cfu/gm in T4. There was no significant effect of T3 on reduction of numbers of cecal SE^{NAR}. A significant reduction of SE^{NAR} was observed in the liver with gall bladder (LGB) from 0.75 in T2 to 0.18 log cfu/gm in T4. In the spleen, T4 significantly reduced (P<0.05) SE^{NAR} to 0.56 log cfu/gm compared to T2 and T3 (0.94 log cfu/gm). There was no significant effect of T3 in reduction of prevalence of spleen SE^{NAR}. By supplementing 0.2% BP (T4), the SE^{NAR} in the ovary was reduced to 0 log cfu/gm. There was no significant effect on fecal SE^{NAR} at 3 dpi. There was a significant reduction (P<0.05) in fecal SE^{NAR} at 6 dpi by T4 (0.71 log cfu/gm) compared to the positive control (1.57 log cfu/gm). The expression levels of immune genes such as interferon (IFN)-Ɣ, interleukin (IL)-6 and IL-10 were significantly increased in the ileum by SE^{NAR} challenge as well as BP compared to the negative control. There was no significant difference observed for toll-like receptor (TLR-4) and IL-1ß. This study suggests that BP can be used as one of the dietary strategies to reduce SE incidence in internal organs as well as feces of laying hens.

Key Words: *Salmonella*, Bacteriophage, White Leghorn, colonization, cytokine

INTRODUCTION

Salmonella Enteritidis **(SE)** and *Salmonella* Typhimurium **(ST)** are major serovars accountable for food-borne illness, causing 74% of human zoonosis cases (EFSA, 2015). Due to the emergence of multiple drug resistant bacteria including *Salmonella enterica*, there has been a search for new alternatives to antimicrobials (Golkar et al., 2014). Bacteriophages **(BP)** have recently been receiving much attention as one of the alternatives to antibiotics to reduce resistant-bacteria (Lee et al., 2015). BP are the viruses that infect and replicate in prokaryotic cells and inject the required amount of components for BP replication into the bacterium (Kim et al., 2013). BP are commonly administered via drinking water, in-feed or *in ovo* (Blankenship et al., 1993; Mead, 2000; Schneitz, 1992). Alteration of the composition of gut microflora is one of the mechanisms of BP (Mead, 2000) and the use of BP to reduce the pathogens such as *Salmonella* has been studied using broiler chickens and their carcasses (Borie et al., 2008b; Higgins et al., 2005; Toro et al., 2005). However, there are not enough published studies that include laying hens challenged with SE and in-feed supplementation of BP as one of the dietary interventions. Therefore, our objectives were to evaluate the role of BP in reducing the number of nalidixic acid resistant *Salmonella* Enteritidis (**SE^{NAR}**) colonizing the ceca and internal organs as well as the effect on ileum immune mRNA gene expression in laying hens.

MATERIALS AND METHODS

Experimental hens and husbandry, bacterial strain and challenge protocol

Thirty-two Single Comb White Leghorns (40 wk old at the beginning of the study) were housed at the Poultry Research Center, University of Georgia in Athens, GA. Hens were kept individually in wire-layer cages and fed a corn-soybean layer control ration for 1 wk and then the treatment diets for 2 wk (Table 1). The feed was formulated to provide crude protein **(CP)** of 16%, metabolizable energy **(ME)** of 2,900 Kcal/kg, calcium **(Ca)** of 4.4% and available

phosphorus **(P)** of 0.5% (NRC, 1994). Hens were provided with water (automatic nipple drinkers) and mash feed *ad libitum* and housed under a 16h light:8h dark schedule throughout the experimental period. Hens were randomly divided into 4 groups: 1) without SE^{NAR} challenge (negative control; T1), 2) SE^{NAR} challenge (SE^{NAR} challenge control; T2), 3) SE^{NAR} challenge + 0.1% BP (CTCBIO, Seoul, Korea) (T3), and SE^{NAR} challenge + 0.2% BP (T4). After 1-wk adaptation to the treatment diets, hens were individually infected with both the oral and intracloacal methods with 10^8 cfu/mL SE^{NAR}. The SE^{NAR} strain used in our study was obtained from Dr. Richard Gast (USDA, Athens). The animal experiment was approved by the Institutional Animal Care and Use Committee (A2014-07-016).

Sampling protocol, bacteriological recovery of SE^{NAR} in feces and internal organs

Fecal samples were collected for SE shedding on 3 and 6 day post-infection (**dpi**). On 7 dpi, all hens including control groups (T1 and T2) were humanely euthanized by electrocution, and the ceca, liver with gall bladder (**LGB**), spleen and ovary were collected aseptically for SE enumeration. All tissue samples were placed into labeled sterile plastic sampling bags (Fisher Scientific, Pittsburgh, PA) and transported on ice to the U.S. National Poultry Research Center, USDA (Athens, GA). Quantitative bacteriology was performed for feces, ceca as well as other organs. Ileum samples were excised aseptically and frozen immediately and stored at -80°C until analyzed for inflammatory cytokines.

The numbers of SE^{NAR} per g of organ samples were estimated using a three swab plating method as suggested by (Blanchfield, et al., 1984). For bacteriological analyses, both feces and organ samples were weighed and diluted in buffered peptone water **(BPW)** 3 × volume to weight. After stomaching for 60s, a cotton-tipped swab was dipped and rotated in the sample bag for 5s. A brilliant green agar with sulphapyridine containing nalidixic acid **(BGS-Nal)** plate was

surface-swabbed (plate A). A second swab was placed into a stomacher bag, transferred and broken off into a 9.9 mL BPW dilution tube. The dilution tube was vortexed for 10s. A second BGS-Nal plate (plate B) was taken and inoculated by dipping a fresh swab into the dilution tube and spreading as above. All plates together with the samples were incubated at 37°C overnight. Negative samples were restreaked from the overnight pre-enrichments onto a BGS-Nal plate and incubated at 37°C overnight.

The swab dipped and rotated took up approximately 0.15 g of sample material and deposited approximately 0.055 g onto a plate A. The sample materials contained on the fresh swab created an approximate 100-fold dilution when vortexed in 9.9 mL of BPW. A fresh swab dipped into the BPW suspension picked up approximately 0.36 g and deposited approximately 0.0005 g of sample material on a plate B. The total factor for the estimation count was 54 (18×3) on the A plate, and the B plate was diluted 100 times. Thus, the multiplier was 5,400 for the B plate. A log count of 1.5 was assigned to the SE^{NAR} detected from pre-enriched samples.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from ileum samples using QIAzol reagents (Qiazen, Life Technologies, Valencia, CA, USA). Tissues were macerated using a mini-bead beater-16 homogenizer (Biospec Products, Fisher Scientific, Bartlesville, OK) for 3 min. RNA pellets were dissolved in 200 µl nuclease-free water (Ambion, Applied BioSystems, Life Technologies, Carlsbad, CA, USA), and concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The purity of RNA was verified at an optical density ratio of 260 to 280 nm. RNA was normalized to a concentration of 2 µg/µl after which it was reverse-transcribed using high capacity cDNA synthesis reverse transcription kits (Applied BioSystems, Life Technologies, Carlsbad, CA) following manufacturer's protocol.

Primers for chicken immune genes such as 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). Real-time quantitative polymerase chain reaction **(qRT-PCR)** was performed in duplicate reaction using both forward and reverse primers, cDNA, SYBR Green (Applied BioSystems, Life Technologies, Carlsbad, CA) and nuclease free water. qRT-PCR was performed using a step one thermo cycler (Applied Biosystem, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase **(GAPDH)** was used as a housekeeping gene and used to normalize the expression of all target genes. Pairs of primers used in this study are shown in Table 5.

Statistical analysis

The positive numbers of SE^{NAR} from feces, ceca and organs were transformed into logarithms (log_{10}cfu). Data were analyzed by one-way ANOVA using PROC GLM of SAS (SAS 9.2). Mean differences in the SE^{NAR} numbers and cytokines among the treatments were determined using Duncan's multiple range test. The percentage positive between treatments were compared using Fisher's Exact Test (GraphPad Prism 6 software). All the values were deemed significant if $P<0.05$.

RESULTS

SE numbers in ceca, LGB, spleen and ovary

Enumeration of SE^{NAR} was performed in the ceca, LGB, spleen and ovary. Negative control was not contaminated and yielded negative results throughout the experiment. Supplementation of BP at 0.2% (T4) significantly reduced ($P<0.05$) cecal SE^{NAR} numbers (2.0 log_{10} cfu/gm) compared to T2 (2.9 log_{10} cfu/gm) and T3 (2.9 log_{10} cfu/gm) at 7 dpi (Table 2). Quantitative bacteriology of LGB showed significantly lower SE^{NAR} in T4 (0.18 log_{10} cfu/gm) compared to T2 group (0.75 log_{10} cfu/gm) (Table 3). All LGB in negative control remained

negative throughout our experiment.

In the spleen, supplementation of 0.1% BP (T3) did not affect the SE^{NAR} numbers compared to T2. However, 0.2% BP (T4) significantly reduced ($P<0.05$) the numbers from 0.94 (T2) to 0.56 $\log_{10}$ cfu/gm (Table 3). There was a 0.4-$\log_{10}$ reduction in T4 group compared to T2 or T3. All negative control hens (T1) remained negative in the spleen. In the ovary, T4 completely reduced ($P<0.05$) the SE^{NAR} numbers to 0 compared to T2. The overall numbers were lower in the ovary (0.19 $\log_{10}$ cfu/gm) than either in the spleen (0.94 $\log_{10}$ cfu/gm) or LGB (0.75 $\log_{10}$ cfu/gm).

SE percentage positive in ceca, LGB, spleen and ovary

In the ceca, negative control had 0% positive whereas SE^{NAR} challenge control had 100% positive SE^{NAR}. T3 and T4 reduced the positive percentage to 88 and 87, respectively (Table 2). In the LGB, spleen and ovary, negative control showed 0% positive SE^{NAR}. In LGB, the positive percentage of SE^{NAR} in SE^{NAR} challenge control was 50% and was significantly reduced ($P<0.05$) to 25 and 12.5% by supplementing T3 and T4, respectively (Table 3). Spleen was 62.5% positive in T2 and T3 but significantly reduced ($P<0.05$) to 37.5% by supplementing T4. In the ovary, 12.5% were positive in T2 and T3, whereas in T4 the percentage was significantly reduced ($P<0.05$) to 0% (Table 3).

SE numbers and percentage positive in feces

The enumeration of SE^{NAR} in feces at both 3 and 6 dpi are shown in Table 4. There were no significant differences in the SE^{NAR} count at 3 dpi among the treatment groups. By 6 dpi, T4 (0.71 $\log_{10}$ cfu/gm) significantly lowered ($P<0.05$) the SE^{NAR} count in fecal samples compared to T2 and T3 (1.57 $\log_{10}$ cfu/gm). There was a 0.86-$\log_{10}$ reduction in fecal samples by 6 dpi. The percent of positive of feces in T2, T3 and T4 were 100, 87.5 and 87.5%, respectively, by 3 dpi.

At 6 dpi, the percent of positive feces in T4 was significantly reduced (P<0.05) to 37.5% compared to T2 (75%) and T3 (75%). Negative control was SE^{NAR} negative in both 3 and 6 dpi (Table 4).

Cytokine mRNA gene expression in the ileum

The relative expression of immune genes in the ileum with or without BP supplementation is shown in Figure 1 (a to e). The SE challenge control (T2) showed significant increase (P<0.05) in most of the immune genes tested such as IFN-Y, IL-6 and IL-10). TLR-4 was significantly upregulated (P<0.05) in hens challenged and fed with BP diets (T3 and T4) compared to the negative control (T1). The relative expression of IFN-Y mRNA was significantly higher (P<0.05) in hens with SE challenge control (T2) or SE challenge with 0.1% BP supplementation (T3) compared to the negative control (T1). However, the expression of IFN-Y mRNA in T4 (0.2% BP) was not different from T3 or T2.

There was a significant increase (P<0.05) in IL-6 mRNA expression in hens SE challenge control (T2) as well as challenged and BP supplemented group (T3) compared to the T1 group. T3 and T4 significantly reduced IL-6 mRNA expression compared to T2. However, in SE challenged with 0.2% BP (T4), the expression of IL-6 was reduced and not different from our unchallenged control group (T1). IL-10 mRNA expression was significantly increased (P<0.05) in all the SE challenged groups (T2, T3 and T4) compared to unchallenged control group (T1). However, there were no significant differences in IL-10 mRNA expression among T2, T3 and T4. IL-1ß mRNA expression was significantly (P<0.05) upregulated in T4 group compared to T1, whereas there were no significantly differences among T1, T2 and T3.

DISCUSSION

SE numbers in ceca, LGB, spleen and ovary

In a study in laying hens, rates of cecal colonization by *Salmonella* were >95% at 5 to 6

dpi given 10^7 cfu cells of *Salmonella* (Gast, et al., 2013b). One study reported that SE^{NAR} colonized as high as 81% of the poultry intestinal tract (Gast et al., 1993). In our study, the SE^{NAR} challenged group had the cecal *Salmonella* positive as high as 100%. Supplementation of 0.2% BP in the present study significantly reduced the prevalence as well as numbers of SE^{NAR} in the ceca. There have been few studies that used supplementation of BP in reducing *Salmonella* or *Campylobacter*, especially in broilers (Atterbury et al., 2007b; Loc Carrillo et al., 2005). According to Loc Carrillo, et al. (2005), high numbers of *Campylobacters* colonize the chicken intestine, and as such are a promising target for phage therapy. Potentially useful BP interact between the genetic contents of target bacteria. Some BP produce progeny without destroying their bacterial host, others have means to temporarily integrate their genome into the bacterium where it is replicated along with the bacterial genome and potentially introduces new traits or modifies the expression of host traits (Joerger, 2003). The use of lytic BP in order to kill different bacterial strains after oral gavage in broilers have proven to be efficient, as therapeutic or prophylactic against *Salmonella* (Atterbury, et al., 2007b; Borie et al., 2008a; Gorski et al., 2005). However, no studies have used BP to reduce the intestinal colonization and fecal shedding of *Salmonella* in laying hens.

A 0.9-$\log_{10}$ reduction of SE^{NAR} in the ceca in the current study was lower than some of the previous studies that used BP to reduce *Salmonella* in broilers (Borie, et al., 2008b; Toro, et al., 2005). In addition, a modest reduction of 1.0 to 1.3 $\log_{10}$ compared to the SE challenged group was observed in studies with SE infection and BP supplementation (Bardina et al., 2012; Joerger, 2003). Similarly, the counts of SE^{NAR} in internal organs including LGB, spleen and ovary in SE challenged control (T2) in the current study were similar to those reported by Toro, et al. (2005). There were less positive SE^{NAR} in the ceca in the SE challenged control group in

the current experiment (2.9 $\log_{10}$ cfu) than in a previous study where the average of 5.67 $\log_{10}$ cfu cells of *Salmonella* was prevalent (Toro, et al., 2005). One reason for this could be due to the older age of Leghorns used in our study that could be more resistant to SE^{NAR} colonization in the gastro-intestinal tract and the primary and secondary lymphoid organs. The incidence of SE^{NAR} positive ovaries in the current study (12.5%) was similar to some of the previous studies where fewer ovaries (6 to 33%) were positive by 5 - 6 dpi (Gast, et al., 2013b; Keller et al., 1995).

SE numbers in fecal shedding

The shedding pattern of SE^{NAR} in feces at 3 dpi was higher (2.8 to 3.45 cfu/gm) than one at 6 dpi (0.71 to 1.57 cfu/gm) in all treatment. The susceptibility of intestinal tract against *Salmonella* is higher in the earlier days of age in birds, and once the gut flora develops, birds are less susceptible to infection (Nurmi, et al., 1973). Whereas *Salmonella* can be shed in the feces for many months after the infection of young chicks, bacterial clearance usually proceeds much more rapidly in adult birds (Gast et al., 2005). Intestinal colonization by *Salmonella* usually declines steadily following experimental infection of mature chickens (Gast, et al., 2005). The reason behind such declination in mature chicks can be explained by the reduction of systemic infection due to greater humoral immune response (Muir et al., 1998). However, in a study, humoral immune response from immunoglobulin A (IgA) may not have provided complete protection against SE colonization nor have the ability to fully combat SE infection of the alimentary tract, as complete clearance of SE from the crop and feces of all the experimentally infected hens did not occur (Vaughn et al., 2008). Another factor likely to act gradually to reduce colonization levels would be direct (for attachment sites) and indirect (via production of metabolites that inhibit *Salmonella*) competition with other gut flora (Durant et al., 2000; Revolledo et al., 2006; Ushijima et al., 1991; van der Wielen et al., 2002). Additionally,

measuring the *Salmonella* load in the gut contents (in orally infected birds) is subject to a gradual dilution effect. These salmonellae would initially be introduced in the contents, some would attach and colonize, and others would be gradually eliminated as the contents are excreted. Some of those salmonellae introduced in the oral dose might still be present in the contents at 3 dpi, but they would presumably have been mostly eliminated by 6 dpi.

A study reported the role of bacterins against shedding of SE in feces (Gast, et al., 1993). Fecal shedding was reduced due to bacterins by 1-wk post SE challenge, whereas by 2-wks post SE challenge, intestinal colonization decreased in all groups including the SE challenge group (Gast, et al., 1993). Reduction of feces SE^{NAR} by supplementation of BP in the current study around 1-wk post challenge shows that it could substantially reduce the production of contaminated eggs. This can provide more information especially to reduce the horizontal SE transmission by laying hens. However, there may be various factors including genetics, strain, infecting dose, and maturity of birds that would affect bacteria shedding differently and responses towards intervention strategies (Barrow et al., 2004; Duchet-Suchaux et al., 1997; Van Immerseel et al., 2004b). In a study, the group of pigeons inoculated with only 10^7 cfu/ml started shedding at 7 dpi whereas those inoculated with 10^9 cfu/mL started shedding at 3 dpi (Albuquerque et al., 2013).

Cytokine mRNA gene expression in the ileum

Toll-like receptors recognize the pathogen associated molecular patterns of *Salmonella* and are a part of innate immunity (Takeda et al., 2005). Such innate immune response relates to the production of pro-inflammatory cytokines and chemokines that eventually initiate the control of bacterial multiplication (Kawai et al., 2006; Weiss et al., 2004). Moreover, in *in vivo* oral challenge experiments with *Salmonella*, TLR-4 has a predominant role for host survival and

containment of *Salmonella* growth (Weiss, et al., 2004). TLR-2, 4, 5, 6 and 10 are expressed and activated at the surface of host cells, mainly recognizes bacterial products unique to bacteria (Mogensen, 2009). Generally, gram-negative bacteria are recognized by TLR-4 via the the lipopolysaccharides present in the cell walls (Poltorak et al., 1998). Similar to our study, *Salmonella* LPS challenge up-regulated more TLR-4 expression in the ileum than in the spleen in broilers (Shang, et al., 2015). In a study that used probiotics in mice increased the expression of TLR-4 compared to their SE challenge control (Castillo et al., 2011). IFN-Y, a major Th1 cytokine produced by T helper cells and natural killer (NK) cells, has been demonstrated to play an important role in protection against *Salmonella* infection in avian hosts (Okamura et al., 2004). The association of IFN-Y production and clearance of primary *Salmonella* infection was suggested previously (Bao, et al., 2000; Withanage et al., 2005). In our study, SE challenge and BP supplemented groups had higher levels of IFN-Y compared to the negative control. A study reported that SE challenge upregulated the RNA expression of IFN-Y in the spleen at 14 dpi (Chappell, et al., 2009).

IL-1ß, a pro-inflammatory cytokine, plays an important role in antimicrobial host defenses (Lee et al., 2010a; Netea et al., 2010). IL-6 is a multifunctional cytokine that has pro-inflammatory activity by the induction of acute phase proteins synthesis as well as aids in the adaptive immune responses (Kaiser et al., 2000). Increase in both IL-1ß and IL-6 after *Salmonella* challenge in the current study was similar to the study that observed high-level production of both cytokines after the *Campylobacter* infection (Smith et al., 2005). The expression of IL-6 being higher after *Salmonella* challenge has been reported in a study in *Salmonella* infected macrophages (Withanage, et al., 2005). The reduction in IL-6 expression after adding BP may be related to decrease number of SE. When there is lower IL-6, there is

lower lipolysis that leads to lower energy expenditure and finally energy saving (Wang et al., 2015). This phenomenon could benefit better growth performance in chickens fed BP.

IL-10, an anti-inflammatory cytokine, can directly regulate both innate and adaptive immune responses by limiting T-cell activation in lymph nodes and suppressing pro-inflammatory responses in tissues (Couper, et al., 2008). Our results correspond to previously published literature in that *S*T significantly increased expression of IL-1ß, IL-18 and IFN-Y (Yasuda, et al., 2002). In a study by Shang, et al. (2015), *Salmonella* LPS challenged to broiler chicks increased the ileum gene expression of major cytokines such as IFN-Y, IL-1ß and IL-10. In the same study, dietary fructoligosaccharides further increased the expression of pro-and anti-inflammatory cytokines under the SE challenged condition, suggesting that there are synergistic effects of diet and *Salmonella* challenge in up-regulating both pro- and anti-inflammatory functions in the host. Furthermore, an increased expression of pro-inflammatory cytokines and chemokine genes was reported to be associated with increased resistance to SE, including higher levels of IL-1ß, IL-6, IL-8, IL-18 and chemokine ligand - 2 **(CCLi2)** in heterophils, monocyte-derived macrophages, the ceca and cecal tonsil (Ferro, et al., 2004; Setta, et al., 2012). Bacterial infections in animals theoretically can be controlled by BP treatment either through two mechanisms: 1) direct BP lysis and 2) immune response via bacterial lysate produced by BP (Borysowski et al., 2008). Some of the cytokines being affected by BP treatment in our study suggest that there is an immune response associated with the BP supplementation.

CONCLUSION

The study has demonstrated that supplementation of 0.2% BP can be useful to reduce the cecal as well as internal organs *Salmonella* in laying hens. The supplementation of BP also reduced the fecal shedding especially at 6 dpi and upregulates the pro- as well as anti-inflammatory cytokines in the ileum. Further work needs to be undertaken to determine the

safety, stability and therapeutic efficacy before we completely implant such a dietary strategy to reduce pathogen colonization in laying hens.

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References

P. Adhikari, D. E. Cosby, N. A. Cox, W. K. Kim. 2017. Colonization of mature laying hens with *Salmonella* Enteritidis by oral or intracloacal inoculation. J. Appl. Poult. Res. 00: 1-9.

Atterbury, R. J., M. A. P. Van Bergen, F. Ortiz, M. A. Lovell, J. A. Harris, A. De Boer, J. A. Wagenaar, V. M. Allen, and P. A. Barrow. 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl. Environ. Microbiol. 73:4543-4549.

Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* Typhimurium infection. Immunol. 99:464-472.

Bardina, C., D. A. Spricigo, P. Cortés, and M. Llagostera. 2012. Significance of the bacteriophage treatment schedule in reducing *Salmonella* colonization of poultry. Appl. Environ. Microbiol. 78:6600-6607.

Blanchfield, B., S. Stavric, T. Gleeson, and H. Pivnick. 1984. Minimum intestinal inoculum for Nurmi cultures and a new method for determining competitive exclusion of *Salmonella* from chicks. J. Food Prot. 47:542-545.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams. 1993. Two-step mucosal competitive exclusion flora treatment to diminish Salmonellae in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Borie, C., I. Albala, P. Sanchez, M. L. Sanchez, S. Ramirez, C. Navarro, M. A. Morales, A. J. Retamales, and J. Robeson. 2008a. Bacteriophage treatment reduces Salmonella colonization of infected chickens. Avian Dis. 52:64-67.

Borie, C., I. Albala, P. Sànchez, M. L. Sánchez, S. Ramírez, C. Navarro, M. A. Morales, J. Retamales, and J. Robeson. 2008b. Bacteriophage treatment reduces *Salmonella* colonization of infected chickens. Avian Dis. 52:64-67.

Borysowski, J., and A. Górski. 2008. Is phage therapy acceptable in the immunocompromised host? Internl. J. Infect. Dis. 12:466-471.

Castillo, N. A., G. Perdigón, and A. de Moreno de LeBlanc. 2011. Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar Typhimurium infection in mice. Bio. Med. Central Microbiol. 11:1-12.

Chappell, L., P. Kaiser, P. Barrow, M. A. Jones, C. Johnston, and P. Wigley. 2009. The immunobiology of avian systemic salmonellosis. Vet Immunol. Immunopathol. 128:53-59.

Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: The master regulator of immunity to infection. J. Immunol. 180:5771-5777.

Durant, J. A., D. J. Nisbet, and S. C. Ricke. 2000. Response of selected poultry cecal probiotic bacteria and a primary poultry Salmonella typhimurium isolate grown with or without glucose in liquid batch culture. J. Environ. Sci. Health B 35:503-516.

EFSA. 2015. European Food Safety Authority and European Centre for

Disease Prevention and Control. Pages 1-162. z. a. The European Union summary report on trends and sources of zoonoses, and a. f.-b. o. i. 2013 eds.

Ferro, P. J., C. L. Swaggerty, P. Kaiser, I. Y. Pevzner, and M. H. Kogut. 2004. Heterophils isolated from chickens resistant to extra-intestinal *Salmonella* Enteritidis infection

express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol. and Infect. 132:1029-1037.

Gast, R. K., J. Guard-Bouldin, and P. S. Holt. 2005. The Relationship between the duration of fecal shedding and the production of contaminated eggs by laying hens infected with strains of *Salmonella* Enteritidis and *Salmonella* Heidelberg. Avian Dis. 49:382-386.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2013. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 92:468-473.

Gast, R. K., H. D. Stone, and P. S. Holt. 1993. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella* Enteritidis by laying hens. Avian Dis. 37:1085-1091.

Golkar, Z., O. Bagasra, and D. G. Pace. 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J. Infect. Dev. Ctries. 8:129-136.

Gorski, A., and B. Weber-Dabrowska. 2005. The potential role of endogenous bacteriophages in controlling invading pathogens. Cell. Mol. Life Sci. 62:511-519.

Higgins, J. P., S. E. Higgins, K. L. Guenther, W. Huff, A. M. Donoghue, D. J. Donoghue, and B. M. Hargis. 2005. Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. Poult. Sci. 84:1141-1145.

Joerger, R. D. 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. Poult. Sci. 82:640-647.

Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella*

Typhimurium, *Salmonella* Enteritidis and *Salmonella* Gallinarum. Microbiol. 146:3217-3226.

Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. Nat. Immunol. 7:131-137.

Keller, L. H., C. E. Benson, K. Krotec, and R. J. Eckroade. 1995. *Salmonella* Enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. and Immun. 63:2443-2449.

Kim, K. H., G. Y. Lee, J. C. Jang, J. E. Kim, and Y. Y. Kim. 2013. Evaluation of anti-SE bacteriophage as feed additives to prevent *Salmonella* Enteritidis (SE) in broiler. Asian-Australasian J. Anim, Sci. 26:386-393.

Lee, H., D. Biswas, and J. Ahn. 2015. In-vitro adhesion and invasion properties of *Salmonella* Typhimurium competing with bacteriophage in epithelial cells and chicken macrophages. Rev. Brasileira de Ciência Avícola. 17:427-432.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Loc Carrillo, C., R. J. Atterbury, A. El-Shibiny, P. L. Connerton, E. Dillon, A. Scott, and I. F. Connerton. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. Appl. Environ. Microbiol. 71.

Mead, G. C. 2000. Prospects for 'competitive exclusion' treatment to control *Salmonellas* and other foodborne pathogens in poultry. Vet. J. 159:111-123.

Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 22:240-273.

Muir, W. I., W. L. Bryden, and A. J. Husband. 1998. Comparison of *Salmonella* Typhimurium challenge models in chickens. Avian Dis. 42:257-264.

Netea, M. G., A. Simon, F. van de Veerdonk, B.-J. Kullberg, J. W. M. Van der Meer, and L. A. B. Joosten. 2010. IL-1β processing in host defense: beyond the inflammasomes. PLoS Pathog. 6:e1000661.

NRC. 1994. Nutrient Requirements of Poultry. Ninth Revised Edition. Natl. Acad. Press, washington, DC.

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

Okamura, M., H. S. Lillehoj, R. B. Raybourne, U. S. Babu, and R. A. Heckert. 2004. Cell-mediated immune responses to a killed *Salmonella* enteritidis vaccine: lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-γ production. Comp. Immunol. Microbiol. and Infect. Dis. 27:255-272.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science (New York, N.Y.) 282:2085-2088.

Revolledo, L., A. J. P. Ferreira, and G. C. Mead. 2006. Prospects in *Salmonella* control: competitive exclusion, probiotics, and enhancement of avian intestinal immunity. J. Appl. Poult. Res. 15:341-351.

Schneitz, C. 1992. Research note: automated droplet application of a competitive exclusion preparation. Poult. Sci. 71:2125-2128.

Setta, A. M., P. A. Barrow, P. Kaiser, and M. A. Jones. 2012. Early immune dynamics following infection with *Salmonella enterica* serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. Comp. Immunol. Microbiol. Infect. Dis. 35:397-410.

Shang, Y., A. Regassa, J. H. Kim, and W. K. Kim. 2015. The effect of dietary fructooligosaccharide supplementation on growth performance, intestinal morphology, and immune responses in broiler chickens challenged with *Salmonella* Enteritidis lipopolysaccharides. Poult. Sci. 94:2887-2897.

Smith, C. K., P. Kaiser, L. Rothwell, T. J. Humphrey, P. A. Barrow, and M. A. Jones. 2005. *Campylobacter jejuni*-Induced cytokine responses in avian cells. Infect. Immun. 73:2094-2100.

Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. Internatl. Immunol. 17:1-14.

Toro, H., S. B. Price, S. McKee, F. J. Hoerr, J. Krehling, M. Perdue, and L. Bauermeister. 2005. Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. Avian Dis. 49:118-124.

Ushijima, T., and A. Seto. 1991. Selected faecal bacteria and nutrients essential for antagonism of *Salmonella* Typhimurium in anaerobic continuous flow cultures. J. Med. Microbiol. 35:111-117.

Van der Wielen, P. W. J. J., L. J. A. Lipman, F. van Knapen, and S. Biesterveld. 2002. Competitive exclusion of *Salmonella* enterica serovar Enteritidis by *Lactobacillus*

crispatus and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68:555-559.

Vaughn, L. E., P. S. Holt, R. W. Moore, R. K. Gast, and K. E. Anderson. 2008. Crop immune response post-*Salmonella* Enteritidis challenge in eight commercial egg-layer strains and specific-pathogen-free White Leghorn chickens. Avian Dis 52:79-87.

Wang, H., and J. Ye. 2015. Regulation of energy balance by inflammation: Common theme in physiology and pathology. Rev. Endocr. Metab. Disord. 16:47-54.

Weiss, D. S., B. Raupach, K. Takeda, S. Akira, and A. Zychlinsky. 2004. Toll-Like receptors are temporally involved in host defense. J. Immunol. 172:4463-4469.

Withanage, G., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. 73:5173 - 5182.

Yasuda, M., S. Tanaka, H. Arakawa, Y. Taura, Y. Yokomizo, and S. Ekino. 2002. A comparative study of gut-associated lymphoid tissue in calf and chicken. Anat. Rec. 266:207-217.

Table 5. 1. Composition of experimental diets in laying hens fed bacteriophage (BP).

Item	Bacteriophage (%)[1]		
	0	0.1	0.2
Ingredients (%)			
Ground corn	60.53	60.45	60.35
Soybean meal	23.13	23.13	23.13
Limestone	9.62	9.61	9.61
Soybean oil	3.00	3.00	3.00
Deflour. phos.	2.13	2.13	2.13
Vitamin premix[2]	0.50	0.50	0.50
Mineral premix[3]	0.15	0.15	0.15
DL-methionine	0.34	0.33	0.33
Common salt	0.30	0.30	0.30
L-lysine HCl	0.30	0.30	0.30
Bacteriophage	0.00	0.10	0.20
Total	100.00	100.00	100.00
Chemical compositions			
ME (kcal/kg)	2.85	2.85	2.85
CP (%)	16	16	16
Lysine (%)	0.97	0.97	0.97
Total Ca (%)	4.4	4.4	4.4
Available P (%)	0.5	0.5	0.5

[1]0= fed to hens either unchallenged (T1) or challenged with *Salmonella* but not supplemented with bacteriophage (T2), 0.1=challenged with *Salmonella* + 0.1% of bacteriophage (T3), 0.2 = challenged with *Salmonella* + 0.2% bacteriophage (T4)

[2]Supplied as 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; D-biotin, 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]Supplied 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.

Table 5. 2. *Salmonella* counts (cfu/gm) in the ceca of hens infected with SE^{NAR} and supplemented with bacteriophage (BP)

Group[1]	cfu/gm log_{10} (Mean±SEM)	Number of hens	% Positive
T1	0	8	0
T2	2.9 ± 0.40^{a}	8	100
T3	2.9 ± 0.54^{a}	8	88
T4	2.0 ± 0.32^{b}	8	87

[1]T1=unchallenged, T2=challenged + 0 % bacteriophage, T3= challenged + 0.1% bacteriophage, T4= challenged + 0.2% bacteriophage

[ab]Means with different superscripts in the same column significantly differ ($P<0.05$)

Table 5. 3. *Salmonella* counts (cfu/gm) in the liver with gall bladder (LGB), spleen and ovary of hens at 7 days post infection infected with SE^{NAR} and supplemented with bacteriophage (BP).

Group[1]	cfu/gm log_{10} (Mean±SEM)	Number of hens	% Positive
LGB			
T1	0	8	0
T2	0.75 ± 0.26^{a}	8	50^{a}
T3	0.40 ± 0.22^{ab}	8	25^{b}
T4	0.18 ± 0.17^{b}	8	12.5^{c}
Spleen			
T1	0	8	0
T2	0.94 ± 0.25^{a}	8	62.5^{a}
T3	0.94 ± 0.25^{a}	8	62.5^{a}
T4	0.56 ± 0.25^{b}	8	37.5^{b}
Ovary			
T1	0	8	0
T2	0.19 ± 0.17^{a}	8	12.5^{a}
T3	0.19 ± 0.17^{a}	8	12.5^{a}
T4	0^{b}	8	0^{b}

[1]T1=unchallenged, T2=challenged + 0% bacteriophage, T3= challenged + 0.1% bacteriophage, T4= challenged + 0.2% bacteriophage

[ab]Means with different superscripts in the same column significantly differ ($P<0.05$)

Table 5. 4. *Salmonella* counts (CFU/gm) in feces samples of hens at 3 and 6 day post-infection infected with SE^{NAR} and supplemented with bacteriophage (BP).

Dpi	Groups[1]	CFU/gm log_{10} (Mean±SEM)	Number of hens	% Positive
3	T1	0	8	0
	T2	3.45 ± 0.43	8	100
	T3	3.17 ± 0.52	8	87.5
	T4	2.80 ± 0.40	8	87.5
6	T1	0	8	0
	T2	1.57 ± 0.37^{a}	8	75^{a}
	T3	1.57 ± 0.37^{a}	8	75^{a}
	T4	0.71 ± 0.34^{b}	8	37.5^{b}

[1]T1=unchallenged, T2=challenged + 0% bacteriophage, T3= challenged + 0.1% bacteriophage, T4= challenged + 0.2% bacteriophage

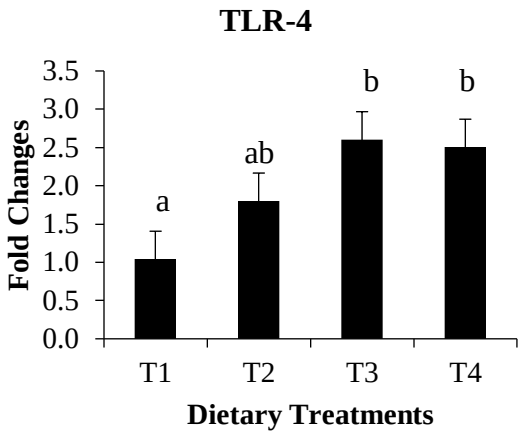
[ab]Means with different superscripts in the same column significantly differ ($P<0.05$)

Table 5. 5. Chicken cytokine primer sequences

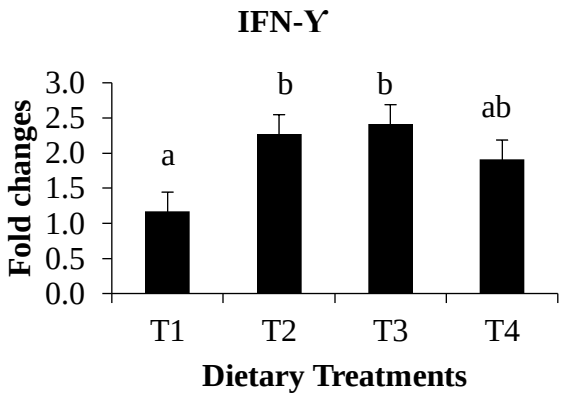
Gene[2]	Primer sequence[3] (5′–3′)	Gene bank accession no.	Fragment size, bp	Annealing Temperature, °C
GAPDH	F: GCTAAGGCTGTGGGGAAAGT	K01458	116	55
	R: TCAGCAGCAGCCTTCACTAC			
TLR-4	F: TCCGTGCCTGGAGGTAAGT	NM001030693	190	56
	R: TGCCTTGGTAACAGCCTTGA			
IL-6	F: TTCGACGAGGCAAGGAACC	NM204628	233	59
	R: AGGTCTGAAAGGCGAACAGG			
IL-10	F: GCTCTCCTTCCACCGAAACC	AJ621614	103	56
	R: GGAGCAAAGCCATCAAGCAG			
IL-1ß	F: CACAGAGATGGCGTTCGTTC	NM204524	118	56
	R: GCAGATTGTGAGCATTGGGC			
IFN-Ɣ	F: GCATCTCCTCTGAGACTGGC	NM205149	159	58
	R: GCTCTCGGTGTGACCTTTGT			

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

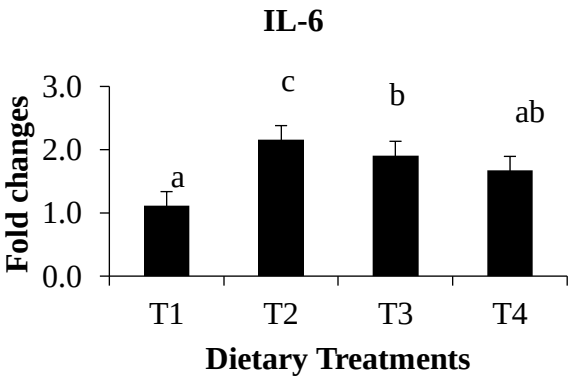
a)


TLR-4
Fold Changes
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
T1
T2
T3
T4
Dietary Treatments

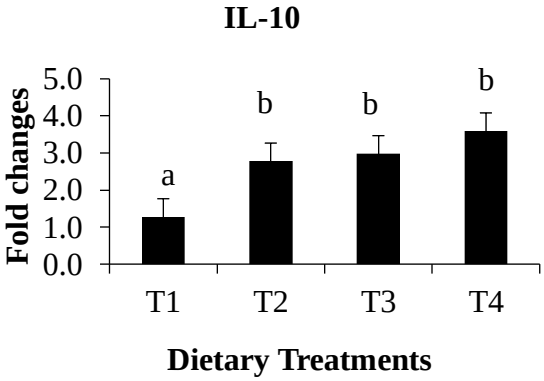
b)


IFN-Y
Fold changes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
ab
T1
T2
T3
T4
Dietary Treatments

d)


IL-6
Fold changes
3.0
2.0
1.0
0.0
a
c
b
ab
T1
T2
T3
T4
Dietary Treatments

c)


IL-10
Fold changes
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
b
T1
T2
T3
T4
Dietary Treatments

e)

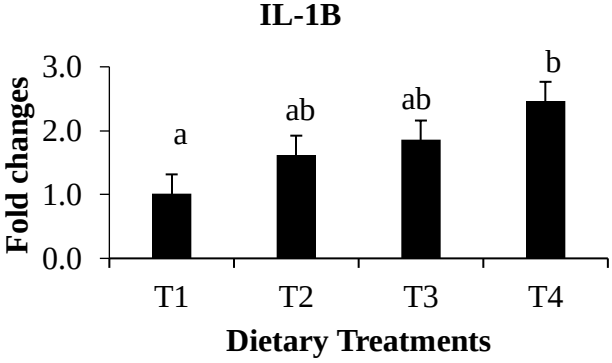

IL-1B
Fold changes
3.0
2.0
1.0
0.0
a
ab
ab
b
T1
T2
T3
T4
Dietary Treatments

Figure 5.1 Ileum gene expression of cytokines: a) toll-like receptor (TLR)-4, b) interferon (IFN) –Y, c) interleukin (IL)-6, d) IL-10 and e) IL-1B of chickens fed BP; T1, T2, T3 and T4 diet. T1=unchallenged, T2=challenged + 0% bacteriophage, T3= challenged + 0.1% bacteriophage, T4= challenged + 0.2% bacteriophage. Gene expressions were calculated relative to the housekeeping gene GAPDH. Error bars represent standard error of means. Bars with different letters (a, b) differ significantly across all 4-treatment groups (P<0.05). N=8

6. EFFECT OF SUPPLEMENTATION OF NITROCOMPOUNDS IN *SALMONELLA* COLONIZATION IN CECA, LIVER WITH GALL BLADDER, OVARY AS WELL AS ILEAL IMMUNE GENE EXPRESSION IN LAYING HENS[4]

[4]P. A. Adhikari, Douglas E. Cosby, Nelson A. Cox and Woo K. Kim. To be submitted to *Poultry Science Journal*.

ABSTRACT

Food-borne disease caused by *Salmonella* Enteritidis (*S*E) is important public health and economic concerns. A study was conducted to determine the effect of supplementation with 2 nitroethanol (NE) and 2 nitropropanol (NP) on *Salmonella* recovery of internal organs as well as on the immune gene expression in the ileum of laying hens. Thirty-six White Leghorns were orally gavaged with nalidixic acid resistant *Salmonella* Enteritidis (SE^{NAR}). Hens were housed individually in wire-laying cages and randomly assigned to six dietary treatments: T1= SE^{NAR} unchallenged (negative control), T2 = SE^{NAR} challenged (positive control), T3 = SE^{NAR} challenged + 100 ppm NE, T4 = SE^{NAR} challenged + 200 ppm NE, T5 = SE^{NAR} challenged + 100 ppm NP and T6 = SE^{NAR} challenged + 200 ppm NP. Hens were sampled at 7-day post-infection (dpi). Ceca, liver with gall bladder (L/GB), and ovary samples were collected for bacteriology, and ileum samples were collected for analysis of immune gene expression. T3 and T6 significantly reduced (P<0.05) cecal SE^{NAR} count, whereas T4 and T5 were not different from T2, the SE^{NAR} challenged control. There was no significant difference in SE^{NAR} reduction in the L/GB or ovary after supplementation of either nitrocompounds. Pro- and anti-inflammatory cytokines such as interferon (IFN)-Ɣ, interleukin (IL)- 1ß, IL-6, toll-like receptors (TLR)-4 and IL-10 all were significantly upregulated (P<0.05) after SE^{NAR} challenge. Supplementation at both levels of NE and NP showed a significant immune gene expression response in the ileum with reduction of IFN-Ɣ, IL-6, TLR-4 and IL-10 mRNA expression. Overall, nitrocompounds such as NE and NP can be used in the intervention strategy to reduce *Salmonella* infection in hens.

Key Words: nitroethanol, nitropropanol, hen, *Salmonella* Enteritidis

INTRODUCTION

Food-borne illness caused by *Salmonella* Enteritidis (SE) is of both public health and economic concerns (CDC, 2014). Alternative feed amendments and interventions have received increased interests. Some of these amendments include feeding prebiotics and probiotics and are already in use in broilers or laying hens to reduce the *Salmonella* infection in their internal organs (Bai, et al., 2013; Bailey, et al., 1991; Donalson, et al., 2008; Lei et al., 2009; Pascual et al., 1999). Nitrocompounds such as 2-nitroethanol **(NE),** 2-nitropropanol **(NP)**, nitroethane and nitropropionic acid have been used as potential alternatives to reduce the ammonia volatilization in poultry manure (Kim, et al., 2006). Nitrocompounds have a broad spectrum of antimicrobial inhibitory effects, particularly, 2-nitro-1 propanol, and have shown bactericidal activity against *Salmonella* Typhimurium, *Campylobacter jejuni*, *Listeria monocytogenes* and *E. coli*. (Jung, et al., 2004). Nitrocompounds have reduced the concentration of bacteria, such as *Salmonella* and *Campylobacter*, in the porcine gut as well as methane-production in bovine and avian gut contents (Anderson et al., 2004; Saengkerdsub, et al., 2006). Also, an *in vitro* study that used NP against *Campylobacter coli* and *jejuni* found that 2-nitro-l-propanol was superior to other tested nitrocompounds (Horrocks, et al., 2007). However, there has been no reported research looking into the effects of NE and NP on the prevalence of *Salmonella* Enteritidis **(SE)** in laying hens. We hypothesized that nitrocompounds should reduce SE in the ceca and other internal organs of laying hens and produce a positive immune response in the ileum. We evaluated dietary NE and NP as potential inhibitors of SE in laying hens. The objective of the study was to evaluate effects of both the nitrocompounds on reducing the *Salmonella* in internal organs and stimulating immune responses in laying hens.

MATERIALS AND METHODS

Salmonella strain and inoculum preparation

Nalidixic acid resistant *Salmonella* Enteritidis **(SE^{NAR})** was chosen and used for the challenge organism. SE^{NAR} was stored at -80°C in Nutrient Broth (Acumedia, East Lansing, MI; **NB**) with 16% glycerol. SE^{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. Hens were orally gavaged with a 1.0 mL of approximately 1.9×10^8 cfu SE^{NAR}.

Hens, housing and dietary treatments

Thirty-six Single-Comb White Leghorns hens (45- wk old at the beginning of the experiment) were used for the study. Hens were housed individually in wire laying cages and housed under a 16h light: 8h dark lightening program. All hens were fed a corn-soybean standard layer ration for one week after which they were switched to the respective treatment diets: T1= SE^{NAR} unchallenged (negative control), T2 = SE^{NAR} challenged (positive control), T3 = SE^{NAR} challenged + 100 ppm NE, T4 = SE^{NAR} challenged + 200 ppm NE, T5 = SE^{NAR} challenged + 100 ppm NP and T6 = SE^{NAR} challenged + 200 ppm NP (Table 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 a week adaptation to the respective treatment diets, each hen except in T1 group was orally gavaged with 1.0 mL of 10^8 cfu SE^{NAR}. Feed was withdrawn from all hens 10 h before challenge and returned immediately after SE^{NAR} challenge. Hens were divided into six replicates per treatment diet. 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 (A2014 04-017).

Sampling protocol and analyses

Ceca, liver with gall bladder and ovary (bacteriological)

All hens were humanely euthanized on 7 days post-infection **(dpi).** Ceca, liver with gall bladder **(L/GB)** and ovary samples were collected aseptically into sterile stomacher bags (VWR, Radnor, PA). All the samples were macerated by a rubber mallet, individually weight and diluted with buffered peptone water (**BPW**; 3X volume/weight), and stomached (Techmar Company, Cincinnati, Ohio) for 60 s. L/GB and ovaries were pre-enriched overnight at 37°C before being streaked for isolation onto BGS-Nal plates and incubated overnight at 37°C for enrichment. The growth of SE^{NAR} was observed and recorded as positive or negative for the samples. Cecal samples were analyzed using the modified 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 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-struck 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^{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 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 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-1ß, IL-6, and IL-10**) and interferon (**IFN)-*Y*** were designed according to National Center for Biotechnology Information (**NCBI**). Pairs of primers used in our study are shown in Table 2. Gene expression data was analyzed by difference in cycle threshold (ΔΔCT) method (Livak, et al., 2001).

Statistical analyses

For L/GB and ovary SE^{NAR} recovery, the prevalence was analyzed with Fisher's exact test. The mean of $\log_{10}$ viable SE^{NAR} counts 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 numbers and prevalence

The SE^{NAR} numbers in ceca were counted as log10 cfu/g of cecal contents and are shown in Figure 1. Unchallenged control (T1) remained negative at 7 dpi. T3 (100 ppm of NE) significantly reduced ($P<0.05$) the SE^{NAR} numbers in the ceca from 3.7 in T2 to 2.7 log10 cfu compared to our SE^{NAR} challenge control. However, T4 (200 ppm of NE) did not differ significantly from SE^{NAR} challenge control, and had similar number of SE^{NAR} compared to SE^{NAR} challenge control (T2). The T6 (200 ppm NP) significantly reduced ($P<0.05$) the ceca SE^{NAR} from 3.7 in T2 to 1.4 log 10 cfu. NP reduced SE^{NAR} numbers in a dose dependent manner.

There was not significant reduction of SE^{NAR} prevalence in L/GB or ovary after supplementing with either level of nitrocompound (Table 2). In L/GB, there was a 50% positive cases in T2 (SE^{NAR} challenged control) and T6, whereas 66% positive in T4, and 33% in both T3 and T5 (Table 2). The recovery of SE^{NAR} in the ovary by 7 dpi was 0 except for T3 which had 16.7% positive cases.

We compared our results with previous studies which used some of the short chain nitrocompounds either *in vitro* or *in vivo* like in pig (Horrocks, et al., 2007; Horrocks et al., 2005). Among some of the widely-used alternatives, nitrocompounds were initially tested against the uric acid utilizing microorganisms (Kim, et al., 2006). In that study, the authors used the nitrocompounds such as nitroethane, nitroethanol, nitropropanol, and nitropropionic acid that had potential to reduce the ammonia volatilization in poultry manure by inhibiting growth of uricase-producing microorganism (Kim, et al., 2006). The cecal SE^{NAR} numbers in birds by supplemented with 200 ppm of NE was not different from our SE^{NAR} challenged control. In the present study, the reduction of SE^{NAR} numbers in most of our treatment groups demonstrate that both nitrocompounds have an effect against cecal colonization of *Salmonella*. In another study

using broilers, NP, supplemented by oral gavage, was effective in significantly reducing cecal *Salmonella* when tested against novobiocin resistant *ST* (Jung, et al., 2004). In that study, 130 ppm of NP reduced the *Salmonella* numbers in the ceca from 6.09 to 3.47 log 10 cfu (challenged control vs. 130 ppm NP).

In an *in vitro* study, 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-propionate (0, 10, and 20 mM) on growth of *Campylobacter jejuni* were tested and the superior inhibitory effect of 2-nitro-l-propanol was observed among all the nitrocompounds (Horrocks, et al., 2007). The data from our study were similar, NP was found more effective in reducing cecal SE^{NAR} numbers. The activity of the nitrocompounds, especially at the higher concentrations, appears to be bactericidal (Horrocks, et al., 2007). The highest level of NP (200 ppm) in the current study showed the better reduction in the cecal SE^{NAR}. Studies that used broilers challenged with *ST* or *SE* and supplemented with probiotics showed reduced numbers of *Salmonella* in the ceca (Haghighi, et al., 2008; Pascual, et al., 1999).

Ileum immune gene expression

There was a significant upregulation in gene expression ($P<0.05$) of all immune cytokines tested, IFN-Y, IL-1ß, IL-6, TLR-4 and IL-10, by SE^{NAR} challenge in our study. IFN-Y was significantly upregulated ($P<0.05$) by SE^{NAR} challenge, but reduced by supplementing both of the NE or NP compounds tested (Figure 2 A). The expression of IFN-Y in T3, T4, T5 and T6 were significantly lower than T2 ($P<0.05$). IFN-Y is the pro-inflammatory cytokine produced by activated T-cells and has a role in host defense for combating against the intracellular pathogens including *Salmonella* (Bao, et al., 2000). It has been shown that the upregulation of IFN-Y mRNA up to 200 fold was observed in cecal tonsils after challenged with *Salmonella* (Withanage, et al., 2005). Expression of pro-inflammatory cytokines like IFN-Y due to

Salmonella challenge in our study is similar to a study where chickens were infected by *Eimeria* (Hong et al., 2006). In that study, chickens were also sampled at 7 dpi, showing an upregulation of IFN-Ɣ in cecal tonsils (Hong, et al., 2006). Our results of reduction in IFN-Ɣ by supplementation of different levels of nitrocompounds can be compared to a previous study where the gene expression of IFN-Ɣ was repressed due to probiotics fed to the chickens (Haghighi, et al., 2008). The effect of diet is observed when the inflammatory genes such as IFN-Ɣ are down-regulated, suggesting there is reduction of inflammation (Vieira et al., 2013). It has been previously demonstrated that probiotic bacteria exert anti-inflammatory functions by reducing IFN-Ɣ production by immune system cells and that this reduction may be important for protection against *Salmonella* serovar Typhimurium (Silva et al., 2004). Benefits of nitrocompounds might include either the bactericidal activity that lower the expression of IFN-Ɣ in the ileum showing the protection against *Salmonella* (Haghighi, et al., 2005) or decreases the pro-inflammatory cytokines. The lower IFN-Ɣ expression in our study suggests that nitrocompounds downregulated the inflammation in the hens. Inflammation slows down the tissue expansion by promoting release of energy from adipocytes in mobilization of energy reserve. In other words, inflammation enhances energy expenditure. Due to the reduced inflammation rate, there is a savings of energy usage for growth and maintenance (Wang, et al., 2015).

The expression of IL-1ß mRNA was significantly upregulated ($P<0.05$) by either only SE^{NAR} challenge or SE^{NAR} challenge and NE (T2, T3 and T4). There was a significant downregulation ($P<0.05$) of IL-1ß mRNA expression in both T5 and T6 (Figure 2 B). IL-1ß is a pro-inflammatory cytokine and has shown protective effects in several bacterial, viral or fungal infections (Sahoo et al., 2011). Pro-inflammatory cytokines are responsible for the recruitment of

immune cells to the site of infection. The elevation in such cytokines provides evidence on the improved and effective immune response (Ferro, et al., 2004). The upregulation of IL-1ß cytokine after *Salmonella* infection in our study was similar to the previous study that used *Salmonella* LPS to challenge broilers (Shang, et al., 2015). There is a correlation between IL-1ß level and amount of intestinal inflammation (Reinecker et al., 1991). The protective action of nitrocompounds particularly NP, is demonstrated by the reduction level of IL-1ß and was similar to some of the previous studies that used dietary alternatives like probiotics and prebiotics (Babu, et al., 2012).

There was a significant upregulation ($P<0.05$) of IL-6 mRNA gene expression in the ileum after SE^{NAR} challenge (Figure 2 C). The effect of T3 was similar to SE^{NAR} challenged control (T2). T4, T5 and T6 showed significant reduction ($P<0.05$) compared to SE^{NAR} challenged control (T2). The lower expression of IL-6 suggests that supplementation with NP has a potential to lower pro-inflammatory cytokines and suppressing the inflammation (Yitbarek et al., 2013). This also indicates that NP interacts with the host either by changing the gut microbiome or direct contact with *Salmonella*, thereby reducing *Salmonella* colonization. IL-6 serves both a pro- and anti-inflammatory cytokine which can secret acute phase proteins and at the same time inhibit the production of IL-1ß (Waititu, et al., 2014). IL-6 upregulation in chickens has been associated with *Salmonella* and *Eimeria* infection (Kaiser, et al., 2000; Wigley, et al., 2003). Supplementation of probiotics, direct fed microbials or bacteriophage has reduced the IL-6 expression when they were added in the diets of *Salmonella* challenged chicken (Chichlowski et al., 2007; Shang, et al., 2015). However, a study reported that there was not any change observed in IL-6 mRNA expression in the cecal tonsil when supplementing with fructoligosachcarides as a method to control *Salmonella* (Janardhana, et al., 2009b).

TLR-4 mRNA expression was significantly higher ($P<0.05$) with SE^{NAR} challenge, whereas T5 (100 ppm NP) significantly reduced TLR-4 compared to T2 (Figure 2 D). Toll-like receptors recognize microbial-associated molecular patterns and stimulate immune system by a chain of reactions (Aderem, et al., 2000). In chickens, TLR-4 has been linked to resistance to *Salmonella* infection (Leveque, et al., 2003). In one study, *Salmonella* LPS challenge elevated expression of TLR-4 which agrees with our results (Shang, et al., 2015).

IL-10 mRNA was significantly upregulated ($P<0.05$) in T2 and T4 (200 ppm NE) compared to our unchallenged control (T1). The expressions in T3, T5 and T6 were not different from T1 (SE^{NAR} unchallenged control) (Figure 2 E). Interleukin-10 is the anti-inflammatory cytokine produced by activated macrophages and T cell. *Salmonella* or its lipopolysaccharide stimulates IL-10 gene expression in chickens (Ghebremicael et al., 2008; Shanmugasundaram, et al., 2015). Chen et al. (2012) reported an elevation of IL-10 in *Lactobacillus* based probiotics supplemented chickens. Also, Bai et al. (2014) showed that at 7 dpi, IL-10 expression was higher than that at 2 dpi and was similar to our results where we observed higher expression compared to our SE^{NAR} challenged control. Elevated expression of IL-10 reduced the inflammation which was also shown in a study that used lactic acid bacteria to reduce *Salmonella* in chickens (Chen, et al., 2012).

CONCLUSION

Both NE and NP are capable of inhibiting the growth of SE^{NAR} particularly in the ceca. Both nitrocompounds significantly reduced SE^{NAR} colonization in the ceca. NP reduced inflammatory cytokines and this can benefit energy utilization without waste. Overall, NP or NE may have application in reducing the food-borne pathogens like *Salmonella*. Future studies may be conducted to find out the mechanism of action and limits of the activity of such compounds.

References

Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. Nature 406:782-787.

Anderson, E. C., G. E. Carstens, R. K. Miller, T. R. Callaway, C. L. Schultz, T. S. Edrington, R. B. Harvey, and D. J. Nisbet. 2004. Effect of nitroethane administration on ruminal VFA production and specific activity of methane production. J. Anim. Feed Sci. 13 (Suppl. 1):23-26.

Babu, U. S., K. Sommers, L. M. Harrison, and K. V. Balan. 2012. Effects of fructooligosaccharide-inulin on *Salmonella*-killing and inflammatory gene expression in chicken macrophages. Vet. Immunol. Immunopathol. 149:92-96.

Bai, S. P., Y. Huang, Y. H. Luo, L. L. Wang, X. M. Ding, J. P. Wang, Q. F. Zeng, and K. Y. Zhang. 2014. Alteration in lymphocytes responses, cytokine and chemokine profiles in laying hens infected with *Salmonella* Typhimurium. Vet. Immunol. Immunopathol. 160:235-243.

Bai, S. P., A. M. Wu, X. M. Ding, Y. Lei, J. Bai, K. Y. Zhang, and J. S. Chio. 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult. Sci. 92:663-670.

Bailey, J. S., L. C. Blankenship, and N. A. Cox. 1991. Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poult. Sci. 70:2433-2438.

Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* Typhimurium infection. Immunol. 99:464-472.

Blanchfield, B., S. Stavric, T. Gleeson, and H. Pivnick. 1984. Minimum intestinal inoculum for Nurmi cultures and a new method for determining competitive exclusion of *Salmonella* from chicks. J. Food Prot. 47:542-545.

CDC. 2014. Incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. Sites, 2006–2013. Morb. Mortal. Wkly. Rep. 63:328-332.

Chen, C.-Y., H.-Y. Tsen, C.-L. Lin, B. Yu, and C.-S. Chen. 2012. Oral administration of a combination of select lactic acid bacteria strains to reduce the *Salmonella* invasion and inflammation of broiler chicks. Poult. Sci. 91:2139-2147.

Chichlowski, M., J. Croom, B. W. McBride, L. Daniel, G. Davis, and M. D. Koci. 2007. Direct-fed microbial PrimaLac and salinomycin modulate whole-body and intestinal oxygen consumption and intestinal mucosal cytokine production in the broiler chick. Poult. Sci. 86:1100-1106.

Donalson, L. M., J. L. McReynolds, W. K. Kim, V. I. Chalova, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* Enteritidis infection, and intestinal shedding in laying hens. Poult. Sci. 87:1253-1262.

Ferro, P. J., C. L. Swaggerty, P. Kaiser, I. Y. Pevzner, and M. H. Kogut. 2004. Heterophils isolated from chickens resistant to extra-intestinal *Salmonella* Enteritidis infection express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol. and Infect. 132:1029-1037.

Ghebremicael, S. B., J. R. Hasenstein, and S. J. Lamont. 2008. Association of interleukin-10 cluster genes and *Salmonella* response in the chicken. Poult. Sci. 87:22-26.

Haghighi, H. R., M. F. Abdul-Careem, R. A. Dara, J. R. Chambers, and S. Sharif. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. Vet. Microbiol. 126:225-233.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, B. Sanei, P. Parvizi, H. Gisavi, J. R. Chambers, and S. Sharif. 2005. Modulation of antibody-mediated immune response by probiotics in chickens. Clinic. Diagnostic Lab. Immunol. 12:1387-1392.

Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. 2006. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following Eimeria maxima infection of chickens. Vet. Immunol. Immunopathol. 114:259-272.

Horrocks, S. M., Y. S. Jung, J. K. Huwe, R. B. Harvey, S. C. Ricke, G. E. Carstens, T. R. Callaway, R. C. Anderson, N. Ramlachan, and D. J. Nisbet. 2007. Effects of short-chain nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli* in vitro. J. Food Sci. 72:M 50-55.

Horrocks, S. M., Y. S. Jung, S. C. Ricke, T. R. Callaway, T. S. Edrington, R. B. Harvey, R. C. Anderson, and D. J. Nisbet. 2005. Effects of Nitroethane and 2-Nitropanol against *Campylobacter* jejuni. Pages 194-196 in SafePork 2005: Sixth international symposium on the epidemiology and control of foodborne pathogens in porkIowa University Digital Press.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. D. Bean. 2009. Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139:1404-1409.

Jung, Y. S., R. C. Anderson, T. S. Edrington, K. J. Genovese, J. A. Byrd, T. R. Callaway, and D. J. Nisbet. 2004. Experimental use of 2-nitropropanol for reduction of *Salmonella* Typhimurium in the ceca of broiler chicks. J. Food Prot. 67:1945-1947.

Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Gallinarum. Microbiol. 146:3217-3226.

Kim, W. K., R. C. Anderson, A. L. Ratliff, D. J. Nisbet, and S. C. Ricke. 2006. Growth inhibition by nitrocompounds of selected uric acid-utilizing microorganisms isolated from poultry manure. J. Environ. Sci. Health B 41:97-107.

Lei, Y., K.-Y. Zhang, X.-M. Ding, S.-P. Bai, and J. S. Choi. 2009. Effect of probiotics on growth performance, development of small intestinal tract and microbial populations in broilers. J. Agric. Sci.Technol. 3:24-31.

Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredo-Osti, K. Morgan, and D. Malo. 2003. Allelic variation in TLR4 is linked to susceptibility to *Salmonella* enterica serovar Typhimurium infection in chickens. Infect. Immun. 71:1116-1124.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods (San Diego, Calif.) 25:402-408.

NRC. 1994. Nutrient Requirements of Poultry. Ninth Revised Edition. Natl. Acad. Press, washington, DC.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga. 1999. *Lactobacillus* salivarius CTC2197 prevents *Salmonella* Enteritidis colonization in chickens. Appl. and Environ. Microbiol. 65:4981-4986.

Reinecker, H. C., M. Steffen, C. Doehn, J. Petersen, I. Pfluger, A. Voss, and A. Raedler. 1991. Proinflammatory cytokines in intestinal mucosa. Immunol. Res. 10:247-248.

Saengkerdsub, S., W.-K. Kim, R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2006. Effects of nitrocompounds and feedstuffs on in vitro methane production in chicken cecal contents and rumen fluid. Anaerobe 12:85-92.

Sahoo, M., I. Ceballos-Olvera, L. del Barrio, and F. Re. 2011. Role of the inflammasome, IL-1, and IL-18 in bacterial infections. Scientific W. J. 11:2037-2050.

Shang, Y., A. Regassa, J. H. Kim, and W. K. Kim. 2015. The effect of dietary fructooligosaccharide supplementation on growth performance, intestinal morphology, and immune responses in broiler chickens challenged with *Salmonella* Enteritidis lipopolysaccharides. Poult. Sci. 94:2887-2897.

Shanmugasundaram, R., M. H. Kogut, R. J. Arsenault, C. L. Swaggerty, K. Cole, J. M. Reddish, and R. K. Selvaraj. 2015. Effect of *Salmonella* infection on cecal tonsil regulatory T cell properties in chickens. Poult. Sci. 94:1828-1835.

Silva, A. M., F. H. Barbosa, R. Duarte, L. Q. Vieira, R. M. Arantes, and J. R. Nicoli. 2004. Effect of *Bifidobacterium* longum ingestion on experimental salmonellosis in mice. J. Appl. Microbiol. 97:29-37.

Vieira, A. T., M. M. Teixeira, and F. S. Martins. 2013. The Role of Probiotics and Prebiotics in Inducing Gut Immunity. Frontiers Immunol. 4:445.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Wang, H., and J. Ye. 2015. Regulation of Energy Balance by Inflammation: Common Theme in Physiology and Pathology. Rev. Endocr. Metab. Disord. 16:47-54.

Wigley, P., and P. Kaiser. 2003. Avian cytokines in health and disease. Revista Brasileira de Ciência Avícola 5:1-14.

Withanage, G., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immunol. 73:5173 - 5182.

Yitbarek, A., J. C. Rodriguez-Lecompte, H. M. Echeverry, P. Munyaka, N. Barjesteh, S. Sharif, and G. Camelo-Jaimes. 2013. Performance, histomorphology, and Toll-like receptor, chemokine, and cytokine profile locally and systemically in broiler chickens fed diets supplemented with yeast-derived macromolecules. Poult. Sci. 92:2299-2310.

Table 6. 1. Diet composition of layer ration fed different levels of 2 nitroethanol (NE) or 2 nitro-propanol (NP)[1]

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

[1] SE^{NAR} unchallenge control (T1), SE^{NAR} challenge control (T2), SE^{NAR} challenge + 100 ppm NE (T3), SE^{NAR} challenge + 200 ppm NE (T4), SE^{NAR} challenge + 100 ppm NP (T5) and SE^{NAR} challenge + 200 ppm NP (T6).

[2]Supplemeted 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; D-biotin, 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.

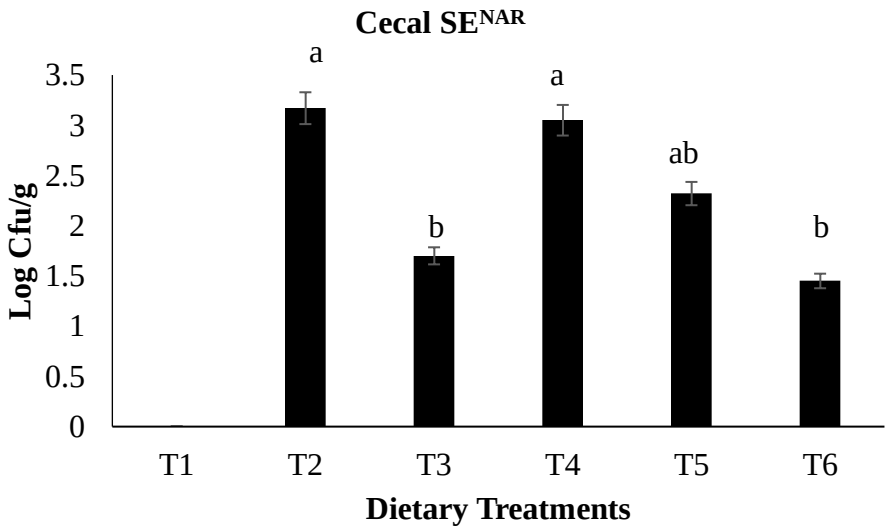


Figure 6.1 Presence of Salmonella number in ceca analyzed at 7 days post-infection (dpi) fed rations with different levels of 2 nitroethanol (NE) and 2 nitropropanol (NP) to laying hens. SE^{NAR} unchallenge control (T1), SE^{NAR} challenge control (T2), SE^{NAR} challenge + 100 ppm NE (T3), SE^{NAR} challenge + 200 ppm NE (T4), SE^{NAR} challenge + 100 ppm NP (T5) and SE^{NAR} challenge + 200 ppm NP (T6).

Table 6. 2. Presence and absence of SE^{NAR} in liver with gall bladder (L/GB) and ovary in laying hens challenged and supplemented with 2 nitroethanol (NE) and 2 nitropropanol (NP)[1].

	Incidence (%) of SE^{NAR} – positives[2]					
Organs	T1	T2	T3	T4	T5	T6
L/GB	0	50[a]	33[a]	66[a]	33[a]	50[a]
Ovary	0	0[b]	16.7[b]	0[b]	0[b]	0[b]

[a-b] Superscripts showing similar letter along the column are in different group.

[1] SE^{NAR} challenge control (T2), SE^{NAR} challenge + 100 ppm NE (T3), SE^{NAR} challenge + 200 ppm NE (T4), SE^{NAR} challenge + 100 ppm NP (T5) and SE^{NAR} challenge + 200 ppm NP (T6). Feed was provided immediate after challenge.

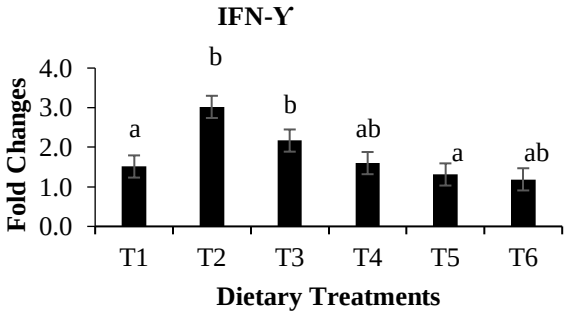
[2]Hens were 6 per treatment group (n=6).

Table 6. 3. Chicken cytokines and toll-like receptor primer sequences

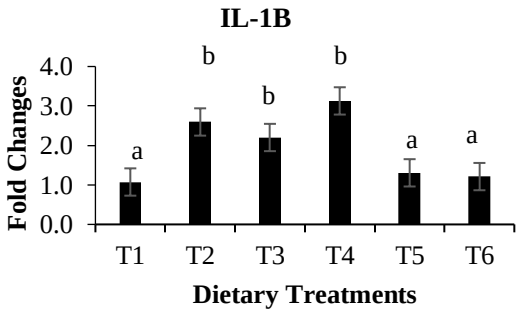
Gene[2]	Primer sequence[3] (5′–3′)	Gene bank accession no.	Fragment size, bp	Annealing temperature, °C
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	K01458	116	56
TLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	190	56
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	AJ309540	233	59
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	NM001004414	103	56
IL-1ß	F: CACAGAGATGGCGTTCGTTC R: GCAGATTGTGAGCATTGGGC	NM204524	118	56
IFN-Y	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	NM205149	159	58

[2]IL = interleukin; IFN = interferon; TLR = Toll-like receptor.

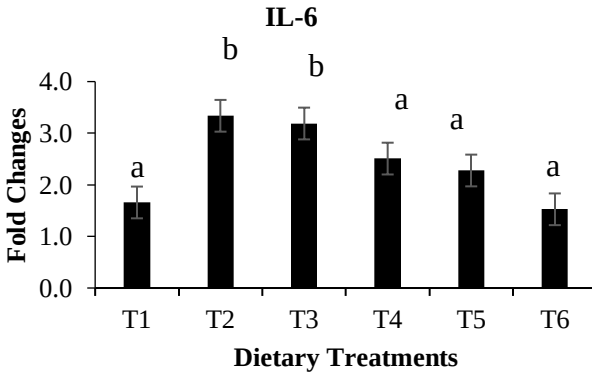
A)


IFN-Y
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
b
ab
a
ab
T1
T2
T3
T4
T5
T6
Dietary Treatments

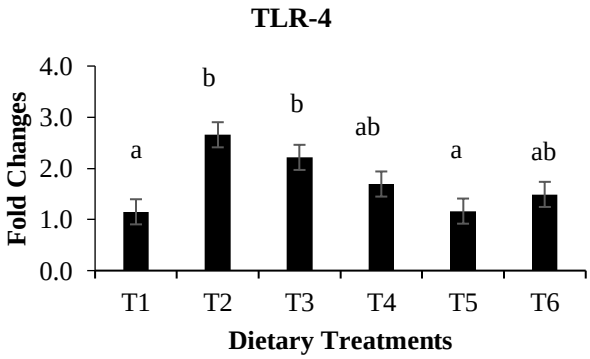
B)


IL-1B
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
b
b
a
a
T1
T2
T3
T4
T5
T6
Dietary Treatments

C)


IL-6
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
b
a
a
a
T1
T2
T3
T4
T5
T6
Dietary Treatments

D)


TLR-4
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
b
ab
a
ab
T1
T2
T3
T4
T5
T6
Dietary Treatments

E)

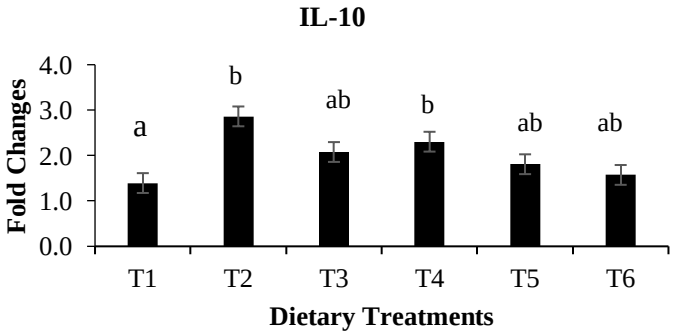

IL-10
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
ab
b
ab
ab
T1
T2
T3
T4
T5
T6
Dietary Treatments

Figure 6.2. Ileal immune gene expressions of cytokines: A) interferon (IFN)–Y, B) Interleukin (IL) – 1B, C) IL-6, D) toll-like receptor (TLR)-4 and D) IL-10 of chickens fed NP diets. Hens were challenged with Salmonella Enteritidis (n = 6/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). SE^{NAR} unchallenged control (T1), SE^{NAR} challenge control (T2), SE^{NAR} challenge + 100 ppm NE (T3), SE^{NAR} challenge + 200 ppm NE (T4), SE^{NAR} challenge + 100 ppm NP (T5) and SE^{NAR} challenge + 200 ppm NP (T6).

7. EFFECT OF SUPPLEMENTATION OF PROBIOTICS IN REDUCING THE *SALMONELLA* ENTERITIDIS COLONIZATION IN CECA, AND INTERNAL ORGANS, AND IMMUNE GENE EXPRESSION IN LAYING HENS[5]

[5]P. A. Adhikari, Douglas E. Cosby, Nelson A. Cox and Woo K. Kim. To be submitted to *Poultry Science Journal*.

ABSTRACT

A study was conducted to evaluate the effect of in-feed supplementation of probiotics in ceca, various internal organs as well as immune response in ileum in laying hens. Thirty-two White Leghorns were housed individually in wire laying cages under 16L:8D lightening schedule. Hens were challenged individually with nalidixic acid resistant *Salmonella* Enteritidis (SE^{NAR}) after which they were grouped into four treatments: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics. All hens, including T1 were euthanized and sampled for liver with gall bladder (L/GB), ovary, spleen and ceca on 7 days post-infection (dpi). Fecal screening was performed from individual hens at 3 and 6 dpi. There were no difference between the treatments in cecal SE^{NAR} enumeration, and the mean log 10 cfu/gm of ceca was averaged at 3.7 for all the treatments. The prevalence of SE^{NAR} was lowest for ovary for all treatments, but was higher in the spleen. However, there were no significant differences between treatments in the internal organs. There was no significant difference between the fecal shedding on either 3 or 6 dpi, with incidence of positive feces higher at 3 dpi compared to 6 dpi (100 vs. 70 - 80%). RNA was extracted from ileum and subjected to real-time quantitative (RT-PCR) for measurement of both pro- and anti-inflammatory cytokines such as interleukin (IL)- 1ß, 6, 10, interferon gamma (IFN-Y) and toll-like receptor (TLR)- 4. SE^{NAR} challenge resulted in significant upregulation ($P<0.05$) of cytokines tested. Highest level of probiotics resulted in a significant decrease in IFN-Y and elevation of IL-10 gene expression in ileum of chickens. For the remaining cytokines tested, the supplementation of probiotics resulted in either higher or similar expression to that of SE^{NAR} challenge. The studies reveal that there was some regulation of immune genes by probiotics supplementation without any effect on internal organs and feces SE^{NAR} shedding.

Key Words: laying hen, probiotic, Lactoplan – B, *Salmonella* Enteritidis

INTRODUCTION

The extensive use of in-feed antibiotics in farm animals whether to improve growth performance or to prevent the intestinal infections have led to growing concerns bacterial resistance (Dibner et al., 2005). Various dietary alternatives like probiotics, prebiotics, and bacteriophage in order to replace the antibiotics by reducing the infection has occurred in the last decade (Bailey, et al., 1991; Huff et al., 2003; Huff et al., 2005; Patterson, et al., 2003; Sims, et al., 2004). However, different products work differently in order to control the disease or infection. Probiotics are defined as the live cultures of beneficial bacteria and have been used in eliminating the *Salmonella* population and enhancing intestinal immunity in chicken (Fuller, 1989). Several mechanisms for probiotics include either competitive exclusion, production of antibacterial substances, or induction of immune and innate responses (Nava et al., 2005).

The concept of probiotics use was initially started from feeding to competitively exclude the pathogens from the chicken gut (Nurmi, et al., 1973). Administration of probiotics in chickens has shown modulation of several cytokines including interleukins **(IL**) or toll-like receptors **(TLR)** providing protection against *Salmonella* (Eckmann, et al., 2001). Commensal bacteria like *Lactobacillus* and *Bifidobacterium* are shown to regulate the cytokines production in gut associated lymphoid cells (Haghighi, et al., 2008). There appears to be a correlation between cytokines production and resistance against *Salmonella* (Haghighi, et al., 2008). *Salmonella* infection are associated with the elevation of the immune genes expression in intestine, cecal tonsil, liver and spleen (Withanage, et al., 2005). The objectives of the current study were to determine wether the in-feed supplementation of probiotics to reduce the *Salmonella* in feces, ceca, and internal organs as well as impacts the mRNA expression of select cytokines.

MATERIALS AND METHODS

Bacterial Strain and Inoculum

Chicken isolate of Nalidixic acid resistant *Salmonella* Enteritidis **(SE^{NAR})** was used as a challenge pathogen. Bacteria were preserved for the longer time in Tryptic soy broth (Acumedia, Neogen Corp., Lansing, MI) with 15% glycerol (Sigma). The frozen culture of SE^{NAR} was revived into brilliant green agar with sulphapyridine (**BGS**; Acumedia, East Lansing, MI) supplemented at 200 ppm nalidixic acid (**Nal**; Sigma Chemical Co., St. Louis, MO) plates. The plates were incubated for 24 h at 37°C.

Isolated SE^{NAR} colonies were transferred to 9 mL of sterile 0.85 % saline solution. The absorbance value was adjusted to an optical density of 0.20 ± 0.01 at 540nm with a spectrophotometer (Spect-20, Milton-Roy, Thermo Spectronics, Madison, WI) which yields approximately 1.0 x 10^8 cfu/mL. Cultures were serially diluted in sterile saline for enumeration. Each hen was orally **(OR**) gavaged with a 1 cc tuberculin syringe (Becton, Dickinson and Co., Franklin Lakes, NJ) and an animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY), whereas intracloacal (**IC**) inoculation was performed using only a 1 cc tuberculin syringe. Hens were challenged with the inoculum dose of 2.8×10^8 cfu of SE^{NAR}.

Hens and Housing

Thirty two laying hens (46 wk old at the beginning of the experiment) were housed individually in a wire layer cage and fed a commercial layer ration. Hens were provided *ad libitum* feed and water, and kept under lightening schedule of 16L:8D. Hens were fed ration that either met or exceeded the requirement of laying hens performance (NRC, 1994). Hens were divided into groups to give 8 replicates per treatment that consisted of: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics. Hens were off-fed for 10 h after which they were

challenged. Feed was provided immediately after challenge. The animal experiment was approved by the Institutional Animal Care and Use Committee.

Sampling plan and Processing, Bacteriological Recovery of SE^{NAR} in Feces and Internal Organs

All hens were screened for fecal SE^{NAR} shedding at 3 and 6 day post-infection (dpi). For feces screening, aluminum foil sheets were placed under each hen for overnight and feces were collected on the next morning. Sterile 50 mL conical centrifuge tubes were used to collect feces and transported in an ice chest for bacteriological analysis. Briefly, feces were weighed and added with BPW 3 times the sample weight and vortexed. A 10 µl portion of each sample was streaked for isolation onto BGS-Nal plates. Plates and sample tubes were incubated for 24 h at 37°C. Plates that were negative by direct plating were again streaked into BGS-Nal plates from the overnight pre-enriched samples. The plates were read as negative or positive.

Hens were humanely euthanized by electrocution on 7 dpi. Internal organs like ceca, liver with gall-bladder (**L/GB**), spleen and ovary were collected aseptically. All the samples were macerated by rubber mallet. Samples were individually weighed and diluted in buffered peptone water (**BPW**) three times their weight. The sample bags were stomached (Techmar Company, Cincinnati, Ohio) for 60 s and pre-enriched overnight at 37°C. Pre-enriched samples for spleen, ovaries and LGB were streaked for isolation onto BGS-Nal plates and incubated overnight at 37°C. The growth of SE^{NAR} was observed and recorded.

Cecal samples were analyzed by a swab plate method according to modified Blanchfield method (Blanchfield, et al., 1984). After stomaching, two sterile cotton swabs were dipped inside the contents of the ceca bags. Swab one was spread plated into the BGS-Nal plate (A plate). Swab two was transferred into a tube containing 9.9 mL of BPW, vortexed and a third swab was

inserted into the tube, soaked and spread plated onto next BGS-Nal plate (B plate). The contents of the tube were poured into the bags with ceca and both ceca bags and plates were incubated at 37°C for 24 h. Any samples that had negative results were re-streaked from the enriched ceca onto a fresh BGS-Nal plate and incubated at 37°C for 24 h. Plate counts were estimated to the nearest $\log_{10}$ and the cfu/gm ceca was calculated.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Section of ileum tissue was aseptically excised and frozen immediately at -80°C until further analyses for cytokines. Total RNA was extracted from ileum samples using TRIzol reagent (Qiazen, Life Technologies, USA). Tissues were disrupted by homogenization using a mini-bead beater-16 homogenizer (Biospec Products, Fisher Scientific, Bartlesville, OK) for 3 mins. RNA pellets were dissolved in 200- µl nuclease-free water (Ambion, Applied BioSystems, Life Technologies, USA) and concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The purity of RNA was verified at optical density ratio of 260 to 280 nm. RNA was normalized to a concentration of 2 ug/ µl after which it was reverse transcribed using High capacity cDNA synthesis Reverse Transcription Kits (Applied BioSystems, Life Technologies, Carlsbad, CA) following manufacturer's protocol. Primers for chicken immune genes such as toll-like receptor (**TLR-4**), interleukins (**IL**-1ß, IL-6, and IL-10) and interferon (**IFN**)-Ɣ were measured. Quantitative real-time PCR (**qRT-PCR**) was performed in duplicate reaction using both forward and reverse primers, cDNA, SYBR Green (Applied BioSystems, Life Technologies, Carlsbad, CA) and nuclease free water. qRT-PCR was performed using Step One thermo cycler (Applied Biosystem, Foster City, State). Glyceraldehyde 3- phosphate dehydrogenase (**GAPDH**) was used as a housekeeping gene and used to normalize the expression of all target genes. Pro- and anti-inflammatory cytokines such

as IL-1ß, IFN-Ɣ, IL-6, IL-10 and TLR-4 were evaluated for the expression. Pairs of primers used in our study are shown in Table 2.

STATISTICAL ANALYSES

The SE^{NAR} prevalence data for feces and internal organs were analyzed using Fisher Exact test. Gene expression data was analyzed by difference in cycle threshold (ΔcT) method. Dietary effects on cecal SE^{NAR} colonization and immune genes were analyzed using a one-way analysis of variance (ANOVA) of General Linear Models (GLM) procedure of SAS 9.2 (SAS Inst., 2001). Significant differences between the means of different treatment groups were determined by Duncan's multiple-range test.

RESULTS

Effects of probiotics and SE^{NAR} in colonization of ceca, L/GB, ovary, spleen and feces

The negative control group, T1 did not show any positive recovery in ceca throughout the experimental period (Fig. 1). There was no reduction in the SE^{NAR} colonization in the chicken ceca after supplementing two levels of probiotics. The mean $\log_{10}$ cfu/gm of cecal contents was 3.7 in T2, T3 and T4. There was no difference between the treatments with supplementation of probiotics. The recovery of SE^{NAR} in L/GB was not different between the treatments and was 78% recovery in all 3 groups (Fig. 2). The pattern of positive recovery of ovary was lower than L/GB or spleen. Ovary was 20% positive for SE^{NAR} in T2 and T3, whereas it was 50% positive in T4. Spleen was 100% positive in T2 while it was 80% positive in T3 and T4, respectively. The percentage of fecal shedding was more positive at 3 dpi than 6 dpi (Fig. 3). The percentage recovery of feces SE^{NAR} was 100% in 3 dpi whereas by 6 dpi, the recovery reduced to 70% in T2 and T3 whereas it was 60% in T4. There was no any positive recovery in T1 group in either of the organs.

Effects of probiotics and SE^{NAR} in immune response in ileum

The expression was detected for all the cytokines tested and there was a significant upregulation (P<0.05) in most of the cytokines tested (Fig. 4). There was a significant upregulation of all cytokines in T2 due to SE^{NAR} challenge. Supplementation of probiotics levels either upregulated or resulted in similar level of expression of IFN-Ɣ as T2 (Fig. 4a). T3 and T4 produced the similar expression to T1. Supplementation of probiotics showed mixed results with IL-1ß in our study. There was a significant upregulation of IL-1ß in T2, T3 and T4 compared to T1 (Fig. 4b). Supplementation of probiotics at T3 level increased the IL-1ß at higher level in ileum. For IL-6, T4 had significantly higher expression (P<0.05) than rest of the treatment group whereas T2 was not different to T3 (Fig. 4c). However, the supplemented levels of probiotics did not reduce the expression, except for T4. The expression of TLR-4 due to SE^{NAR} challenge (T2) was higher but not different to T1 (Fig. 4d). There was significant upregulation (P<0.05) of TLR-4 in both T3 and T4. Expression of IL-10 was significantly higher (P<0.05) in T2, T3 and T4 compared to T1 with the highest expression in T4 (Fig. 4e).

DISCUSSION

Probiotics based especially on *Lactobacillus* have been used in several studies that were used to reduce or control the pathogens like *Salmonella* and *Campylobacter* (Haghighi, et al., 2008). The efficacy of the probiotics can be evaluated either by looking into the effect they provide for growth performance (Bai, et al., 2013), modulating the intestinal mucosa (Perdigon et al., 2002), immune gene expression (Plaza-Diaz et al., 2014; Sato et al., 2003) or directly controlling the pathogens (Chenoll et al., 2011; Pascual, et al., 1999). However, as the mechanism of action to work for a probiotic depends upon various factors like age of animals, environment, strains of *Lactobacillus* to antagonize the pathogenic bacteria (Jin et al., 1998).

Immune genes expression in ileum

Infection with *Salmonella* results in production of cytokines by host cells. Induction of pro-inflammatory cytokines due to *Salmonella* infection has already been well reported (Haghighi, et al., 2008). There have been previous reports on the expression of several cytokines and chemokines that can occur in several cells and organs like cecal tonsil, spleen, ileum, liver, macrophages due to *Salmonella* challenge (Beal et al., 2004; Withanage, et al., 2005). Some cytokines such as IL-4 and IL-10 help in down regulation of infection while some cytokines like IFN-Y , IL -12, and IL-18 are involved in protection against *Salmonella* infection (Eckmann, et al., 2001). Also, administration of probiotics to chickens enhances antigen-specific antibodies and has revolved around the gut associated lymphoid tissues (**GALT**) (Haghighi, et al., 2008; Haghighi, et al., 2005; Haghighi, et al., 2006).

IFN-Y is the pro-inflammatory cytokine that usually upregulates after *Salmonella* infection (Withanage, et al., 2005). INF- Y is also important for clearance of *Salmonella* in mice (Bao, et al., 2000). In a previous study, probiotic bacteria showed anti-inflammatory functions by reducing IFN-Y production by immune system cells and the reduction is important to protect against *Salmonella* infection (Haghighi, et al., 2008; Silva, et al., 2004). In our study, the down regulation of IFN-Y by supplementing probiotics, especially in T4, was similar to a previous study that used chicken as model and were sampled in 5 dpi (Haghighi, et al., 2008). Similarly, due to the development of cellular mediated immunity by IFN-Y, induction of IL-10 expression is pronounced and this is similar to a previous study that reported *Eimeria* infection and probiotics supplementation (Chen et al., 2016).

IL-1ß is the pro-inflammatory cytokine that provide early innate immune response and mediator of acute phase protein (Cassatella, 1995). In the current study, there was an increase in IL-6 expression due to SE^{NAR} challenge and it was similar to a study that used *S*T to look into the

effect in ileum, cecal tonsil and spleen (Hu et al., 2015). Also, increase in the expression of IL-1ß due to both *Salmonella* challenge and *Lactobacillus* based probiotics in our study was similar to a previous study (Brisbin et al., 2010).

IL-6 is produced from monocytes and macrophages, and serves as both pro-inflammatory and anti-inflammatory cytokine (Waititu, et al., 2014). IL-6 is the indicative of the initiation of acute phase response occurring in avian cells in response to *Salmonella* cells (Kaiser, et al., 2000). The increased expression of IL-6 due to *Salmonella* infection has been observed in some previous studies (Withanage, et al., 2005). A study showed that pretreatment of chickens with combination of four *Lactobacillus* spp. could reduce IL-6 production caused by *Salmonella* infection (Chen, et al., 2012). Our result of higher expression of IL-1ß, especially in T4 corresponds to a study where IL-6 was potentiated in probiotics treated group in cultured enterocytes (Reilly et al., 2007).

TLRs are pathogen recognition receptors and play a crucial role in activating T cells in the intestinal immune system, especially via MyD88 dependent signaling pathway (Higgs et al., 2006). TLR-4 is the principle receptor of lipopolysaccharide, which is a major component of the outer membrane of gram negative bacteria (Kannaki et al., 2010). The augmented expression of TLR-4 in probiotics supplemented groups in our study was similar to a study that used broiler chickens challenged with *Salmonella* and supplemented with 0.1 and 0.2% *Lactobacillus* and *Saccharomyces* containing probiotics (Bai, et al., 2013). Due to augmentation of TLR-4 signaling, there is regulation of local mucosal cell mediated immunity and promotion of gut barrier integrity (Gao, et al., 2008; Ng, et al., 2009). However, there was a downregulation of TLR-4 in intestine observed in a study that used broilers and fed with probiotics (Lei, et al., 2009). The reason behind such downregulation would be due to the lower pathogenic population

of *Coliform* hence possible due to probiotics supplementation (Lei, et al., 2009).

IL-10 is an anti-inflammatory and immune-regulatory cytokine that is involved in B-cell activation and antibody production (Saraiva et al., 2010). Infection with SE^{NAR} upregulated the expression of IL-10 in the current study and this was similar to a study that used *S*T as challenge pathogen, thus upregulated the IL-10 expression (Brisbin, et al., 2010). Our results on higher expression in T4 group is similar to a study in broilers where dietary supplementation of yeast cells upregulated IL-10 expression and produced anti-inflammatory effects (Alizadeh et al., 2016).

CONCLUSION

We confirmed that the effect of probiotics used in our study was effective more at the tissue level rather than direct inhibiting or controlling the *Salmonella* in the organs. This correlates with the protection against the *Salmonella* infection. Further study needs to be performed in order to find out detail in the mechanism of action of probiotics.

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References

Alizadeh, M., J. C. Rodriguez-Lecompte, H. Echeverry, G. H. Crow, and B. A. Slominski. 2016. Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on antibody-mediated immune response and gene expression of pattern recognition receptors and cytokines in broiler chickens immunized with T-cell dependent antigens. Poult. Sci. 95:823-833.

Bai, S. P., A. M. Wu, X. M. Ding, Y. Lei, J. Bai, K. Y. Zhang, and J. S. Chio. 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult. Sci. 92:663-670.

Bailey, J. S., L. C. Blankenship, and N. A. Cox. 1991. Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poult. Sci. 70:2433-2438.

Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* Typhimurium infection. Immunol. 99:464-472.

Beal, R., C. Powers, P. Wigley, P. Barrow, and A. Smith. 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella* enterica serovar Typhimurium. Avian Pathol. 33:25 - 33.

Blanchfield, B., S. Stavric, T. Gleeson, and H. Pivnick. 1984. Minimum intestinal inoculum for Nurmi cultures and a new method for determining competitive exclusion of *Salmonella* from chicks. J. Food Prot. 47:542-545.

Brisbin, J. T., J. Gong, P. Parvizi, and S. Sharif. 2010. Effects of *Lactobacilli* on cytokine expression by chicken spleen and cecal tonsil cells. Clinic. and Vacc. Immunol. 17:1337-1343.

Cassatella, M. A. 1995. The production of cytokines by polymorphonuclear neutrophils. Immunol. Today 16:21-26.

Chen, C.-Y., L.-T. Chuang, Y.-C. Chiang, C.-L. Lin, Y.-Y. Lien, and H.-Y. Tsen. 2016. Use of a probiotic to ameliorate the growth rate and the inflammation of broiler chickens caused by *Eimeria tenella* infection. J. Anim. Res. Nutr.

Chen, C.-Y., H.-Y. Tsen, C.-L. Lin, B. Yu, and C.-S. Chen. 2012. Oral administration of a combination of select lactic acid bacteria strains to reduce the *Salmonella* invasion and inflammation of broiler chicks. Poult. Sci. 91:2139-2147.

Chenoll, E., B. Casinos, E. Bataller, P. Astals, J. Echevarría, J. R. Iglesias, P. Balbarie, D. Ramón, and S. Genovés. 2011. Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter* pylori. Appl. Environ. Microbiol. 77:1335-1343.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634-643.

Eckmann, L., and M. F. Kagnoff. 2001. Cytokines in host defense against *Salmonella*. Microbes and Infect. 3:1191-1200.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gao, J., H. J. Zhang, S. H. Yu, S. G. Wu, I. Yoon, J. Quigley, Y. P. Gao, and G. H. Qi. 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. Poult. Sci. 87:1377-1384.

Haghighi, H. R., M. F. Abdul-Careem, R. A. Dara, J. R. Chambers, and S. Sharif. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. Vet. Microbiol. 126:225-233.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, B. Sanei, P. Parvizi, H. Gisavi, J. R. Chambers, and S. Sharif. 2005. Modulation of antibody-mediated immune response by probiotics in chickens. Clinic. Diagnostic Lab. Immunol. 12:1387-1392.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, H. Zhou, B. Sanei, J. R. Chambers, and S. Sharif. 2006. Probiotics stimulate production of natural antibodies in chickens. Clinic. Vacc. Immunol. : CVI 13:975-980.

Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly. 2006. Induction of a Novel Chicken Toll-Like Receptor following *Salmonella* enterica Serovar Typhimurium Infection. Infect. Immun. 74:1692-1698.

Hu, J.-L., H. Yu, R. R. Kulkarni, S. Sharif, S. W. Cui, M.-Y. Xie, S.-P. Nie, and J. Gong. 2015. Modulation of cytokine gene expression by selected *Lactobacillus* isolates in the ileum, caecal tonsils and spleen of *Salmonella*-challenged broilers. Avian Pathol. 44:463-469.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2003. Bacteriophage treatment of a severe *Escherichia* coli respiratory infection in broiler chickens. Avian Dis. 47.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2005. Alternatives to antibiotics: utilization of bacteriophage to treat colibacillosis and prevent foodborne pathogens. Poult. Sci. 84:655-659.

Jin, L. Z., Y. W. Ho, N. Abdullah, M. A. Ali, and S. Jalaludin. 1998. Effects of adherent *Lactobacillus* cultures on growth, weight of organs and intestinal microflora and volatile fatty acids in broilers. Anim. Feed Sci.Technol. 70:197-209.

Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Gallinarum. Microbiol. 146:3217-3226.

Kannaki, T., M. Reddy, M. Shanmugam, P. Verma, and R. Sharma. 2010. Chicken toll-like receptors and their role in immunity. World's Poult. Sci. J. 66:727-738.

Lei, Y., K.-Y. Zhang, X.-M. Ding, S.-P. Bai, and J. S. Choi. 2009. Effect of probiotics on growth performance, development of small intestinal tract and microbial populations in broilers. J. Agric. Sci.Technol. 3:24-31.

Nava, G. M., L. R. Bielke, T. R. Callaway, and M. P. Castaneda. 2005. Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. Anim. Health Res. Rev. 6:105-118.

Ng, S. C., A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: recent advances. Inflamm. Bowel Dis. 15:300-310.

NRC. 1994. Nutrient Requirements of Poultry. Ninth Revised Edition. Natl. Acad. Press, washington, DC.

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

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga. 1999. *Lactobacillus* salivarius CTC2197 prevents *Salmonella* enteritidis colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.

Perdigon, G., C. M. Galdeano, J. C. Valdez, and M. Medici. 2002. Interaction of lactic acid bacteria with the gut immune system. European J. Clinic. Nutr. 56:S21-S26.

Plaza-Diaz, J., C. Gomez-Llorente, L. Fontana, and A. Gil. 2014. Modulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver by probiotics. W. J. Gastroenterol. 20:15632-15649.

Reilly, N., V. Poylin, M. Menconi, A. Onderdonk, S. Bengmark, and P. O. Hasselgren. 2007. Probiotics potentiate IL-6 production in IL-1beta-treated Caco-2 cells through a heat shock-dependent mechanism. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293:R1169-1179.

Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10:170-181.

Sato, M., H. Sano, D. Iwaki, K. Kudo, M. Konishi, H. Takahashi, T. Takahashi, H. Imaizumi, Y. Asai, and Y. Kuroki. 2003. Direct binding of Toll-Like Receptor 2 to Zymosan, and Zymosan-induced NF-κB activation and TNF-α secretion are down-regulated by lung collectin surfactant protein A. J. Immunol. 171:417-425.

Silva, A. M., F. H. Barbosa, R. Duarte, L. Q. Vieira, R. M. Arantes, and J. R. Nicoli. 2004. Effect of *Bifidobacterium longum* ingestion on experimental salmonellosis in mice. J. Appl. Microbiol. 97:29-37.

Sims, M. D., K. A. Dawson, K. E. Newman, P. Spring, and D. M. Hoogell. 2004. Effects of dietary mannan oligosaccharide, bacitracin methylene disalicylate, or both on the live performance and intestinal microbiology of turkeys. Poult. Sci. 83:1148-1154.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Withanage, G., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. 73:5173 - 5182.

Table 7. 1. Ingredient composition and values of diet fed either control or probiotics to laying hens

Item	Diet[1]		
	T1/T2	T3	T4
Ingredient (% of the diet)			
Corn, Grain	59.53	59.53	59.53
Soybean Meal -48%	23.13	23.13	23.13
Limestone	9.62	9.62	9.62
Soybean OiL	3.00	3.00	3.00
Defluor. Phos.	2.13	2.13	2.13
Vitamin Premix[2]	0.50	0.50	0.50
Mineral Premix[3]	0.15	0.15	0.15
DL-Methionine	0.34	0.34	0.34
Common Salt	0.30	0.30	0.30
L-Lysine HCl	0.30	0.30	0.30
Lactoplan-B[4]	0.00	0.05	0.10
Sand	1.00	0.50	0.00
Calculated composition			
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]Hens were fed corn-soybean control diet for treatment 1 and 2 (T1 and T2), whereas for T3 and T4, probiotics was supplemented at 0.05% and 1.0%, respectively.

[2]Supplemeted 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; D-biotin, 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] Lactoplan – B

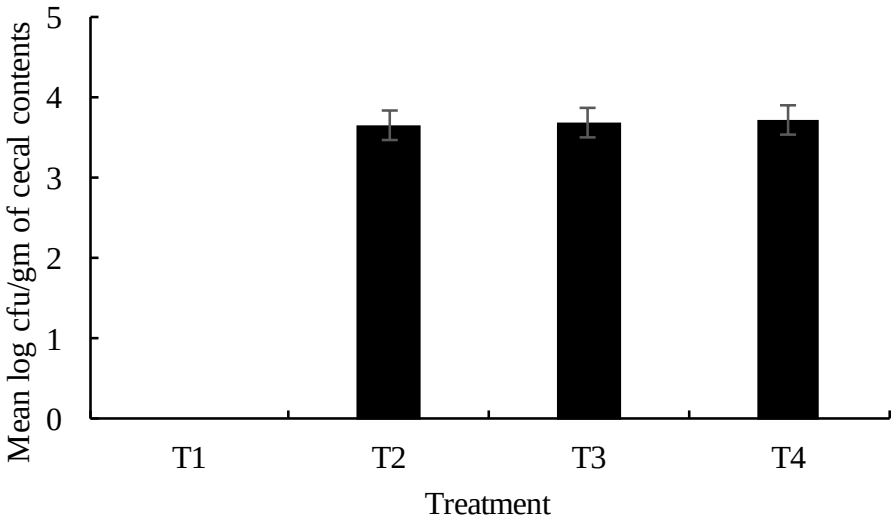


Figure 7.1 Salmonella count in ceca of laying hens supplemented with or without probiotics. The treatment groups were: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics

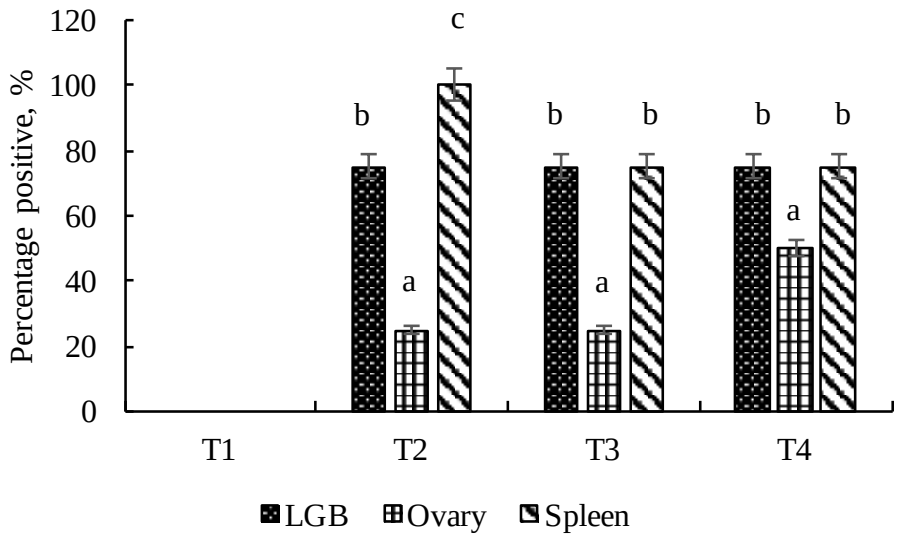


Figure 7.2 Presence or absence of SE^{NAR} in liver with gall-bladder (LGB), ovary and spleen in laying hens challenged with SE^{NAR} and supplemented with or without probiotics. The treatment groups were: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics

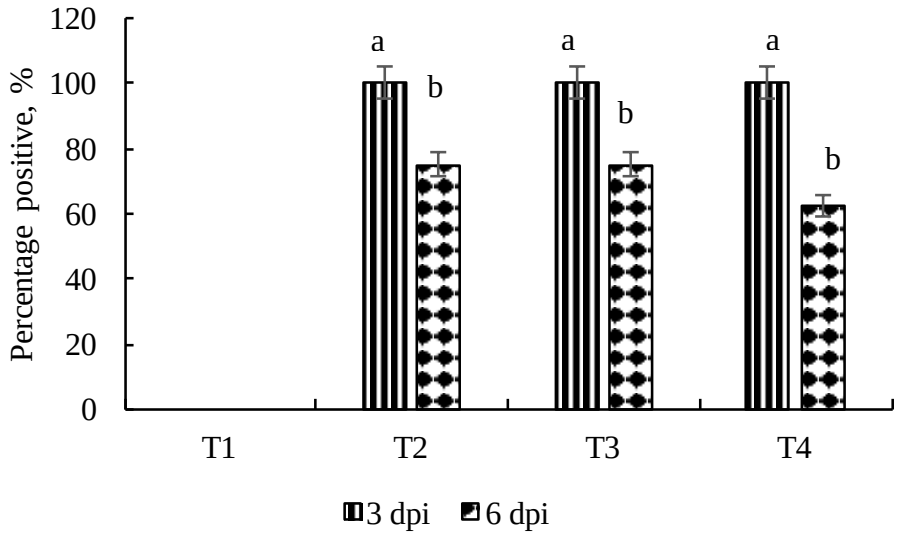


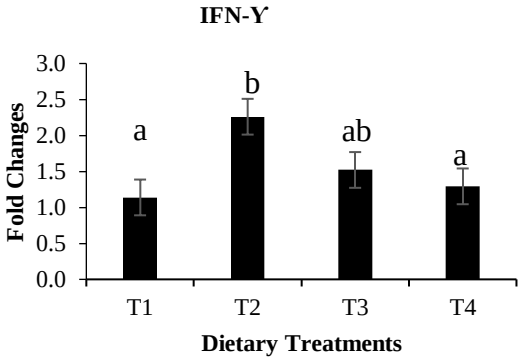
Figure 7.3 Presence or absence of SE^{NAR} in feces of laying hens challenged with SE^{NAR} and supplemented with or without probiotics. The treatment groups were: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics

Table 7. 2. Chicken cytokine primer sequences

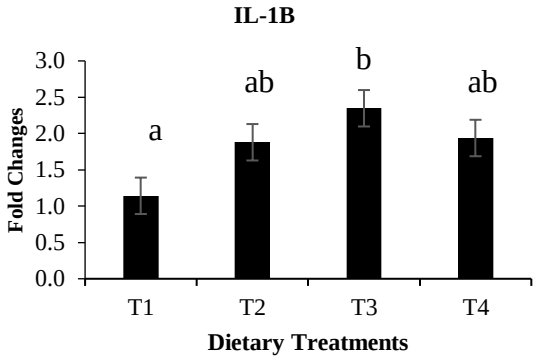
Gene[2]	Primer sequence[3] (5′–3′)	Gene bank accession no.	Fragment size, bp	Annealing Temperature, °C
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	K01458	116	55
TLR-4	F: TCCGTGCCTGGAGGTAAGT R: TGCCTTGGTAACAGCCTTGA	NM001030693	190	56
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	AJ309540	233	59
IL-10	F: GCTCTCCTTCCACCGAAACC R: GGAGCAAAGCCATCAAGCAG	AJ621614	103	56
IL-1ß	F: CACAGAGATGGCGTTCGTTC R: GCAGATTGTGAGCATTGGGC	NM204524	118	56
IFN-Y	F: GCATCTCCTCTGAGACTGGC R: GCTCTCGGTGTGACCTTTGT	NM205149	159	58

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

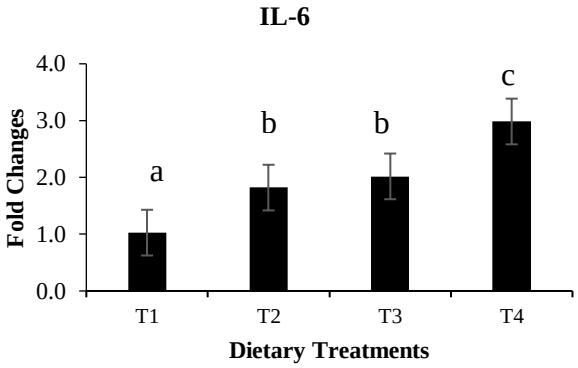
a)


IFN-Y
Fold Changes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
ab
a
T1
T2
T3
T4
Dietary Treatments

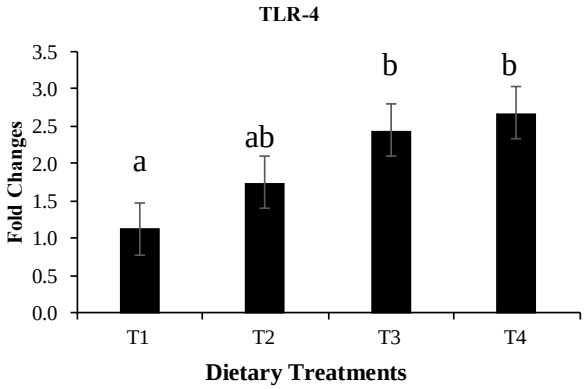
b)


IL-1B
Fold Changes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
ab
T1
T2
T3
T4
Dietary Treatments

C)


IL-6
Fold Changes
4.0
3.0
2.0
1.0
0.0
a
b
b
c
T1
T2
T3
T4
Dietary Treatments

D)


TLR-4
Fold Changes
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
T1
T2
T3
T4
Dietary Treatments

E)

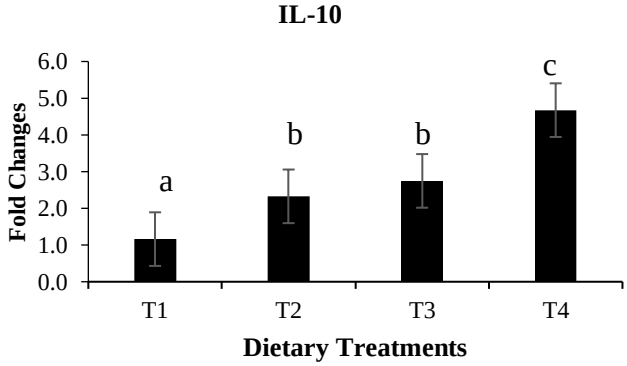

IL-10
Fold Changes
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
c
T1
T2
T3
T4
Dietary Treatments

Figure 7.4 Relative expression of cytokines in probiotics fed to laying hens. Cytokines are: a) IFN-Y, b) IL-1B, c) IL-6, d) TLR-4 and e) IL-10 in ileum of laying hens fed probiotics and challenged with SE^{NAR}. The treatment groups were: T1 = SE^{NAR} unchallenged, T2 = SE^{NAR} challenged, T3= SE^{NAR} challenged + 0.05% probiotics (Lactoplan-B) and T4 = SE^{NAR} challenged + 0.1% probiotics

8. SUMMARY AND CONCLUSION

Salmonella Enteritidis is a global foodborne problem that is associated with poultry and their products such as meat and egg. The prevention of *Salmonella* with dietary alternatives is important especially since the world is moving towards an antibiotic free era of animal production. These supplementations with different alternatives might provide insight into the use of new products. In our study, supplementing with prebiotics (FOS), bacteriophages, nitro compounds and probiotics have all shown to positively reduce SE^{NAR} in chicken's internal organs as well as reduce fecal shedding in laying hens. The most effective dietary supplemented among all the ones tested was BP, where SE^{NAR} recovery was significantly reduced in feces, ceca, and all the internal organs. Also, there were pronounced effects observed in both pro- and anti-inflammatory cytokines mRNA expression using these dietary supplements. Immune modulation observed by the inflammatory response due to SE^{NAR} challenge as well as such dietary strategies shows the immunity in these hens. However, it also shows that SE recovery in mature hens would not exceed than 14 days post-infection and we need to find the best window to supplement with dietary alternatives to minimize the SE infection.

This research is beneficial to the egg industry in that it shows *Salmonella* growth and transmission can be minimized in layers through the use of dietary feed supplements, although timing of initiation of the dietary supplements needs to be defined . The impact of dietary supplements on reducing SE^{NAR} colonization was pronounced in the ceca compared to the other internal organs. Overall, the pattern of *Salmonella* recovery relative to dietary supplementation was similar between the ceca and feces. As federal and state pressure on the poultry industry to

eliminate antibiotic usage in animal feed increases, employing alternative dietary supplements becomes more attractive. However, more studies are needed to understand the specific mechanisms of action of these alternative dietary supplements on inhibiting pathogens, such as *Salmonella.* Also, since this research focused only SE, it would be beneficial to evaluate the patterns of *Salmonella* reduction in other serovars and other bacterial pathogens affecting chickens.