Utilization of Synbiotics, Acidifiers, and a Polyanhydride Nanoparticle Vaccine in Enhancing the Anti-*Salmonella* Immune Response in Laying Hens Post-*Salmonella* Challenge

Dissertation

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ABSTRACT

Salmonellosis, a zoonotic disease caused by the bacterium *Salmonella*, is most commonly attributed to the consumption of poultry eggs and meat. The current project examined the effects of drinking water synbiotics, in-feed acidifiers, and a polyanhydride nanoparticle *Salmonella* vaccine in enhancing the anti-*Salmonella* immune response and decreasing *Salmonella* infection in laying hens. The synbiotic experiment was conducted to study the effects of drinking water supplementation of synbiotic product in laying hens with and without a *Salmonella* challenge. A total of 384 one-day-old layer chicks were randomly distributed to the drinking water synbiotic supplementation or control groups. At 14 wk of age, the birds were vaccinated with a *Salmonella* vaccine, resulting in a 2 (control and synbiotic) X 2 (non-vaccinated and vaccinated) factorial arrangement of treatments. At 24 wk of age, half of the birds in the vaccinated groups and all the birds that were not vaccinated were challenged with *Salmonella Enterica* serotype Enteritidis, resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. At 8 d post-*Salmonella* challenge, synbiotic supplementation decreased ($P = 0.04$) cecal *S*. Enteritidis in the challenge group compared to the un-supplemented challenge group. At 17 d post-*Salmonella* challenge, synbiotic supplementation increased bile anti-*Salmonella* IgA in the challenge group compared to the birds in the challenge group without synbiotic supplementation. The acidifier experiment was conducted to study the effects of

acidifier supplementation in laying hens with and without a *Salmonella* challenge. A total of 384 one-day-old layer chicks were randomly distributed to the acidifier supplementation or control groups. At 14 wk of age, the birds were vaccinated with a *Salmonella* vaccine, resulting in a 2 (control and acidifier) X 2 (unvaccinated and vaccinated) factorial arrangement. At 24 wk of age, half of the birds in the vaccinated groups and all the birds that were not vaccinated were challenged with *Salmonella Enterica* serotype Enteritidis, resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement. At 8 d post-*Salmonella* challenge, birds supplemented with acidifier in the challenge group had decreased ($P = 0.04$) cecal *S.* Enteritidis percentage compared to the challenge group without acidifier supplementation. At 22, 24, and 30 d post-*Salmonella* challenge, acidifier supplementation had higher ($P < 0.01$) plasma anti-*Salmonella* IgA titers in the challenge group compared to the challenge group without acidifier supplementation. The polyanhydride nanoparticle experiment analyzed the immunological effects of an oral *Salmonella* nanoparticle vaccine (OMPs-F-PNPs) loaded with *Salmonella* outer membrane proteins (OMPs) and flagellin proteins. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded OMPs-F-PNPs suspension in 1 mL of sterile PBS (OMPs-F-PNPs). The same dose and route of delivery were repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1×10^9 CFU of live *S.* Enteritidis. Birds that were vaccinated with OMPs-F-PNPs had significantly ($P < 0.01$) higher serum and bile OMPs-specific IgG titers compared to that from the mock + challenge and the mock treatment groups. The OMPs-F-PNPs vaccinated

birds had significantly fewer positive samples for cecal *S.* Enteritidis compared to that of the mock + challenge group. In conclusion, drinking water synbiotic supplementation, in-feed acidifier supplementation, and the polyanhydride *Salmonella* nanoparticle vaccine each resulted in significant increases in the anti-*Salmonella* immune response as measured by *Salmonella*-specific antibody titers and decreased *Salmonella* colonization in layer chickens.

Dedication

I dedicate my dissertation to my father, mother, and sister, who gave me constant encouragement in pursuing my goals in education.

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Chapter 1: Introduction

Introduction

Salmonella enterica serotype Enteritidis is one of the most commonly isolated non-typhoidal serotypes of *Salmonella.* When spread to humans, it can lead to a salmonellosis infection, with symptoms including diarrhea, fever, and vomiting. In the United States, there are over 40,000 cases of salmonellosis and 400 deaths caused by acute salmonellosis reported annually (Fabrega and Vila, 2013). *Salmonella* is most commonly spread from chicken intestinal contents contaminating meat or from egg shell contamination (Braden, 2006; Pires et al., 2014). The increase of laying hens raised in cage-free systems is further increasing risk of *Salmonella* contamination in eggs, since eggs are more likely to come in contact with the hens' feces (Whiley and Ross, 2015). In the studies reported herein, a drinking water synbiotic (probiotic plus prebiotic combination), in-feed acidifier, and a novel nanoparticle *Salmonella* vaccine were used to reduce *Salmonella* following a challenge and to enhance the anti-*Salmonella* immune response in layer chickens.

Salmonella

Salmonella is a Gram-negative, non-spore-forming, rod-shaped bacteria from the *Enterobacteriaceae* family. The individual species have diameters ranging from 0.7 to 1.5 μm, lengths from 2 to 5 μm, and multiple flagella that surround the entire cell body (Coburn et al., 2007). *Salmonella* can be divided into two species, *Salmonella bongori* and *Salmonella enterica.*

Salmonella enterica can further be divided into six subspecies which include *S.enterica* subsp.*enterica* (I), *S.enterica* subsp. *salamae* (II), *S.enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica*subsp. *indica* (VI) (Lan et al., 2009).

Almost all *Salmonella* that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica (I). S. enterica* serotypes can be typhoidal or nontyphoidal. The typhoidal serotype can cause fever in humans, whereas the more common nontyphoidal serotypes cause diarrheal disease and can infect a wide range of animal hosts (Ohl and Miller, 2001). *S.* Enteritidis and *S.* Typhimurium are the most commonly isolated non-typhoidal serotypes and rarely cause clinical disease in chickens despite frequently colonizing the gastrointestinal tract of poultry (Barrow et al., 1987).

Salmonella is shed in the feces and can be spread by horizontal transmission to other birds by fecal-oral transfer. *Salmonella* can also be spread by infected intestinal contents or feces contaminating the meat. The transfer of *Salmonella* from the hen to its offspring, vertical transmission, is another route of infection as *Salmonella* can colonize the reproductive tract, leading to the subsequent contamination of eggs (Humphrey et al., 1988).

Both *S.* Enteritidis and *S.* Typhimurium have similar virulence mechanisms with regard to cellular invasion, survival, and growth within the host (Guard-Petter, 2001). *Salmonella* infection begins when the bacteria is ingested by the host. To protect from the acidity of the stomach, *S.* Enteritidis activates an acid tolerance response that maintains the intracellular pH at values higher than the extracellular environment (Foster and Hall, 1991). *Salmonella* can invade

the intestinal mucus layer before adhering to intestinal epithelial cells. After the adhesion, the infected host cell initiates signaling pathways that leads to cytoskeletal rearrangement (Francis et al., 1992) and disruption of the epithelial brush border, thus inducing the formation of membrane ruffles that engulf adherent bacteria and form vesicles called *Salmonella*-containing vacuoles (SCVs) where the *Salmonella* can survive and replicate. The SCVs are transported to the basolateral membrane where *Salmonella* cells are released into the submucosa. Additionally, phagocytic cells such as macrophages act as SCVs by internalizing the *Salmonella* and transporting the bacteria through the lymph and bloodstream, thus facilitating a systemic infection (Ohl and Miller, 2001).

Immune Responses to *Salmonella* Enteritidis in Chickens

The mucosal immune system of the intestine, which includes mucosal immunoglobulin A (IgA) and mucosa-associated lymphocytes and leukocytes, forms the first line of defense against *S*. Enteritidis infection. Humoral and cell-mediated responses are critical for the resistance and clearance of *S*. Enteritidis infection. Chicken immune response to *Salmonella* include an influx of inflammatory immune cells such as macrophages and T lymphocytes, and cytokines such as LITAF (lipopolysaccharide-induced tumor necrosis factor alpha factor) and IL-1ß. B lymphocytes have a major role during a *Salmonella* infection as well, as immune suppression of B cells results in an increase in the rate of *S*. Enteritidis intestinal shedding (Arnold and Holt, 1995). Secretory IgA, the most prevalent immunoglobulin secreted by B lymphocytes within the intestinal tract, limits mucosal colonization of *S*. Enteritidis by preventing adherence of the bacteria (Shroff et al., 1995).

Probiotics

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Grimont and Weill, 2007). Probiotics can improve the health of the host through several mechanisms, including: (1) inhibition of colonization and proliferation of pathogenic bacteria through competitive exclusion (Lawley and Walker, 2013), (2) production of bacteriostatic and bactericidal substances against pathogens (Van Der Wielen et al., 2000), (3) enhancement of intestinal barrier (Yang et al., 2012), and (4) enhancement of host immunity (Yang et al., 2012).

To successfully colonize within the intestines, probiotics are required to withstand the low pH of the stomach and bile acids in the intestine (Kailasapathy and Chin, 2000). Additionally, probiotics should effectively compete against pathogens for adhesion of the intestinal mucosa. Koenen et al. (2004) observed that supplementing broilers and laying hens with *Lactobacillus paracase* LW12 resulted in increased humoral and cell-mediated immune responses. However, Stephenson et al. (2010) studied the colonization ability of several chicken-derived *Lactobacillus* strains and found that out of over 20 strains, only three strains persistently colonized the intestines. It is therefore necessary to analyze the effects of distinct species and strains of probiotics despite having a well-studied genus, such as *Lactobacillus* or *Bifidobacterium.*

Lactobacillus reuteri

L. reuteri, a probiotic isolated from the gastrointestinal tract of chickens, possesses several characteristics which yield beneficial effects in the host. *In vitro* studies with *L. reuteri*

have demonstrated its capability in withstanding high heat, low pH, and bile salts; these traits result in effective intestinal colonization (Yu et al., 2007). Additionally, *L. reuteri* possesses surface-binding proteins such as mucus-binding proteins, located on the outside of the bacterial cell. These proteins can adhere to the intestinal enterocytes of the mucosal layer, which may further contribute to intestinal colonization (Mackenzie et al., 2010). Yu et al. (2007) observed that L. *reuteri* significantly inhibited *S.* Typhimurium and *E. coli in vitro* compared to *L. acidophilus*, another probiotic commonly used in chickens. *L. reuteri* produces antimicrobial compounds such as lactic acid, hydrogen peroxide, and reuterin, which have inhibitory effects on pathogenic bacteria (Cleusix et al., 2007).

Pediococcus acidilactici

P. acidilactici are facultative anaerobes that can inhibit growth of enteric pathogens through production of lactic acid and bacteriocins such as pediocins (Bhunia et al., 1990). Abbasiliasi et al. (2017) reported that *P. acidilactici* inhibited growth *Listeria monocytgenes, S. enterica, Shigella sonnei,* and *Streptococcus pyogenes.* Additionally, *P. acidilactici* is tolerant against bile salts and can survive acidic conditions as low as a pH of 2 (Abbasiliasi et al., 2017).

Bifidobacterium animalis

B. animalis is a Gram-positive, anaerobic, rod-shaped bacterium that colonizes naturally in the intestinal tract of chickens, rabbits, and humans (Scardovi and Zani, 1974). Li et al. (2010) reported that *B. animalis* had the greater oxygen tolerance compared with *Bifidobacterium infantis, Bifidobacterium longus*, and *Bifidobacterium thermophilus*. Meile et al. (1997) reported that the optimum growth temperature of *B. animalis* was from 39 °C to 42 °C and detected no

growth above 46 °C. *B. animalis* can survive at a low pH and in the presence of bile acid (Sanders, 2006).

Enterococcus faecium

E. faecium is a lactic acid bacteria gaining popularity for use in animal feeds (Kreuzer et al., 2012). *E. faecium* is a facultative anaerobe, Gram-positive, non-motile bacteria (Schleifer and Kilpper-Balz, 1984). Extremely robust, *E. faecium* persists in temperatures ranging from 10 to 45°C and survives in both basic and acidic environments (Huycke et al., 1998). *E. faecium* produces bacteriocins which exhibit inhibitory growth of both gram-positive and gram-negative bacteria, including *S.* Typhimurium, *E. coli*, and *Streptococcus thermophilus* (Kang and Lee, 2005).

Organic Acids

Organic acids are weak acids formed through microbial fermentation of carbohydrates (Lueck, 1980). Organic acids can affect the integrity of the microbial cell membrane and cause a bactericidal effect by changing the cytoplasmic pH of the cell and interfering with nutrient transport and energy metabolism (Davidson and Taylor et al., 2007). The antimicrobial effect of an acid depends on the dissociation constant (pKa) or pH, which is when 50% of the total acid is non-dissociated. The non-dissociated part of the acid can penetrate into cells, allowing for the antimicrobial effect (Davidson and Taylor, 2007).

Vaccines and Nanoparticle Polymer Delivery Systems

Current vaccines available against *Salmonella* in poultry include bacterins, attenuated, and subunit vaccines. Bacterins are vaccines consisting of killed vaccines and have had varying

results in effectiveness (Davison et al., 1999). Live, attenuated vaccines are often preferable because they are easy to administer and can induce mucosal, cellular, and humoral responses (Barrow, 2007). However, live attenuated *Salmonella* vaccines have also had varying results in protection in chickens. Lalsiamthara et al. (2016) observed an increase in mucosal, cellular, and humoral response in male Leghorn chickens that were administered a live attenuated *Salmonella* vaccine, biologically modified to secrete detoxified heat labile toxins as an adjuvant. In contrast, Groves at al. (2016) administered commercial live *Salmonella* vaccine to laying hens and found that the oral vaccine failed to exhibit lasting protection against *Salmonella* in the ceca. Additionally, live vaccines possess safety concerns for the well-being of the consumers (Zhang-Barber et al., 1999; Lauring et al., 2010).

An alternative to live vaccines are acellular vaccines, which contain immunodominant components of the bacteria. These components include lipopolysaccharide, outer-membrane proteins, flagellae epitopes, and fimbriae (Ochoa et al., 2007). Acellular vaccines vary in levels of protection, although low levels of resistance against *Salmonella* have been observed quite frequently (Tennant and Levine, 2015). However, when acellular components are used with adjuvants or delivery systems, results are more effective in protection against *Salmonella* (Ochoa et al., 2007).

Vaccine adjuvants and delivery systems enhance the immune system by stimulating humoral, cellular, and mucosal immune responses when administered with a vaccine (Tiwari et al., 2012). Nanoparticles are submicron-sized colloidal delivery systems that protect antigens against chemical, enzymatic or immunological degradation, thus facilitating targeting and

presentation of antigens to specific sites of the mucosal immune system (Tiwari et al., 2012). Delivery systems should induce minimal side effects in the animal, be biodegradable, economical, and simple to manufacture (Tiwari et al., 2012). In addition to protecting against enzymatic degradation (nucleases and proteases), nanoparticles can adhere to the mucosa and prolong its time in the gastrointestinal tract (Delgado et al., 1999). However, conventional nanoparticles have low site-specificity within the gastrointestinal tract. To improve site specificity, ligands that are able to bind to specific receptors within the intestine can be used in addition to the nanoparticle polymers. Examples of these ligands include lectin, invasins, and vitamin B12 derivatives (Harokopakis et al., 1998; Young et al., 1992).

Salman et al. (2005) designed "*Salmonella*-like" nanoparticles by attaching *Salmonella* Enteritidis flagellin to the nanoparticle vaccine. This was done to mimic the natural colonization ability of *Salmonella* Enteritidis in the gastrointestinal tract. Flagellin proteins conjugated to the surface of *Salmonella* nanoparticles successfully enhanced specific uptake of the vaccine antigens through mucosal tissues in mice (Salman et al., 2005).

The objectives of the present study are as follows:

Specific Aim 1

To study the effects of drinking water synbiotic supplementation on the anti-*Salmonella* immune response and cecal bacterial colonization in laying hens

Specific Aim 2

To study the effects of acidifier supplementation on the anti-*Salmonella* immune response and cecal bacterial colonization in laying hens

Specific Aim 3

To study the effects of a nanoparticle-based *Salmonella* vaccine on the anti-*Salmonella* immune response in layer chickens

Chapter 2: Review of Literature

Introduction

The foodborne illness salmonellosis is estimated to affect over one million people in the United States annually. Salmonellosis causes fever, abdominal pain, diarrhea, and vomiting. Each year, the disease in the United States results in approximately 19,000 hospitalizations and over 300 deaths (Fabrega and Vila, 2013). *Salmonella,* the bacterium accountable for salmonellosis infection, colonizes within the gastrointestinal tract of poultry and can be transferred to humans by consumption of contaminated poultry meat or eggs (Braden, 2006; Pires et al., 2014). Beginning in the 20th century, sub-therapeutic antibiotics were supplemented to poultry resulting in a significant decrease in poultry intestinal bacteria, thereby improving feed efficiency in the birds (Landers et al., 2012). However, due to the emergence of antibiotic-resistant bacteria, subclinical antibiotic-use is declining and alternative approaches for decreasing pathogenic bacteria are becoming increasingly prevalent. This review investigates the effects of synbiotics (probiotic plus prebiotic combination), organic acids, and *Salmonella* vaccines in enhancing the anti-*Salmonella* immune response in chickens.

Innate and Adaptive Immune Response- Brief Overview

The innate immune system forms the first line of defense against pathogens (Akira et al., 2006). Immune cells that make up the innate immune system include phagocytic cells such as heterophils, macrophages, and dendritic cells (Hansell et al., 2007). Phagocytic cells, also known

as “phagocytes”, engulf and destroy bacteria and viruses. Heterophils are also classified as granulocytes, as they contain granules that are toxic to bacteria (Genovese et al., 2013). Macrophage and dendritic cells are antigen presenting cells that bridge together the innate immune system and the adaptive immune system (Akira et al., 2006).

The immune cells of the innate immune system utilize a limited number of germ-line encoded pattern recognition receptors (PPRs), such as toll-like receptors that recognize pathogen-associated molecular patterns (PAMPs; Akira et al., 2006). The recognition of PAMPs by PRRs results in direct activation of immune cells to produce pro-inflammatory cytokines and chemokines (Akira et al., 2006). However, the innate immune system can fail to recognize pathogens and does not always completely eliminate infection (Janeway et al., 2001). The adaptive immune system provides increased protection against pathogens. Yet because the adaptive immune system can take four to seven days to respond, the role of the innate immune system is crucial for the initial defense of infection (Janeway et al., 2001).

Lymphocytes of the adaptive immune system respond to recognition of the antigen peptide, presented by the antigen presenting cell, by dividing into identical daughter cells. The daughter cells differentiate into effector cells (Janeway et al., 2001). Effector cells of B lymphocytes produce antibodies and are defined as plasma cells. Effector cells of T lymphocytes are comprised of either cytotoxic T lymphocytes, which kill infected cells, or helper T lymphocytes, which activate other immune cells. Helper T lymphocytes further mature into Th1 or Th2 cells. Induction of Th1 cells leads to a pro-inflammatory response, whereas Th2 cells involves a predominantly anti-inflammatory role (Alberts et al., 2002). Effector cells have a

limited life-span and go into apoptosis after the antigen is removed. The cells that do persist after the antigen has been eliminated are known as memory cells (Janeway et al., 2001). Memory cells play a major role in "immunological memory", which ensures a more rapid and effective response on the next encounter with the same antigen, thereby providing lasting protective immunity (Janeway et al., 2001).

Cytokines are proteins secreted by cells and are involved in cell signaling for regulation and activation of immune cells (Wigley and Kaiser, 2003). Interleukin-1ß (IL-1ß) is a highly inflammatory cytokine produced by mainly macrophages. IL-1ß stimulates T cells and the production of acute phase proteins (Corwin, 2000). IL-1ß production occurs at sites of infections where inflammatory response is induced, such as infections elicited by *S. enterica* and *Eimeria tennella* in chickens (Wigley and Kaiser, 2003). IFN-γ is a pro-inflammatory, anti-viral cytokine produced primarily by Th1 cells, leading to the activation of macrophages and increased MHC presentation transcription (Tizard, 2009). TNF-α (homologous to LITAF in chickens) is a potent inflammatory cytokine produced primarily by macrophages and T cells (Corwin, 2000; Hong et al., 2006). IL-10 is a major anti-inflammatory cytokine that inhibits cytokine production by Th2 cells and down-regulates transcription of major histocompatibility antigens in immune cells (Corwin, 2000). Cytokines such as IL-4 and IL-13 have additional anti-inflammatory properties. IL-4 and IL-13 activates the Th2 response, and stimulates proliferation and differentiation of B cells, resulting in increased antibody production (Corwin, 2000).

Salmonella

Salmonella is an intracellular, Gram-negative, non-spore-forming, rod-shaped bacterium from the family *Enterobacteriaceae. Salmonellae* are motile, facultative anaerobic bacilli, and have an optimal growth temperature between 8 to 45 ℃ in a pH range of 4 to 9 (Guthrie, 1992). *Salmonellae* have diameters of 0.7 to 1.5 μm, lengths from 2 to 5 μm, and multiple flagella that surround the entire cell body (Coburn et al., 2007). They are heat-sensitive and can be killed at temperatures of 70℃ and above. However, *Salmonella* can also be very robust and has been shown to survive in dust and dirt for longer than two years (Davies and Wray, 1996). *Salmonella* can be divided into two species, which are *Salmonella bongori* and *Salmonella enterica. Salmonella enterica* can further be divided into six subspecies. These subspecies are*: S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) (Lan et al., 2009).

Salmonella isolates are described most commonly by its serotype name (Foley and Lynne, 2008). Serotyping is based on the identification of O (somatic) and H (flagellar) antigens (Brenner et al., 2000). The somatic O antigen is the oligosaccharide component of the liposaccharide, located on the outer membrane of *Salmonella*, whereas the flagellar H antigens are found in the bacteria's flagella (Eng et al., 2015). *Salmonella enterica* serotype Enteritidis is one of the most commonly isolated serovars of *Salmonella* (Tarabees et al., 2017). *Salmonella*

Enteritidis have few clinical effects in poultry but can lead to the disease salmonellosis when spread to humans (Tarabees et al., 2017).

Salmonellosis affects an estimated 1.4 million people in the United States and more than 90 million people worldwide annually (Braden et al., 2006; Majowicz et al., 2010). Symptoms for salmonellosis include fever, diarrhea, and even death in immunocompromised humans. Furthermore, salmonellosis can occur through many disease symptoms including gastroenteritis, bacteremia, and typhoid fever (Darwin and Miller, 1999). The incubation period for developing salmonellosis is 6 to 72 hours after ingestion of the bacteria (Guthrie, 1992). Fever of 38 to 39 °C is common, accompanied by an initial chill. Abdominal pain frequently occurs with salmonellosis, causing mild to severe discomfort. In uncomplicated cases of salmonellosis, symptoms can be resolved within 48 hours. However, the illness can occasionally last for 10 to 14 days, alongside with persistent diarrhea and low-grade fever (Guthrie, 1992).

Salmonella is commonly spread to humans by chicken intestinal contents spilling onto the meat or from egg shell contamination (Braden, 2006; Pires et al., 2014). There are two routes of egg contamination by *Salmonell*a Enteritidis. One route is through horizontal transmission; eggs can be contaminated by penetration through eggshell from the colonized chicken intestine or from contaminated feces during or after oviposition (de Reu et al., 2006). The other route is through vertical transmission, such as direct contamination of the yolk, albumen, eggshell membranes or eggshells before oviposition, originating from the infection of reproductive organs with *Salmonella* Enteritidis (Okamura et al., 2001). The United States and European Union have developed egg quality assurance programs (EQAPs), which outlines specific guidelines for

reducing *Salmonella* Enteritidis contamination of shell eggs (Denagamage et al., 2017). The EQAPS outlines risk factors associated with *Salmonella,* including lack of cleaning and disinfection, previous *Salmonella* infection in chickens, presence of rodents, flock sizes greater in cage houses with greater than 20,000 birds compared to cage houses with less than 20,000 birds, and unvaccinated flocks (Denagamage et al., 2017).

Immune response to *Salmonella*

Salmonella enters the host through the oral route and colonizes within the gastrointestinal tract. Attachment of *S.* Enteritidis to the intestinal epithelial cells is the first step of successful *S.* Enteritidis colonization and is mediated by a group of proteins known as adhesins (Beachey, 1981). Blocking intestinal adhesion of *S.* Enteritidis to intestinal epithelial cell receptors may be the most effective strategy to prevent bacterial infection (Wizemann et al., 1999).

The cellular component of the innate system in chickens, which includes macrophages and heterophils, is crucial in protecting against intestinal infection (Fasina et al., 2010; Van Immerseel et al., 2002). When intestinal microorganisms such as *Salmonella* invade the intestinal epithelial barrier, innate immune cells are recruited to the site of infection, where they kill pathogens using mechanisms such as phagocytosis and oxidative burst (Brisbin et al., 2008). The greatest population of *Salmonella* within the intestines are in the ceca, two pouches connected to the junction between the ileum and colon (Sivula et al., 2008). The lamina propria is the thin layer of connective tissue, rich in lymphocytes, residing directly under the epithelial cell lining of the intestine (Boudry et al., 2004). Immunohistochemical analysis of the caecal lamina propria revealed that heterophils infiltrate the caecal lamina propria from 12 h post *Salmonella*

inoculation (Van Immerseel et al., 2002). *Salmonella* colonization in chickens involves an influx of inflammatory cytokines including LITAF, IL-1ß, and IFN- γ (Sheela et al., 2003; Matulova et al., 2013). Although the exact mechanism for *Salmonella* to colonize and withstand clearance from host immune cells within the gastrointestinal tract is unclear, it is likely partly due to the ability of *Salmonella* to manipulate the host defense system by increasing the suppressive cytokine, IL-10 (Ghebremicael et al., 2008).

The adaptive immune response to *Salmonella* infection in chickens includes both humoral and cell-mediated responses. Van Immerseel et al. (2002) reported an increase in T-lymphocyte infiltration in the cecal lamina propria from 20 h post-*S.* Enteritidis inoculation in three-day old chicks. Berndt et al. (2007) additionally detected rapid increases in cecal T-cell populations after inoculation of *S.* Enteritidis in day-old chicks. B-lymphocytes were reported in the cecal lamina propria from two d post-*S.* Enteritidis inoculation in chicks (Van Immerseel et al., 2002).

The mucosal immune system, which includes mucosal immunoglobulin A (IgA) and mucosa-associated lymphocytes and leukocytes, forms the first adaptive immune defense against *S.* Enteritidis infection. Secretory IgA limits mucosal colonization of *S.* Enteritidis by preventing adherence of the bacteria to intestinal epithelial cells (Shroff et al., 1995). Desmidt et al. (1997) reported that the administration of *Salmonella* to day-old chickens elicited LPS-specific antibody responses starting from 18 d post-infection. Sheela et al. (2003) reported increased levels of *S.* Enteritidis-specific mucosal IgA and serum *S.* Enteritidis-specific IgG titers in chickens inoculated with live *S.* Enteritidis compared to the control. Immune suppression of B cells

correlates with an increase of *S*. Enteritidis intestinal shedding rate in chickens at 5 wk of age (Arnold and Holt, 1995).

Intestinal morphology is an important indicator of intestinal health. Increased villus height and decreased crypt depth correlates with increased absorption of nutrients within the small intestine due to increased surface area and reduced tissue turnover rate (Munyaka et al., 2012). Borsoi et al. (2011) reported lower cecal villus height:crypt depth ratios in broilers from 0 to 3 d post-*Salmonella* inoculation compared to the control group. Fasina et al. (2010) also observed that chicks challenged with *Salmonella* Typhimurium had decreased jejunal villus height, crypt depth, and height:crypt depth ratio compared to unchallenged chicks. Reductions in villi height:crypt depth may be caused by reduced cell migration and proliferation rates and increased rates of cell loss (Ferraris and Carey, 2000).

Salmonella vaccines and Nanoparticle Delivery Systems

Salmonella vaccines are commonly utilized to decrease *Salmonella* infection in chickens. The efficacy of vaccines is determined by the level of intestinal and systemic colonization of the pathogen, and the morbidity and mortality rates of the birds after vaccination and subsequent experimental infection (Barrow, 2007). Vaccine efficacy can be affected by the challenge strain, route of administration, infection dose, age of birds, and species or line of birds. Therefore, it can be difficult to compare the efficacy of vaccine preparations that are currently available (Barrow, 2007).

Current vaccines that are available against *Salmonella* include bacterins, attenuated, and subunit vaccines. Bacterins consist of killed whole bacteria and have had varying results in

protection against *Salmonella* (Davison et al., 1999). Liu et al. (2001) reported that vaccinating chickens at 2 wk of age with formalin-inactivated *S.* Enteritidis encapsulated in biodegradable microspheres resulted in decreased S. Enteritidis fecal shedding and organ colonization after challenging the birds with 10^9 CFU of *Salmonella* at 6 wk of age. Miyamoto et al. (1999) reported that vaccinating chickens with an oil-emulsion bacterin of S. Enteritidis at 38 weeks (booster 4 weeks later) reduced S. Enteritidis shedding and colonization in the ovary and spleen (Miyamoto et al., 1999). However, other studies using bacterins without adjuvants have shown no effects in decreasing *Salmonella* colonization. Davison et al. (1999) reported that layer flocks vaccinated subcutaneously with *S.* Enteritidis bacterins between 14 to 20 wk of age showed no differences in percentage of positive samples with *Salmonella* compared to the control flock. Berghaus et al. (2011) studied the effects of killed *Salmonella* vaccine administration on commercial chicken breeding farms and found that although vaccinated birds had higher antibody titers, vaccine administration did not significantly decrease *Salmonella* in the environment.

Live attenuated (weakened) vaccines are often preferable to bacterins because they are easy to administer and can induce mucosal, cellular, and humoral responses (Lalsiamthara et al., 2016). Live attenuated *Salmonella* vaccines undergo attenuation through negative mutations of essential enzymes, resulting in prolonged generation times and reduction in virulence (Linde et al., 1998). When modifying live vaccine strains, it is essential to aim at reducing the risk of spread or persistence of the strain in the environment while also inducing an adaptive immune response. Genes that have been mutated for vaccine attenuation include ompR (regulation of

expression of outer membrane proteins) and galE (involved in the biosynthesis of bacteria lipopolysaccharide) (Kong et al., 2011).

Attenuated *S.* Enteritidis vaccine orally inoculated (10^9 CFU) in chickens at 1, 2, 3, and 7 d post-hatch resulted in fewer challenge bacteria in the cecal contents, liver, and spleen 14 d post-*Salmonella* challenge (Cerquetti and Gherardi, 2000). In contrast, Groves at al. (2016) administered commercial oral live *Salmonella* vaccine to laying hens and found that the vaccine failed to exhibit lasting protection against *Salmonella* in the ceca. Live vaccines additionally possess safety concerns for the well-being of the consumers (Zhang-Barber et al., 1999, Lauring et al., 2010).

Acellular or subunit vaccines may offer a more effective alternative to bacterins and live attenuated vaccines (Sharma and Hinds, 2012). Acellular vaccines consist of immunogenic components or antigens of the bacteria. *Salmonella* antigens were identified using electrophoresis, after measuring antigens that stimulated strong B and T lymphocyte responses in immunoblot and western blot assays (Vordermeier and Kotlarski, 1990). These components include lipopolysaccharides, outer-membrane proteins, flagellae epitopes, and fimbriae (Ochoa et al., 2007). Acellular vaccines vary in levels of protection, although low levels of resistance against salmonellosis have been observed quite frequently (Tennant and Levine, 2015). However, acellular/subunit vaccines consisting of outer membrane proteins have successfully been used to decrease *S.* Enteritidis infection in poultry. Khan et al. (2003) subcutaneously vaccinated 9 week-old chickens with two outer membrane proteins, followed by two boost vaccinations with time intervals of 2 weeks. Vaccination of either of the outer membrane

proteins decreased *Salmonella* colonization in the ceaca at 48 hours post-*Salmonella* challenge with $8x10^4$ CFUs of S. Enteritidis (Khan et al., 2003).

When developing an effective vaccine, it is crucial to understand the interactions between the *Salmonella* and the host. As discussed earlier, the *Salmonella* genus can be divided into two major groups. One group includes serovars that produce systemic disease, but are rarely involved in human food poisoning. The other group of serovars produces food-poisoning and only produces systemic disease in immunocompromised animals (for example, in the very young or old animals) (Barrow, 2007). *Salmonella* vaccines that prevent food-poisoning are much less successful compared to serotypes that produce "typhoid-like" diseases (Barrow, 2007). Most studies analyzing *Salmonella* pathogenesis have been based on experimental infection in mice with *Salmonella* Typhimurium, resulting in systemic infection. However, a model studying the local intestinal effects of *Salmonella* is not as clear (Barrow, 2007).

Vaccine adjuvants and delivery systems enhance the immune system by stimulating humoral, cellular, and mucosal immune responses. Successful delivery systems have minimal side effects in the animal and are biodegradable, cost-efficient, and simple to manufacture (Tiwari et al., 2012). Nanoparticles are submicron-sized co-polymer delivery systems that protect antigens against chemical, enzymatic or immunological degradation, thus facilitating targeting and presentation of antigens to specific sites of the mucosal immune system (Tiwari et al., 2012). Nanoparticles are receiving attention as delivery vehicles for vaccine antigens, as they have the ability to both stabilize the vaccine antigens and act as adjuvants (Gregory et al., 2013). These properties especially make nanoparticles suitable candidates as orally administered vaccines.

Nanoparticle characteristics impact the type of immune response depending on the nanoparticle size, composition, shape, and charge. The nanoparticle shape can affect the ability of macrophages to internalize the nanoparticles by manipulating the actin-driven movement of the macrophage membrane. Spherical particles have been well-studied due to its efficient uptake and homogenous antigen-loading and antigen-releasing properties (Richards and Endres, 2016). However, computer stimulations of nanoparticles have predicted that rod-shaped particles may be more efficiently endocytosed than spherical particles, as they often represent more biologically-relevant shapes such as bacteria and dividing cells (Richard and Endres, 2016). In general, cationic particles are taken up into cells more readily than those with an overall negative surface charge due to the anionic nature of cell membranes (Foged et al., 2005). The delivery of antigens to dendritic cells is central to the development of a protective immune response. The efficiency of antigen uptake into dendritic cells using nanoparticles with antigens is significantly increased compared to vaccinating with the antigens alone (Uto et al., 2011).

The vaccine antigen is either encapsulated within or attached to the surface of the nanoparticle. Encapsulating the nanoparticle helps prevent the antigen from rapidly degrading upon administration to the host (Delgado et al., 1999). Conjugation of the antigens onto the nanoparticle surface can additionally target the vaccine to specific immune sites within the gastrointestinal tract. Examples of antigens that can be conjugated onto the nanoparticle surface include lectins, invasins, flagellin proteins, and vitamin B12 derivatives (Young et al., 1992; Salman et al., 2005). Salman et al. (2005) designed "*Salmonella*-like" nanoparticles by conjugating *Salmonella* Enteritidis flagellin to the surface of the nanoparticle vaccine. This was

done to mimic the natural colonization ability of *S.* Enteritidis in the gastrointestinal tract. Salman et al. (2005) demonstrated that the flagellin ligands associated with nanoparticles successfully enhanced specific uptake of mucosal tissues, including Peyer's patches. Peyer's patches are aggregates of immune cells, including macrophages, dendritic cells, and T and B lymphocytes, located throughout the intestines, beneath the epithelial cellular layer (Jung et al., 2010).

Salman et al. (2005) designed the "*Salmonella*-like" nanoparticles by using a biocompatible copolymer between poly methyl vinyl ether and maleic anhydride for the nanoparticle coating. Maleic anhydride co-polymers react strongly with primary amines and proteins, forming stable amide bonds able to withstand highly acidic conditions (Schmidt et al., 2003). Nanoparticles were prepared by the solvent displacement method and then cross-linking by 1,3-diaminopropane for further stabilization of the nanoparticles to resist the acidity and enzymes within the gastrointestinal tract (Salman et al., 2005). Salman et al. (2009) demonstrated that oral *Salmonella* nanoparticle vaccine administration in mice resulted in increased *Salmonella*-specific humoral systemic and mucosal immune responses beginning at four wk post-vaccination.

In addition to protecting against enzymatic degradation (nucleases and proteases), nanoparticles can adhere to the mucosal layer and prolong its time in the gastrointestinal tract (Delgado et al., 1999). The basic colloidal and degradation properties of the nanoparticle depend on its copolymer composition. Several polymers can degrade by hydrolysis, including esters, anhydrides, acetyls, carbonates, and amides (Carrillo-Conde et al., 2010). The biodegradable

properties of polymeric nanoparticles are advantageous for delivering both drugs and vaccines within the host (Li et al., 2001). The release kinetics of loaded drugs or vaccine antigens from the polymeric nanoparticles can be controlled by compositional changes to the copolymer (Li et al., 2001).

Polymeric nanoparticles can be prepared from a variety of polymers including poly(a-hydroxy acids), poly(amino acids)s, and polysaccharides. The most commonly used poly(a-hydroxy acids) for preparing polymeric nanoparticles are poly(lactic-co-glycolic acids) (PLGA) or poly(lactic acid) PLA. Poly(lactic-co-glycolic acids) and PLA nanoparticles are synthesized using a double emulsion-solvent evaporation technique (Sahoo et al., 2002). For the preparation, the polymer is dissolved in an organic solvent such as ethyl acetate or methylene chloride. Next, the antigen is added to the solution and vortexed to get a primary emulsion. Emulsifying agents such as polyvinyl alcohol or polyvinylpyrrolidine are then added, forming a water-in-oil-in-water emulsion. As a result, the polymer precipitates around the antigen. The solvent is then left to evaporate and dried to prevent degradation of the polymer, due to water-catalyzed ester hydrolysis (Florindo et al., 2010). This method, however, results in low antigen entrapment and the possibility of antigen denaturation. The addition of stabilizers such as surfactants or sugars (such as trehalose and sucrose) can provide stability against denaturation by keeping the antigen hydrated in its native form (Brogan and Hallett, 2016).

An alternative method for encapsulating and stabilizing the antigens uses poly(amino acids) such as poly(γ -glutamic acid) (γ -PGA), poly(E-lysine), poly(L-arginine), or poly(L-histine) which does not require an emulsion step in their synthesis (Holowka et al., 2007). The

amphiphilic copolymers arrange themselves through hydrophobic interactions, forming polymeric structures consisting of a hydrophobic core and hydrophilic outer shell (Lu et al., 2009). γ -linked glutamic acids in y-PGA are not easily recognized by common proteases and therefore have additional stability (Obst and Steinbuchel, 2004).

Polyanhydrides are polymers that can minimize exposure of vaccine antigens to moisture and can retain the vaccine antigens' structure and immunogenicity (Determan et al., 2004). In addition, polyanhydride nanoparticles have been shown to activate dendritic cells and enhance the innate immune response through upregulation of toll-like receptors such at TLR-4 and TLR-2 (Petersen et al., 2011). Polyanhydrides contain two carbonyl groups bound together by an ether bond (Petersen et al., 2011). Originally developed as textile fibers in the 1930s, polyanhydrides began to be researched for biomedical use in the 1980s (Rosen et al., 1983). Polyanhydrides were approved by the FDA as drug delivery vehicles in 1996 (Katti et al., 2002). The degradation of the anhydride bond is highly dependent on its polymer backbone chemistry. The degradation rate can vary by over six orders of magnitude depending on its backbone chemistry (Katti et al., 2002). Polyanhydrides have been used for the delivery of chemotherapeutics, antibiotics, vaccines, and proteins (Agueros et al., 2009; Brin et al., 2009; Salman et al., 2005; Sun et al., 2009).

For successful nanoparticle vaccine preparation, it is essential to characterize the structure and composition of the nanoparticle formulations to avoid any variation between or within the batches (Gregory et al., 2013). Variations could arise from contamination, heterogeneous size distribution of the particles, the accumulation of toxic components, or

incomplete particle formation. Spatial uniformity is crucial for ensuring homogenous antigen loading efficiency between each nanoparticle. The antigen dose of the vaccine could vary if the nanoparticles are not homogenous (Gregory et al., 2013). The size and shape of the particles are characterized using a variety of methods including electron microscopy, dynamic light scattering, and density gradient centrifugation (Gregory et al., 2013).

Interactions between Chicken Gastrointestinal Tract and lntestinal Microflora

Advances in technology have allowed for precise analyses of bacterial populations in chickens. These advances encompass sequencing of 16s rRNA genes by first the Sanger sequencing technology and, more recently, next-generation sequencing (NGS) (Pan and Yu, 2014). Beginning in the 1970s, Sanger sequencing method was the first to give scientists the ability to sequence DNA. Next-generation sequencing, developed several decades later, uses high throughput sequencing to analyze samples in a much more cost efficient and timely manner (Liu et al., 2012). Research has used these techniques to demonstrate that extensive interactions occur between the poultry host and its intestinal microbiome. These interactions occur through exchange of nutrients, modulation of host gut morphology, physiology, and immunity (Pan and Yu, 2014).

The gastrointestinal tract of poultry consists of the esophagus, crop, proventriculus, gizzard, and small intestine (duodenum, jejunum and ileum) (Pan and Yu, 2014). Within the intestinal tract there is a diverse and critical microbial ecosystem. The ceca are two pouches attached to the small intestines that have the slowest passage rate, and as a result is the region containing the most populated and diverse intestinal bacteria (Pan and Yu, 2014). The three most

abundant phyla in the intestines are the Bacteroidetes (gram-negative), Firmicutes (gram-positive) and Actinobacteria (gram-positive). The bacteroidetes produce acetate and propionates, whereas the Firmicutes produce mainly butyrate (Pan and Yu, 2014).

The major bacterial metabolic routes are the Embden-Meyerhof-Parnas pathway and the pentose-phosphate pathway, both of which converts monosaccharides into the metabolite phosphoenolpyruvate (PEP) (Miller and Wolin, 1996). The Embden-Meyerhof-Parnas pathway, known as glycolysis, metabolizes glucose to generate the main products, ATP, NADH, and pyruvate (Miller and Wolin, 1996). The pentose-phosphate pathway has two main steps; the first is oxidizing glucose to generate NADPH, and the second is the synthesis of five-carbon sugars (Miller and Wolin, 1996). The metabolite produced from both these pathways, PEP, is converted into fermentation products such as SCFAs (organic acids) or alcohols (Miller and Wolin, 1996). The SCFAs can be absorbed by the host and used for energy. Fermentation of sugars into SCFAs can take place from the crop to the cecum, but occurs mostly in cecum, due to having the highest density of bacteria (Van Der Wielen et al., 2000).

Although nutrient exchange between the intestinal bacteria and their host can be mutually beneficial, intestinal bacteria can also compete with the host for nutrients. This competition can especially occur during dysbiosis of the intestinal microbiota. For example, some bacteria have the ability to deconjugate bile acids, resulting in decreased lipid digestion by the host. Examples of these species include *Clostridium perfringens*, *Streptococci*, and some species of *Bifidobacteria* and *Lactobacilli* isolated from chickens (Miyata et al., 2011). However, few studies have analyzed the significance of the degree to which these bacteria can decrease lipid

digestion in chickens. Torok et al. (2011) utilized PCR to identify over 26 bacterial species that positively correlate with improved broiler performance, including *Lactobacillus salivarius*, *Lactobacillus aviarius*, and *Lactobacillus crispatus*, but further studies are needed to assess if these bacteria are the cause or consequence of improved feed utilization efficiency.

Intestinal microbiota can also affect intestinal digestive enzymes. Amylase and protease activities, as measured by digestive enzyme assays, were increased in broilers fed diets containing 4.0 g/kg of fructooligosaccharides (Xu et al., 2003). Xu et al. (2003) additionally observed that that 4.0 g/kg of fructooligosaccharide supplementation increased *Bifidobacterium* and *Lactobacillus* populations and decreased *E. coli* in the small intestine. *Bifidobacterium* and *Lactobacillus* stimulate digestive enzyme activity, whereas bacteria such as *E. coli* may impair digestive enzyme secretion (Xu et al., 2003).

The type of diet can additionally affect the intestinal microbiota. Diets containing high levels of indigestible, water-soluble, non-starch polysaccharides favor the proliferation of *C. perfringens*, predisposing young chicks to necrotic enteritis compared to corn-based diets (Annett et al., 2002). This may be because high intake of non-starch polysaccharides can increase digesta viscosity, decrease digesta passage rate, and a decrease nutrient digestibility, which in turn favors the growth of *C. perfringens* (Annett et al., 2002).

Sub-therapeutic Antibiotic Use

For more than 50 years, dietary antibiotics have been used at sub-therapeutic levels to improve feed efficiency and maintain animal health (Danzeisen et al., 2011). It is widely accepted that the feed efficiency effects of antibiotics occur through the modulation of the

intestinal microbiome. Adverse and pathogenic bacteria in the intestinal tract of chickens, such as *E. coli, Salmonella ssp.*, and *C. perfringens,* compete with the host for nutrients and may damage the intestinal epithelium, resulting in decreased digestion and absorption within the host (Adil et al., 2010). Sub-therapeutic antibiotics inhibit enteric pathogens, thus reducing the incidence of disease, which in turn promotes the growth of the birds (Danzeisen et al., 2011). However, due to concerns of antibiotic resistant bacteria, sub-therapeutic antibiotics are decreasing in use (Danzeisen et al., 2011).

Probiotics

Probiotics are defined by The World Health Organization as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Mack, 2005). Probiotics benefit the host through various mechanisms and can depend on the specific probiotic species and strain. These mechanisms include competitive exclusion (Lawley and Walker, 2013), production of bacteriostatic and bactericidal substances against pathogens, (Van Der Wielen et al., 2000), enhancement of intestinal mucosal barrier (Yang et al., 2012), and regulation of host immune response (Yang et al., 2012).

Daily supplementation of several species of *Lactobacilli* strains, including *Lactobacillus reuteri* and *Lactobacillus salivarius,* can decrease the population of *Salmonella* and *Campylobacter* in the intestine of chickens at 21 d of age (Nakphaichit et al., 2011). Daily dietary supplementation of *Bacillus subtilis* significantly decreased *E. coli* in the ileum of broiler chickens at 21 d of age (Molnar et al., 2011). Yang et al. (2012) found that daily supplementation of *C. butyricum* HJCB998, beginning at d of hatch, significantly decreased cecal *Salmonella* and

C. perfringens population while increasing *Lactobacillus* and *Bifidobacterium* populations in the cecum of broilers from 21 to 42 d of age. The protective effects of multispecies probiotics have also been investigated. Broilers supplemented daily, beginning at d of hatch, with a multispecies probiotic product containing *Enterococcus faecium, Bifidobacterium animalis, Pediococcus acidilactici, Lactobacillus salivaris* and *Lactobacillus reuteri*, decreased cecal coliform population (Mountzouris et al., 2010). Ghareeb et al. (2012) found that the multispecies probiotic containing *E. faecium, Bifidobacterium animalis, Pediococcus acidilactici, L. salivaris* and *L. reuteri* significantly reduced cecal colonization of C. *jejuni* in broiler chickens at 8 and 15 d post-challenge. The mode of supplementation can additionally affect bacteria colonization within the gastrointestinal tract. Several studies have reported greater efficacy of probiotics supplemented in water when compared to in-feed probiotics (Karimi Torshizi et al., 2010; Ritzi et al., 2014).

The phenomenon known as "competitive exclusion" within the intestinal tract is the competition between microorganisms for resources such as nutrients and attachment sites within the intestines (Nurmi et al., 1992). To cause infection in birds, pathogenic bacteria such as *E. coli* and *Salmonella* are required to first attach to the intestinal epithelial barrier (Lan et al., 2005). In healthy birds, commensal bacteria colonize the intestinal mucosa, forming a complex layer of bacteria covering the mucosal surface, thereby effectively blocking the attachment and subsequent colonization by invading pathogenic bacteria (Lan et al., 2005). Furthermore, some bacteria gain competitive advantages within the intestine by producing bacteriostatic or bactericidal substances that are hostile to competitors. Murry et al. (2004) demonstrated that

lactic acid bacteria, including strains from the genus *Lactobacillus,* ferment carbohydrates and produce lactic acid, which decreases environmental pH and inhibits pathogen growth including *E. coli, C. perfringens,* and *S.* Typhimurium *in vitro*. An *in vivo* study demonstrated a negative correlation between concentrations of SCFAs (acetate, propionate, and butyrate) and abundance of the family *Enterobacteriaceae,* including many pathogenic species such as *Salmonella* and *E. coli,* in the ceca of broilers (Van Der Wielen et al., 2000). It is proposed that in addition to lowering extracellular pH, SCFAs in non-dissociated form can diffuse freely across the pathogenic bacterial cell membrane into the cell where they dissociate, resulting in bactericidal and bacteriostatic effects on the pathogen (Van Der Wielen et al., 2000).

Many probiotic strains produce bacteriocins, which are a group of antimicrobials that can inhibit other bacteria (Dobson et al., 2012). Various strains of *Lactobacillus salivarius* isolated from the chicken intestinal tract produce bacteriocins which can inhibit both gram-negative and gram-positive bacteria such as *S.* Enteritidis and *C. jejuni* (Svetoch et al., 2011). Bacteriocins produced by strains of *Enterococcus faecium, Pediococcus pentosaceus* and *Bacillus subtilis,* isolated from broiler chickens, were reported to inhibit *C. perfringens* (Shin et al., 2008). Additionally, several strains of *E. faecium* produce bacteriocins against the oocysts of poultry *Eimeria* spp (Strompfova et al., 2010). Bacteriocin production is a frequently considered trait in selection of probiotics, due to its inhibitory effect on various adverse bacteria and pathogen.

Fermentation of carbohydrates by intestinal bacteria results in the production of SCFAs (Morrison and Preston, 2016). The SCFAs are important energy sources of intestinal epithelial cells and can affect host metabolism (den Besten et al., 2013), stimulate enterocyte growth and

proliferation (Blottiere et al., 2003), regulate mucin production (Willemsen, 2003), and regulate intestinal immune responses (Correa-Oliveira et al., 2016). Cook and Bird (1973) reported that intestinal villi are shorter in germ free birds or birds colonized with low loads of bacteria than in conventionally-raised birds. As the bird matures, fermentation increases. Van der Wielen et al. (2000) studied SCFA production in broilers and observed that cecal acetate, propionate and butyrate are undetectable in 1 d old broilers, whereas at 15 d of age, the cecal microflora stabilizes, and the SCFAs reach high concentrations and remains stable as well.

Additionally, probiotics stimulate the host immune system. Microorganisms such as probiotics help prime the host immune system by interacting with immune cells within the intestine (Kamada and Nunez, 2014). Several studies have demonstrated that the manipulation of the intestinal microbiota can influence the antibody-mediated immune response. For example, birds receiving probiotics containing *L. acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecali*s had higher systemic antibody response to sheep red blood cells compared to unsupplemented birds (Haghighi et al., 2006). The effects bacteria have on the immune response depends on the strain of bacteria used, the type of chicken (layer vs broiler), and the age of the chicken (Brisbin et al., 2008). It is speculated that probiotics stimulate the production of Th2 cytokines (e.g., IL-4 and IL-10), which may subsequently enhance the immune response mediated by antibodies (Haghighi et al., 2006). Haghighi et al. (2006) observed that broilers treated with *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis* had increased levels of serum IgG and IgM at 14 d of age.

There is an abundance of evidence that the intestinal microbiota affects cell-mediated response as well. Mwangi et al. (2010) reported significant differences in gut associated T cell differentiation in germ-free chickens compared to conventional-raised chickens. Brisbin et al. (2012) reported that various *Lactobacillus* species had the capacity to induce differential cytokine expression in T cells of chicken cecal tonsils which could contribute to intestinal homeostasis. The study also demonstrated that broiler chickens treated with probiotics containing *L. acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis* post-*Salmonella* challenge had a significant decrease in gene transcription of IL-12 and IFN- γ (Haghighi et al., 2006). In addition to pathogenic and probiotic species of bacteria, commensal bacteria may also affect the immune response. However, more studies are need to determine the importance of these commensal bacteria on the immune response in poultry.

Some poultry producers use competitive exclusion cultures to help newly hatched chicks rapidly establish a healthy gut microbiome. Competitive exclusion cultures are suspensions of intestinal contents obtained from healthy adult birds (McReynolds et al., 2007). The intestinal tract of a newly hatched chick is sterile, but is immediately colonized by microorganisms present in the surrounding environment (Brisbin et al., 2008). In the wild, the intestinal tract is rapidly colonized by bacteria, protecting the chick form pathogen invasion (Lutful, 2009). However, in the hatchery, the surrounding environment is relatively clean and has a different microbial community than that found in a healthy adult chicken's intestine (Dahiya et al., 2006). This may leave newly hatched chicks more vulnerable to disease, since enteric pathogens have a greater opportunity to attach to and breach the intestinal mucosal layer (Dahiya et al., 2006).

Administration of competitive exclusion cultures has decreased *Salmonella* and *C. perfringens* infection in new hatchlings (McReynolds et al., 2007).

To successfully colonize within the intestines, probiotics are required to withstand the low pH of the stomach and the bile acids in the intestine (Kailasapathy and Chin, 2000). Additionally, probiotics should effectively compete against pathogens for adhesion of the intestinal mucosa (Kailasapathy and Chin, 2000). Horizontal gene transfer is an additional concern that should be considered when using probiotics in chickens. Horizontal gene transfer is defined as "the non-genealogical transmission of genetic material from one organism to another" (Goldenfeld and Woese, 2007). The predominant commensal intestinal microorganisms usually possess certain traits which enable them to outcompete other bacteria; however, these traits may be acquired by pathogens via horizontal gene transfer, making those pathogens more competitive. Thus, horizontal gene transfer of antibiotic resistance genes can lead to wide spread antimicrobial resistance among adverse and pathogenic bacteria (Goldenfeld and Woese, 2007).

Lactobacillus reuteri

L. reuteri is a heterofermentative species of bacteria that can produce multiple products including carbon dioxide, ethanol, acetate, lactic acid, hydrogen peroxide, reuterin and retericylin (Yu et al., 2007). *L. reuteri* can inhibit the growth of many enteric pathogens including *E. coli, S.* Typhimurium, *Staphylococcus epidermidis, Staphylococcus aureus, Helicobacter pylori*, and rotavirus (Seo et al., 2010). *L. reuteri* are resistant to heat, low pH, and bile salts (Yu et al., 2007). *L. reuteri* possess mucus binding proteins on the surface of its cells, which facilitates adherence to intestinal epithelial cells (Mackenzie et al., 2010).

Cao et al. (2012) supplemented broiler chickens with 10^8 cfu/mL *L.* reuteri and reported reduced lesions in birds challenged with *Clostridium perfringens,* suggesting that *L. reuteri* can decrease necrotic enteritis infection. *L. reuteri* can modulate innate immune response through regulation of inflammatory cytokines. *L. reuteri* strains are either immunosupressive or immunostimulatory. *L. reuteri* has been found to decrease mRNA transcription of IL-1ß in the ileum in neonatal piglets (Liu et al., 2015). Furthermore, *L. reuteri* suppressed TNF-α production of lipopolysaccharide-activated monocytes and macrophages *in vitro* (Lin et al., 2008). Quinteiro-Filho et al. (2015) reported upregulation of inflammatory cytokines IL-1ß and IL-12 in chicken macrophages treated with *L. reuteri in vitro.* Analysis of 14 published trials of *L. reuteri* supplementation in infants and children found no safety concerns of its use (Urbanska and Szajewska, 2014).

Pediococcus acidilactici

P. acidilactici are facultative anaerobes that have ideal growth conditions with a pH of 6.2, overnight at 37 degrees celsius. *P. acidilactici* can also grow at higher temperatures up to 65 degrees celsius (Lin et al., 2006). *Pediococci* can inhibit growth of enteric pathogens through production of lactic acid and bacteriocins such as pediocins (Daeschel and Klaenhammer, 1985). Whereas most *Lactobacillus* and *Bifidobacterium* strains are sensitive to room temperature (and therefore sensitive to storage conditions), *Pediococci acidilactici* can survive much more variable temperatures (Lin et al., 2006). Furthermore, *Pediococci acidilactici* are less sensitive to acidic environments than *Lactobacillus* and *Bifidobacterium,* and are therefore more resistant to the low pH of the stomach (Lin et al., 2006).

Enterococcus faecium

E. faecium is a facultative anaerobe, Gram-positive, non-motile bacterium. The bacteria have pili and flagella. *E. faecium* can grow in temperatures ranging from 10 to 45 degrees Celsius and can survive in both basic and acidic environments. *E. faecium* produces bacteriocins, which are antibacterial peptides. *E. faecium* have been commonly used in fermenting foods such as cheese and vegetables, and is also gaining popularity as a feed additive for animals to inhibit growth of unwanted microbes (Kang and Lee, 2005).

Bifidobacterium animalis

B. animalis is a Gram-positive, anaerobic, rod-shaped bacterium that colonizes naturally in the intestines of chickens, rabbits and humans (Scardovi and Zani, 1974). Simpson et al. (2005) tested the survival of *Bifidobacterium* species to heat and oxygen and determined that *B. animalis* had the highest tolerance to both traits compared with *B. adolescentis, B. angulatum, B. longum,* and *B. gallinarium*. Oxygen and heat tolerance is especially important for surviving manufacturing and storing processes of probiotic feed additives. Meile et al. (1997) reported that the optimum growth temperature of *B. animalis* was from 42 to 49 degrees Celsius. *B. animalis* is also highly resistant to bile salts (Sanchez et al. 2007).

Prebiotics

Prebiotics are indigestible nutrients that can be selectively metabolized by beneficial intestinal bacteria (Cummings and Macfarlane, 2002). Ao and Choct (2013) observed that broilers supplemented with mannanoligosaccharides or fructooligosaccharides daily for 35 d had increased body weight and increased feed conversion ratios compared to the control. Shang et al.

(2015) analyzed the effects of dietary fructooligosaccharide supplementation on performance, intestinal morphology, and immune responses in broilers challenged with *Salmonella* Enteritidis lipopolysacharides and found that fructooligosaccharide supplementation increased ileal mucosa thickness and elevated the transcription of ileal IL-1ß, IL-10, and interferon (IFN)-γ mRNA. Additionally, fructooligosaccharide supplementation resulted in increased leukocyte counts and serum IgY levels in response to LPS challenge (Shang et al., 2015).

Fructooligosaccharides, one of the most common types of prebiotics, consist of short-chain and non-digestible carbohydrates (Gibson and Roberfroid, 1995). Previous studies have shown that fructooligosaccharide supplementation can improve growth performance, enhance innate and adaptive immune response, increase small intestinal villi length, and increase beneficial intestinal bacteria colonization in broiler chickens (Xu et al., 2003). Fructooligosaccharide supplementation shifts intestinal microbiota towards more beneficial bacteria, thus increasing production of short chain fatty acids and increasing anti-*Salmonella* immune response (Shang et al., 2015).

Studies analyzing fructooligosaccharide effect on body weight and feed conversion in chickens have had differing results. Kim et al. (2011) observed that 0.5% fructooligosaccharide-supplemented chickens did not differ in feed conversion of body weight gain compared to control group. Yet other studies reported an increase in body weight gain and feed conversion in broilers supplemented with 0.25% to 0.5% fructooligosaccharides compared to the control (Xu et al., 2003; Shang et al., 2015). Factors affecting the differing body weight and feed conversion

results may include age, sex, health status of the birds, environmental hygiene and inclusion level of the fructooligosaccharide (Shang et al., 2015).

Synbiotics

Synbiotics are feed additives containing probiotics and prebiotics combined (Pandey et al., 2015). The combination of probiotics and prebiotics is theorized to act in synergy in improving animal health (Pandey et al., 2015). The prebiotic acts as a nutrient source for the probiotic, resulting in increased survival of the probiotic in the intestinal tract (Pandey et al., 2015). Several studies have found that synbiotics are more efficient than probiotics and prebiotics used separately. Fukata et al. (1999) found that the combination of competitive exclusion production and 0.1% fructooligosaccharide was more effective in decreasing intestinal *Salmonella* Enteritidis colonization in one-day-old and seven-day-old chicks than when used separately. Radu-Rusu et al. (2010) supplemented laying hens with a synbiotic product containing *E. Faecium* and fructooligosaccharide and found significantly higher egg production and egg shell quality compared to the control at 57 wk of age.

Organic Acids

Organic acids are short chain fatty acids (C1-C7) formed by microbial fermentation of carbohydrates within the intestine (Lueck, 1980). Organic acids have been used for decades to decontaminate beef, pork, and poultry products from bacteria such as *Salmonella* and *E. coli*. Organic acid treatment on meat has shown to be cost effective, quick, and efficient (Lueck, 1980). Organic acids are considered weak acids. Weak acids dissociate in water in a pH-dependent manner. The antimicrobial activity of organic acids increases as the pH of its

surrounding lowers to that of, or below the pKa of the acid (Skrivanová and Marounek, 2007). The pKa is defined as the acid dissociation constant. A decrease in the pH of its surrounding results in increased protonated acid, thus decreasing the polarity of the acid molecule, resulting in increased diffusion of the acid against the bacterial membrane and into the cytoplasm (Davidson and Taylor, 2007).

Organic acids affect microbial activity by two mechanisms. Organic acids decrease the pH of the bacterial cytoplasm, resulting in uncoupling of energy production and regulation. Additionally, the dissociated acid anions accumulate to toxic levels in the cytoplasm (Davidson and Taylor, 2007). The diffusion of a non-dissociated acid through a microbial membrane where the pH of the cytoplasm is higher than the environment establishes a transmembrane gradient (Warnecke and Gill, 2005). As protonated acid diffuses across the membrane, an alkaline environment is encountered, causing the acid to dissociation into the acid anion and free proton (Eklund, 1983). The bacteria cell then works to expel the protons by exchanging the protons for cations (such as Na+ or K+). This is known as the chemiosmotic theory (Mitchell and Moyle, 1969). It has been proposed that the microbial membrane is impermeable to protons, requiring active transport to efflux protons and maintain pH homeostasis in the cellular interior (Hirshfield et al., 2003). Russell (1992) reported that the accumulation of anions hinders the proton motive force, resulting in the cell's inability to extrude protons across the membrane. Consequently, the cell is unable to re-alkalinize the cytoplasm. Additionally, protein, DNA, and RNA synthesis are adversely affected by increased pH levels in the cytoplasm (Cherrington et al., 1990). Recent

research has suggested that an interplay of multiple mechanisms likely leads to the inhibition of bacteria by organic acids (Koczon, 2009).

Byrd et al. (2001) reported that the addition of 0.5% acetic, lactic, or formic acid in drinking water decreased *Salmonella* Typhimurium in the crop compared to the control groups. In addition to decreased pathogenic bacterial colonization, many studies have reported enhanced performance in animals supplemented with organic acids. Organic acids can lower gastric pH, resulting in increased proteolytic enzyme activity, improved protein digestibility, and inhibition of pathogenic bacteria (Ragaa and Korany, 2016). Fascina et al. (2012) reported improved weight gain, feed intake and feed conversion ratio in broilers supplemented with a mixture of benzoic, acetic and formic acids. Dahiya et al. (2016) reported that supplementation of organic acid in salt form (0.5% of sodium propionate or calcium propionate) improved egg production, egg weight, and FCR in laying hens.

Ahmed et al. (2014) found that citric acid supplementation in piglets challenged with *S.* Typhimurium and *E. coli* had higher average daily gain than unsupplemented groups. Additionally, citric acid supplementation decreased pathogenic bacteria, increased *Lactobacillus,* and increased serum IgG concentrations (Ahmed et al., 2014). IgM and IgA concentrations were not significantly affected (Ahmed et al., 2014). Lower intestinal pH produces a more favorable environment for *Lactobacilli* (Fuller, 1989). It is possible that the decrease in pathogenic bacteria may be the result of beneficial bacteria blocking intestinal receptors and secreting antimicrobial metabolites (Fuller, 1989).

The effects of organic acids on the immune system remains unclear, although there is an abundance of evidence that organic acids can modulate intestinal microflora, which is necessary for the development of the immune system (Khan et al., 2016). It is therefore likely that organic acids stimulate the immune system by modulating the composition of the intestinal microbiota and decreasing pathogenic bacteria (Van der Wielen et al., 2000). Namkung et al. (2004) reported higher plasma IgG titers in unchallenged pigs supplemented with organic acids compared to unchallenged pigs supplemented with antibiotics at 14 d post-weaning. There were no differences between cytokines IL-1β and TNF-α. Lee et al. (2017) fed day old broilers with either a control diet, organic acid blend, containing lactic, citric and formic acids, then vaccinated birds with H9N2 vaccine. Broilers fed organic acids had increased regulatory T cells and decreased H9N2-specific antibodies compared to unsupplemented broilers at 14 d of age (Lee et al., 2017).

Acetic Acid

Acetic acid is a monocarboxylic acid with a pKa value of 4.76. It is a principal component of vinegars and is highly soluble in water. However, acetic acid has a pungent odor and taste which often limits its use in foods. Acetic acid is generally regarded as safe for miscellaneous and general-purpose usage. Alvarez-Ordonez et al. (2010) compared growing *Salmonella* in a culture medium with a pH of 4.25 with acetic, citric, lactic, and hydrochloric acids and reported that acetic acid had the highest antimicrobial activity (followed by lactic, citric and hydrochloric acid). Zhou et al. (2007) reported a synergistic effect of acetic acid (0.10%) and essential oils thymol and carvacrol (100ul/l each) for *in vivo* inhibition of *S.*

Typhimurium. Acetic acids come in several derivatives for use as antimicrobial agents. The sodium and calcium salt forms, despite having different handling and utilization procedures as the acid, have similar antimicrobial properties as acetic acid at the same pH values (Hoffman et al., 1939).

Lactic Acid

Lactic acid (2-hydroypropanoic acid) is a monocarboxylic acid with a pKa of about 3.8. It is produced during anaerobic respiration by fermentation of several bacteria, most predominately lactic acid bacteria (Axelsson, 1998). Lactic acid occurs in two isomeric forms (D-, L-). The L isomer has been reported to be more effective in inhibiting pathogens (Leitch and Steward, 2002). Genera of bacteria the produce lactic acid include *Lactobacillus, Bifidobacterium, Streptococcus,* and *Pediococcus* (Kim et al., 2005).

Propionic Acid

Propionic acid and its salts, calcium and sodium propionate, are approved as generally recognized as safe substances for miscellaneous and general purpose usage. Propionic acid has a pKa of 4.88 (Serjeant and Dempsey, 1979). Goepfert and Chung (1970) observed that propionic acid at pH 5.5 was more effective in inhibiting *Salmonella* than acetic, succinic, lactic, fumaric, and citric acids. Cherrington et al. (1990) observed that at a concentration of 5 mM, proprionic acid decreased the rate of RNA, DNA, protein, lipid, and cell-wall synthesis of *Salmonella.*

***Salmonella* Resistance to Organic Acids**

Many studies have identified the benefits of organic acids in inhibiting or decreasing *Salmonella* counts in poultry meat and eggs. The bactericidal or bacteriostatic effects of these

weak acids depends on the concentration, pH of the environment, and the dissociation constant of the acids. Non-optimal acid treatments risk the development of adapted or resistant strains of pathogenic bacteria (Foster and Hall, 1991). *Salmonella* has the ability to adjust to acidic environments and survive in extreme conditions of pH (Foster and Hall, 1991). *Salmonella's* acid tolerance response can protect against the effects of organic acids especially at low pH values. The inducible acid tolerance response is a two stage process involving overlapping acid protection systems that are triggered at different levels of acidity. The first stage occurs with encounters with external pH 6. The second stage (post-acid shock) is triggered when the external pH falls below 4.5. During this stage, about 50 of the bacterium's acid shock proteins are activated to repair cell damage (Foster and Spector, 1995). *Salmonella* requires different signals to induce the synthesis of acid shock proteins among both internal and external pH (Foster and Spector, 1995). Several inducible amino acid decarboxylases contribute to the *Salmonella* acid induced tolerance response, which subsequently contributes to pH internal maintenance. These decarboxylases include lysine decarboxylase, lysine cadaverine antiporter, and regulatory proteins such as Rpos, Fur and PhoP (Foster and Spector, 1995).

Cinnamaldehyde

Cinnamaldehyde is an aldehyde present in the bark of cinnamon trees that gives cinnamon its flavor and odor (de Cássia da Silveira e Sá et al., 2014). Cinnamaldehyde has anti-fungal, anti-inflammatory, and anti-bacterial properties (de Cássia da Silveira e Sá et al., 2014). Cinnamaldehyde possesses antimicrobial activity against both gram-positive and gram-negative bacteria. Gill and Holley (2004) reported bactericidal effects of 30 mM cinnamaldehyde against

the pathogenic gram-positive *Listeria monocytogenes* and no significant effects on *Lactobacillus sakei in vitro.* Additionally, they found that cinnamaldehyde prevented an increase of cellular ATP concentration in *Listeria monocytogenes* upon supplementation of glucose. Possible mechanisms of cinnamaldehyde's antimicrobial activity include inhibition of glucose uptake or utilization, and disruption of membrane permeability (Gill and Holley, 2004). Kollanoor-Johny et al. (2010) observed the antibacterial effects of cinnamaldehyde on *S.* Enteritidis and found that 10 mM cinnamaldehyde reduced *S.* Enteritidis populations in chicken cecal content by approximately 6.0 log10 cfu/mL after 8 h and >8.0 log10 cfu/mL after 24 h of incubation. Dietary inclusion of cinnamaldehyde has also been used to protect chickens from enteric disease. Kollanoor-Johny et al. (2012) supplemented 0.5% or 0.75% cinnamaldehyde supplemented daily to broiler chicks (d of hatch to 20 d of age) and reported significantly reduced cecal *S.* Enteritidis colonization at 10 d post-challenge (Kollanoor-Johny et al., 2012).

Conclusion

Due to the emergence of antibiotic resistant bacteria, sub-therapeutic antibiotic use is decreasing in the poultry industry (Van Immerseel et al., 2004). Consequentially, alternatives for decreasing pathogenic intestinal bacteria are increasing. Various studies have reported significant reduction in intestinal pathogens in birds supplemented with prebiotics, probiotics, and acidifiers. However, because use of these feed additives are fairly recent compared to sub-therapeutic antibiotic use, further studies are needed to confirm optimal dosage and usage. Another topic which has had varying results in previous studies is the interactions between the intestinal bacteria and immune system. My dissertation analyzes the effects of drinking water synbiotic

supplementation and in-feed acidifier supplementation in laying hens with and without a *Salmonella* challenge. *S.* Enteritidis is used as the challenge pathogen due to the magnitude of effects it has on foodborne illness in humans and its significant costs to the poultry industry (Majowicz et al., 2010). Lastly, my dissertation will assess the effects of a novel, oral polyanhydride nanoparticle *Salmonella* vaccine in layer chickens. Oral vaccines are beneficial due to ease of administration and more direct targeting of the mucosal immune response compared to conventional injected vaccines (Ochoa et al., 2007). Past literature has demonstrated that polyanhydride coating of nanoparticles can protect the vaccine antigen from degradation within the gastrointestinal tract (Ochoa et al., 2007). Moreover, previous studies reported that the polyanhydride coating and the surface-conjugated flagellin proteins can act as an adjuvant in enhancing the anti-*Salmonella* immune response (Salman et al., 2009). Future studies would be beneficial for optimizing the multi-faceted approach of combining multiple anti-bacterial feed additives with *Salmonella* vaccines to fully eliminate *Salmonella* in poultry farm settings.

Chapter 3: Effects of Drinking Water Synbiotic Supplementation in Laying Hens Challenged with *Salmonella*

ABSTRACT

This experiment was conducted to study the effects of drinking water supplementation of synbiotic product PoultryStar®sol (containing *Lactobacillus reuteri, Bifidobacterium animalis, Pediococcus acidilactici, Enterococcus faecium,* and fructo-oligosaccharide) in laying hens with and without a *Salmonella* challenge. A total of 384 one-day-old layer chicks were randomly distributed to the drinking water synbiotic supplementation or control groups. At 14 wk of age, the pullets were vaccinated with a *Salmonella* vaccine, resulting in a 2 (control and synbiotic) X 2 (non-vaccinated and vaccinated) factorial arrangement of treatments. At 24 wk of age, half of the hens in the vaccinated groups and all the hens that were not vaccinated were challenged with *Salmonella Enterica* serotype Enteritidis, resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. At 8 d post-*Salmonella* challenge, synbiotic supplementation decreased (P = 0.04) cecal *S.* Enteritidis in the challenge group compared to the unsupplemented challenge group. Hens that were supplemented with synbiotic in the vaccine + challenge group had significantly greater cecal *B. animalis* and *P. acidilactici* percentage at 10 d post-*Salmonella* challenge than the hens in the vaccine + challenge group without synbiotic supplementation. At 3 d post-*Salmonella* challenge, hens that

were supplemented with synbiotic in the challenge group had significantly greater cecal *L. reuteri* percentage than the hens in the challenge group without synbiotic supplementation. At 17 d post-*Salmonella* challenge, synbiotic supplementation increased bile anti-*Salmonella* IgA in the challenge group compared to the hens in the challenge group without synbiotic supplementation. At 10 d ($P < 0.01$) and 30 d ($P = 0.05$) post-*Salmonella* challenge, synbiotic supplementation decreased LITAF mRNA transcription compared to the un-supplemented groups. At 3 d post-*Salmonella* challenge, synbiotic supplementation in the vaccine group had longer jejunal villi compared to the vaccine group without synbiotic supplementation. This experiment demonstrated that drinking water supplementation of the synbiotic product evaluated can significantly manipulate immune response and intestinal microbiota of laying hens post-*Salmonella* challenge to handle the challenge effectively.

INTRODUCTION

Salmonella infection in humans, also known as salmonellosis, includes symptoms such as diarrhea, fever, vomiting, and in severe cases, even death (Crum-Cianflone, 2008). In the United States, there are over 40,000 reported cases of *Salmonella* infection in humans and 400 deaths reported annually (Fabrega and Vila, 2013). *Salmonella* colonizes within the intestine of poultry and can be transferred to humans through chicken meat or eggs via contamination from chicken intestinal contents (Braden, 2006; Pires et al., 2014). *Salmonella enterica* serotype Enteritidis represents one of the most common serotypes of *Salmonella* associated with salmonellosis (Andino and Hanning, 2015).

S. Enteritidis is most commonly transmitted between poultry via fecal-oral ingestion, although vertical transfer from the hen to egg is also possible (Gantois et al., 2009). A *S.* Enteritidis infection in chickens has few observed clinical effects in chickens, but the immunological responses have been thoroughly reported (Andino and Hanning, 2015; Sheela et al., 2003). *S.* Enteritidis activates the innate immune system, resulting in the proliferation and migration of immune cells such as macrophages, heterophils, granulocytes and dendritic cells to the site of infection (Van Immerseel et al., 2002). The adaptive immune system is also crucial for minimizing *S.* Enteritidis colonization (Sheela et al., 2003). In chickens, *S.* Enteritidis infection is correlated with increased levels of mucosal IgA antibodies, serum IgG antibodies, and intestinal-associated T cells (Sheela et al., 2003). Correspondingly, *S.* Enteritidis infection is positively correlated with increased mRNA transcription of inflammatory cytokines lipopolysaccharide-induced tumor necrosis factor-alpha (LITAF) and IL-12 in the cecum (Fasina et al., 2008).

Furthermore, *S.* Enteritidis can activate the anti-inflammatory cytokine, IL-10, as a strategy to manipulate the host's defense system to allow the bacteria to colonize and withstand clearance from host immune cells (Ghebremicael et al., 2008).

In the egg industry, the recent increase in laying hens reared in cage-free environments has further increased risk of bacterial contamination (Whiley and Ross, 2015). Vaccines have become one strategy for decreasing *Salmonella* colonization in chickens. Yet *Salmonella* vaccines are not fully effective and are therefore often used in conjunction with feed additives such as probiotics and or prebiotics (Davies and Breslin, 2003; Patterson and Burkholder, 2003). Probiotics are defined by the World Health Organization as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" (Mack, 2005). Probiotics can benefit the host through several mechanisms which include inhibition of pathogenic bacteria through competitive exclusion, production of bacteriostatic and bactericidal substances against pathogens, and enhancement of host immunity (Van Der Wielen et al., 2000; Yang et al., 2012; Lawley and Walker, 2013). The term prebiotic is defined by the Food and Agriculture Organization of the United Nations as "a non-viable food component that confers a health benefit to the host associated with modulation of the microbiota" (Pineiro et al., 2008). Synbiotics are feed additives that are a combination of both probiotics and prebiotics which work synergistically to improve animal health (Dunislawska et al., 2017).

Despite consensus that both probiotics and prebiotics can benefit the health of animals, the effects of differing combinations of prebiotics and probiotic species on the chicken immune system and the intestinal microbiota remains unclear. Furthermore, effects of synbiotics can vary

depending on supplementation method, such as in-feed or drinking water supplementation (Karimi Torshizi et al., 2010). The current study analyzed the effects of supplementation of a drinking water synbiotic product, PoultryStar®sol (containing *Lactobacillus reuteri, Bifidobacterium animalis, Pediococcus acidilactici, Enterococcus faecium*, and fructo-oligosaccharide), in laying hens with and without a *Salmonella* challenge. Cecal microbiota, *Salmonella*-specific IgA and IgG antibodies, cecal tonsil LITAF and IL-10 cytokine mRNA transcription, jejunal villi length, and crypt depth were analyzed to assess the intestinal health of the poultry.

MATERIALS AND METHODS

Birds

Day old White Leghorn chicks (n = 384; Hy-Line North America; Johnstown, OH) were provided *ad libitum* intake of water and feed, housed in cages (pullet and layer), and raised using standard animal husbandry practices. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. The birds were housed in pullet cages (eight birds per cage) for the first 12 wk, after which they were transferred to layer cages (216 sq. in.; two birds per cage). At wk 18, the light hours were increased gradually from six h to 16 h to stimulate production.

Treatments

The birds were fed a pullet starter diet from 0 to 8 wk of age, a pullet grower diet from 8 to 18 wk of age, and a layer diet from 18 to 28 wk. The chicks were randomly distributed to the drinking water synbiotic supplementation or control groups. Each treatment was replicated in 24

water basins of eight chicks per replication (n = 24). All birds were fed a basal diet based on corn and soybean meal. Birds allotted to the drinking water synbiotic treatment groups received synbiotic product (20 mg per bird per day) from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge (PoultryStar® sol, BIOMIN America, Kansas City, KS).

At 14 wk, the pullets were weighed and 32 pullets per treatment were vaccinated with a *S.* Enteritidis vaccine resulting in a 2 (control and synbiotic supplementation) X 2 (vaccinated and non-vaccinated group) factorial arrangement of treatments. During the pre-*Salmonella* challenge portion of the study, there were 16 replicate chicks for the vaccinated group (n = 16) and 8 replicate chicks for the unvaccinated groups (n = 8). Each replication had eight pullets. Birds were vaccinated subcutaneously with 0.3 cc of *S.* Enteritidis vaccine (Poulvac® SE, Zoetis, Florham Park, NJ) at 14 wk of age, with a booster dose at 17 wk of age. Body weight was measured at 14 to 28 wk of age. Eggs were collected and recorded daily from day of first egg until the end of the study.

At 24 wk of age, half the hens in the vaccinated groups and all the hens that were not vaccinated were challenged with 250 µl of 1 X 10^9 CFU *S.* Enteritidis. This resulted in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control, synbiotic) factorial arrangement. The number of replicates was eight for all the treatment groups post-*Salmonella* infection (n = 8) and each replication had eight hens.

A pure culture of wild-type *S.* Enteritidis was used for the challenge (Luoma et al., 2017). *S.* Enteritidis was grown in tryptic soy broth for 12 hours. The cells were washed three times with

PBS followed by centrifugation (3,000xg), and the concentration of the bacteria in the media was estimated spectrophotometrically at 600 nm until the concentration of the bacteria reached approximately 1 x 10^9 CFU/ml. The concentration of the bacteria was further confirmed by serial dilution plating of the inoculum on Xylose Lysine Tergitol-4 (XLT) agar plates. At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, one bird was randomly chosen from six out of the eight replications for sample collection.

Effect of Drinking Water Synbiotic Supplementation on Bacterial Analysis of Cecal Content

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). Cecal content samples (0.10 g) were diluted individually in 1 mL of sterile PBS and centrifuged at 18,000xg for two minutes to remove debris. The pellet was resuspended in EDTA and treated with lysozyme (20 mg/ml) for 45 minutes at 37°C. After incubation, samples were re-centrifuged at 18,000xg for two minutes and the supernatant discarded. Samples were subsequently treated with lysis buffer and Proteinase K (10 mg/ml) for five minutes at 80°C. NaCl (6M) and isopropanol were added to the cell lysate and centrifuged at 18,000xg for two minutes. The DNA pellets was re-suspended and washed in 70% ethanol and then re-suspended in TE buffer. Extracted DNA samples were stored at -20°C (Luoma et al., 2017). Cecal microflora was analyzed by real-time PCR (Amit-Romach et al., 2004) using the primers shown in Table 2. The annealing temperature for cecal microbiota primers was 55.0°C.

Effect of Drinking Water Synbiotic Supplementation on cecal tonsil LITAF and IL-10 mRNA Transcription

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). The RNA collected from cecal tonsils was reverse transcribed into cDNA as described by Selvaraj and Klasing, (2006). The mRNA was analyzed for LITAF and IL-10 by real-time PCR (iCycler, BioRad, Hercules, CA) using SyBr green after normalizing for β-actin mRNA. The housekeeping gene was verified by analyzing the average Ct value for β-actin in each treatment group and using ANOVA to confirm that the P –value for differences between groups was less than 0.05. Primers are shown in Table 1. The annealing temperature for IL-10 was 57.5°C and 57°C for LITAF and β-actin. Fold change from the reference vaccine treatment was calculated using the $2^{(\text{Ct Sample - Housekeeping})}/2^{(\text{CtReference – Housekeeping})}$ comparative Ct method, where Ct is the threshold cycle (Schmittgen and Livak, 2008). The Ct was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background.

Effect of Drinking Water Synbiotic Supplementation on Anti-Salmonella IgA and IgG Antibody Titers

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). *Salmonella*-specific IgA and IgG titers in the bile and plasma were analyzed using an enzyme-linked immunosorbent assay (ELISA). Reagent concentrations were established using checkerboard titrations with dilutions of plasma, bile, antigens, and conjugates. *Salmonella* from

a pure culture was lysed twice by glass beads size 425 to 600 µm (Sigma, St. Louis, MO) in a TissueLyser LT (Qiagen Hilder, Germany) for 5 min at 50 1/s, for use as an antigen to coat the wells of the microtiter plates. Flat-bottomed 96-well microtitration plates were coated with 80.0 µl of the antigen (1.09 ug/ml) diluted in 0.1M carbonate buffer and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (50mM Tris, pH 7.4, containing 150mM sodium chloride and 0.05% Tween 20). To prevent non-specific binding, wells were blocked with PBS-Tween 20 – 2.5% nonfat dry milk incubated for one h at 37°C. For IgA analysis, the plasma was diluted 1:10 and the bile was diluted 1:200 in PBS-Tween 20 – 2.5% nonfat dry milk and added to the plates (100 µl/well) in duplicates (two wells per sample) and incubated for 1 h at room temperature. After washing, HRP-conjugated anti-chicken IgA diluted 1:100,000 in PBS-Tween 20 – 2.5% nonfat dry milk was added to each well (100 µl/well) and incubated for 1 h at room temperature. For IgG analysis, HRP-conjugated anti-chicken IgG was diluted 1:25,000 in PBS-Tween 20 – 2.5% nonfat dry milk, added to each well (100 µl/well), and incubated for 1 h at room temperature. Plates were washed three times and 50 µl/well of TMB peroxidase substrate (1:1 mixture of TMB peroxidase substrate and TMB peroxidase substrate solution B) (KPL, MD) were added to each well. The reaction was stopped after 15 min by adding 1 M phosphoric acid. The OD was measured at 450 nm using the ELISA plate reader. The corrected OD was obtained by subtracting the treatment group OD from blank control OD. The baseline OD value was set at 0 for anti-*Salmonella* IgA and 0.02 for anti-*Salmonella* IgG, based on the average OD values from chicken bile and plasma samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge.

Effect of Drinking Water Synbiotic Supplementation on Jejunal Villi Length and Crypt Depth

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds from each treatment group were randomly chosen for sample collection (n = 6). Six jejunum samples per treatment group were collected from the end of the duodenal loop and before the Meckel's diverticulum and stored in 10% formalin. Samples were dehydrated at room temperature in a graded series of alcohol concentrations (15 min in 50% ethanol, 15 min in 70% ethanol, 15 min in 96% ethanol, 30 min in 100% ethanol with one change at 15 min), cleared in Pro-par (Anatech, Battle Creek, MI) for 45 min with two changes at 15 and 30 min, and infiltrated with paraffin at 60 °C overnight with one change at 15 min using a Leica TP 1020 tissue 45 processor (GMI Inc., Ramsey, MN). Paraffin blocks were cut into 5 μm cross-sections and mounted on frosted slides. Slides were then stained with hematoxylin and eosin (Velleman et al., 1998). Cross sections were viewed using CellSens Imaging software (Olympus America, Central Valley, PA) to measure villi length and crypt depth. Five villi and crypts per section and five sections per sample were analyzed, resulting in a total of 25 villi and crypts average measurements per bird.

Statistical Analysis

A two-way ANOVA was used to examine the interaction effects of vaccination X synbiotic on dependent variables collected from 14 to 24 wk of age. A two-way ANOVA was used to examine the interaction effects of vaccination/challenge X synbiotic on dependent variables collected from 24 to 28 wk. When the interaction effects were not significant ($P > 0.05$), the main

effects of synbiotic were analyzed. When interaction or main effects were significant ($P \leq 0.05$), differences between means were analyzed by Tukey's Honest Significant Difference test. In addition, a repeated measures ANOVA was used to analyze the effect of time (wk) on weekly HDEP production.

RESULTS

Effect of Drinking Water Synbiotic Supplementation on Body Weight Gain and Weekly Hen Day Egg Production (HDEP)

There were no significant interaction or main effects of synbiotic supplementation ($P > 0.05$) on body weight gain or weekly HDEP between 14 to 28 wk of age.

Effect of Drinking Water Synbiotic Supplementation on Relative Percentage of S. Enteritidis in Cecal Content

S. Enteritidis was detected in the cecal content at 3 and 8 d post-*Salmonella* challenge (Fig. 1). There were no significant interaction effects between the synbiotic supplementation and treatments at 3 d ($P = 0.28$) post-*Salmonella* challenge. At 8 d post-*Salmonella* challenge, synbiotic supplementation in the challenge treatment decreased ($P = 0.04$) cecal *S.* enteritidis compared to the unsupplemented challenge treatment.

Effect of Drinking Water Synbiotic Supplementation on Relative Percentage of *L. reuteri, B. animalis, P. acidilacti* and *E. faecium* in Cecal Content

There were significant interaction effects between synbiotic supplementation and treatment on cecal *L. reuteri* percentage at 3 d post-*Salmonella* challenge ($P = 0.05$), cecal *B. animalis* percentage at 10 d post-*Salmonella* challenge ($P < 0.01$), and cecal *P. acidilactici*

percentage at 10 d post-*Salmonella* challenge (P = 0.03; Table 3). Hens that were supplemented with synbiotic in the vaccine + challenge group had significantly greater cecal *B. animalis* percentage and cecal *P. acidilactici* percentage at 10 d post-*Salmonella* challenge than the birds in the vaccine + challenge group without synbiotic supplementation. At 3 d post-*Salmonella* challenge, hens that were supplemented with synbiotic in the challenge group had significantly greater cecal *L. reuteri* percentage than the hens in the challenge group without synbiotic supplementation. There were no significant interaction or main effects of synbiotic supplementation on *E. faecium* in the cecal content.

Effect of Drinking Water Synbiotic Supplementation on Bile and Plasma Anti-*Salmonella* IgA Titers

There were significant interaction effects between the synbiotic supplementation and treatments at 17 and 22 d post-*Salmonella* challenge on bile anti-*Salmonella* IgA titers (P = 0.01; P < 0.01) (Fig. 2). At 17 d post-*Salmonella* challenge, synbiotic supplementation increased bile anti-*Salmonella* IgA in the challenge group compared to the hens in the challenge group without synbiotic supplementation by 76.0%. At 22 d post-*Salmonella* challenge, synbiotic supplementation increased bile anti-*Salmonella* IgA in the vaccine + challenge group compared to the hens in the vaccine + challenge group without synbiotic supplementation by 57.7%.

There were significant (P < 0.01) interaction effects between the synbiotic supplementation and treatment at 8, 10, 24, and 30 d post-*Salmonella* challenge on plasma anti-*Salmonella* IgA titers (Table 4). At 8, 10, 24, and 30 d post-*Salmonella* challenge, synbiotic supplementation resulted in an increased in plasma anti-*Salmonella* IgA in the challenge group

compared to the hens in the challenge group without synbiotic supplementation by 94.4%, 94.3%, 73.7% and 80.8%, respectively. At 8 and 10 d post-*Salmonella* challenge, synbiotic supplementation increased plasma anti-*Salmonella* IgA in the vaccine group compared to the vaccine group without synbiotic supplementation by 93.8% and 98.5%, respectively.

Effect of Drinking Water Synbiotic Supplementation on Plasma Anti-*Salmonella* IgG Titers

There were no significant interaction ($P > 0.05$) effects between the synbiotic supplementation and treatments at 22 and 30 d post-*Salmonella* challenge on plasma anti-*Salmonella* IgG titers (Table 5). At 22 and 30 d post-*Salmonella* challenge, there was a significant main synbiotic effect, in that synbiotic supplementation had increased ($P < 0.01$) IgG titers compared to the un-supplemented groups by 22.4% and 14.3%, respectively.

Effect of Drinking Water Synbiotic Supplementation on Cecal Tonsil LITAF and IL-10 mRNA Transcription

At 10 and 30 d post-*Salmonella* challenge, there were no significant interaction ($P > 0.05$) effects between synbiotic supplementation and treatment on cecal tonsil LITAF mRNA transcription (Fig. 3). At 10 ($P < 0.01$) and 30 ($P = 0.05$) d post-*Salmonella* challenge, there were significant synbiotic main effects, in that synbiotic supplementation decreased overall LITAF mRNA transcription compared to the un-supplemented groups by 98.5% and 89.9%, respectively. At 10 d post-*Salmonella* challenge, there were no significant interaction ($P > 0.05$) effects between synbiotic supplementation and treatment on IL-10 mRNA transcription (Fig. 4). At 10 d post-*Salmonella* challenge, there was a significant synbiotic main effect ($P = 0.04$), in

that synbiotic supplementation decreased overall IL-10 mRNA transcription compared to the un-supplemented groups by 56.8%. At 30 d post-*Salmonella* challenge, there was a significant interaction effect, in that synbiotic supplementation in the vaccine + challenge group had decreased ($P < 0.01$) IL-10 mRNA transcription by 87.2% compared to the vaccine + challenge group without supplementation (Fig. 4).

Effect of Drinking Water Synbiotic Supplementation on Jejunal Villi Length and Crypt Depth

There was a significant interaction ($P = 0.05$) effect between the synbiotic supplementation and treatments at 3 d post-*Salmonella* challenge on jejunal villi length (Fig. 5). At 3 d post-*Salmonella* challenge, synbiotic supplementation in the vaccine group resulted in longer villi compared to the vaccine group without synbiotic supplementation by 28.6%. There were no significant interaction or main effects on crypt depth.

DISCUSSION AND CONCLUSION

The results of this experiment suggest that drinking water synbiotic supplementation significantly modulates the anti-*Salmonella* immune response and decreases *Salmonella* colonization in laying hens. Drinking water synbiotic supplementation numerically decreased relative percentage of cecal *S.* Enteritidis in laying hens. In addition, our analysis found that three out of the four supplemented probiotics (*L. reuteri, B. animalis,* and *P. acidilactici)* were consistently present within the cecal content. Our experiment also analyzed the immunological effects of drinking water synbiotic supplementation and observed increased plasma and bile IgA

antibody titers, decreased plasma IgG titers, and decreased LITAF and IL-10 mRNA transcription in laying hens supplemented with drinking water synbiotic product.

Our study utilized real-time PCR to analyze the relative percentage of *S.* Enteritidis in the cecal content and identified *S.* Enteritidis at 3 and 8 d post-challenge. The results showed that the synbiotic supplementation decreased ($P = 0.04$) the relative percentage of cecal *Salmonella* in the challenge treatment group compared to the challenge treatment without supplementation. Probiotics have previously shown to be effective in decreasing *Salmonella* in chickens (Tellez et al., 2012). This effect is likely due to a combination of mechanisms of probiotics, including competitive exclusion, enhancement of immune response, and production of antimicrobial substances (Van Der Wielen et al., 2000; Yang et al., 2012; Lawley and Walker, 2013). However, the effects of probiotics can vary depending on probiotic strains and concentration (Lutful Kabir, 2009).

Our study analyzed the relative percentage of the supplemented probiotics (*L. reuteri, B. animalis, P. acidilactici,* and *E. faecium*) in the cecal content. We chose to analyze the cecal content, because the cecum contains the greatest density of bacteria within the intestinal tract (Pan and Yu, 2014). We found that *L. reuteri, B. animalis, and P. acidilactici* were consistently present in the cecal content of the birds supplemented with drinking water synbiotic product. However, *E. faecium* was not present in the cecal content at any of the collection time points. The relative percentages of probiotic species in the cecal content were detected at levels similar to previous studies when probiotics were supplemented via the feed in laying hens (Luoma et al., 2017).

Drinking water synbiotic supplementation significantly increased bile and plasma anti-*Salmonella*-specific IgA titers in laying hens with and without *Salmonella* infection. It is particularly interesting that at 8, 10, 24, and 30 d post-challenge, the challenge group supplemented with synbiotics had the highest *Salmonella*-specific plasma IgA titers. It is plausible that the lower IgA titers in the vaccine + challenge group may be because the vaccinated + challenge group had decreased cecal *Salmonella* colonization compared to the challenge group. Van Immerseel et al. (2002) observed increases in lymphocyte proliferation as early as 48 h post-*Salmonella* challenge in chickens. Therefore, it is possible that we may have observed an increase in IgA titers in the vaccine + challenge group if we had measured the titers before 8 d post-challenge.

Revolledo et al. (2009) found that broiler chickens infected with *Salmonella* and supplemented with a probiotic combination of 12 strains of *Lactobacilli,* five strains of *Enterococcus* and one strain of *Bifidobacteria* had increased IgA titers within the intestine compared to both challenged and unchallenged birds without probiotic supplementation. Secretory IgA serves as the first line of defense against pathogens within the intestinal tract by preventing pathogenic bacteria from adhering to the intestinal mucosal layer and colonizing (Mantis et al., 2011). Increased IgA titers observed in our experiment likely contributed to the decreased *Salmonella* infection within the ceca.

In addition to analyzing the local humoral response, our study also assessed the plasma anti-*Salmonella*-specific IgG titers effect of drinking water synbiotic supplementation. Our results found that synbiotic supplementation significantly ($P < 0.05$) decreased plasma anti-

Salmonella-specific IgG titers at 22 and 30 d post-*Salmonella* challenge. Revolledo et al. (2009) observed similar results in their study analyzing the effects of probiotics supplemented to broilers, in that despite increased IgA titers within the intestine, the IgG titers were decreased in the serum.

Our experiment analyzed mRNA transcription of inflammatory and anti-inflammatory cytokines within the cecal tonsils and found significant main effects ($P < 0.05$) in that the drinking water synbiotic supplementation decreased LITAF and IL-10 transcription in laying hens with and without *Salmonella* infection. The effects of probiotics decreasing inflammation has been well-studied (Lin et al., 2008). However, in addition to decreased mRNA transcription of the inflammatory cytokine LITAF, our results showed decreased mRNA transcription of the anti-inflammatory IL-10 cytokine at 10 and 30 d post-challenge. Many species of lactic acid bacteria can inhibit NF-κB activation, resulting in an increase in the anti-inflammatory cytokine IL-10 (de Moreno de LeBlanc et al., 2011). A possible reason for the decrease of IL-10 mRNA transcription observed in the current study may be due to the decrease of *Salmonella* infection. *Salmonella* infection in chickens is correlated with an increase in IL-10 due to a defense mechanism against the host by *Salmonella* (Ghebremicael et al., 2008).

Our study also examined the effects of drinking water synbiotic supplementation on jejunal villi length and crypt depth to further analyze intestinal health post-*Salmonella* challenge. Borsoi et al. (2011) measured cecal villus height and crypt depth in broiler chicks at 3 d post-*Salmonella* challenge and found shorter villi lengths and greater crypt depths compared to unchallenged chicks. Our study observed significant interaction effects ($P < 0.05$) at 3 d post-

Salmonella challenge, in that the synbiotic supplementation in the vaccinated chickes resulted in longer villi compared with the vaccinated treatment without supplementation. Aliakbarpour et al. (2012) observed that in-feed supplementation of lactic acid bacteria correlated with increased average villi length in 6-week old broilers. Longer villi lengths and shorter crypt depths are positively correlated with absorption due to increased epithelial surface area and reduced turnover rate (Shang et al., 2015).

The combination of prebiotics and probiotics (synbiotics) is theorized to act in synergy in improving animal health, because prebiotics act as nutrients for probiotics, thereby increasing probiotic survivability (Dunislawska et al., 2017). Several studies analyzing probiotic supplementation on intestinal infection in broilers reported that drinking water probiotic supplementation was more effective than in-feed probiotic supplementation in enhancing health (Karimi Torshizi et al., 2010; Ritzi et al., 2014). The combined results of our experiment demonstrate that drinking water supplementation of the synbiotic product PoultrySol® (containing *L. reuteri, B. animalis, P. acidilactici, E. faecium* and fructo-oligosaccharide) can significantly regulate immune response and intestinal microbiota of laying hens with and without a *Salmonella* challenge.

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Chapter 4: Effects of Acidifier Product Supplementation in Laying Hens Challenged with *Salmonella*

ABSTRACT

This experiment was conducted to study the effects of acidifier supplementation (Biotronic® Top3), consisting of propionic, formic and acetic acids combined with cinnamaldehyde, in laying hens with and without a *Salmonella* challenge. A total of 384 one-day-old layer chicks were randomly distributed to the acidifier supplementation or control treatment groups. At 14 wk of age, the birds were vaccinated with a *Salmonella* vaccine, resulting in a 2 (control and acidifier) X 2 (unvaccinated and vaccinated) factorial arrangement of treatments. At 24 wk of age, half of the hens in the vaccinated treatment and all the hens that were not vaccinated were challenged with *Salmonella Enterica* serotype Enteritidis, resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. At 17 through 25 wk of age, hens supplemented with the acidifier had increased ($P < 0.05$) body weights compared to the un-supplemented treatment groups. At 19 wk of age, hens supplemented with acidifier had higher weekly hen day egg production in the unvaccinated group compared to the unvaccinated group without acidifier supplementation. At 8 d post-*Salmonella* challenge, hens supplemented with acidifier in the challenge group had decreased ($P = 0.04$) cecal *S.* Enteritidis percentage compared to the challenge group without acidifier supplementation. At 3 ($P = 0.03$), 8

($P < 0.01$), and 17 ($P < 0.01$) d post-*Salmonella* challenge, hens supplemented with acidifier had increased cecal *Bifidobacteria* percentage by 98.2%, 99.7%, and 93.2% compared to the un-supplemented groups, respectively. At 24 d post-*Salmonella* challenge, hens supplemented with acidifier lower anti-*Salmonella* IgG titers in the vaccine + challenge group by 42.3% compared to the vaccine + challenge group without acidifier supplementation. At 3 and 10 d post-*Salmonella* challenge, hens supplemented with acidifier had decreased ($P < 0.01$) LITAF mRNA transcription compared to the un-supplemented groups by 84.8% and 98.7%, respectively. The combined analysis of performance, immune response, and cecal microbiota in laying hens indicates that acidifier supplementation can significantly manipulate immune response post-*Salmonella* challenge and decrease *Salmonella* infection.

INTRODUCTION

Salmonella is a zoonotic bacterium that can result in foodborne illness in humans, with symptoms including fever, diarrhea, vomiting, and death (Scallan et al., 2011; Hale et al., 2012)). *Salmonella* infection is most frequently caused by consumption of contaminated poultry meat and eggs (Andino and Hanning, 2015). *Salmonella enterica* serotype Enteritidis is one of the most common serovars associated with foodborne illness (Andino and Hanning, 2015). *S.* Enteritidis often has no clinical effects in chickens and can therefore easily go unnoticed on poultry farms (Suzuki, 1994). The reason for the asymptomatic effects of *S.* Enteritidis in chickens is not fully understood, though it has been reported that *S.* Enteritidis can stimulate IL-10 expression in chickens, which is associated with a suppressed immune response against the infection (Ghebremicael et al., 2008).

S. Enteritidis infection is most frequently transmitted between chickens through fecal-oral route (Kramer et al., 2001). As *S.* Enteritidis colonizes within the intestinal enterocytes, the innate immune response is triggered, resulting in infiltration of phagocytic cells to the site of infection (Desmidt et al., 1997). The adaptive immune system plays an important role in response to *S.* Enteritidis as observed by increased intestinal T cell populations and levels of mucosal IgA and serum IgG during infection (Sheela et al., 2003; Tran et al., 2010). Because *S.* Enteritidis is an intracellular bacterium, clearance of the bacteria requires a cell-mediated immune response in addition to a humoral immune response (Sheela et al., 2003). *S.* Enteritidis infection correlates with increased expression of Th1 cytokines including lipopolysaccharide-

induced tumor necrosis factor (LITAF), IL-12, and IFN- γ, in addition to Th2 cytokines IL-4 and IL-10 (Berndt et al., 2007; Wigley, 2014).

Acidifiers are organic acids that inhibit intestinal pathogens by interfering with bacterial cell membranes and decreasing the cytoplasmic pH (Hedayati et al., 2014). Acidifiers can additionally exist in salt forms with similar bactericidal and bacteriostatic properties (Hedayati et al., 2014). Acidifiers diffuse across the cell membrane and dissociate within the cytoplasm into protons and anions (Eklund, 1983). The bacterial cell reacts by actively expelling the protons out of the cell (Mitchell and Moyle, 1969). This activity causes the cell to lose energy, resulting in cell death. Cherrington et al. (1990) reported decreased rates of protein, RNA, and DNA synthesis in gram-negative bacteria cultured *in vitro* with propionic and acetic acids.

Previous studies have observed that organic acids can decrease total gram-negative bacterial counts in poultry (Mani-López et al., 2012). The antimicrobial effect of an acid depends on the dissociation constant (pKa), which is the pH value when 50% of the total acid is non-dissociated. The non-dissociated acid can penetrate into cells, allowing for the antimicrobial effect (Davidson and Taylor, 2007). As a result, the combination of acid types can have varying effects within the gastrointestinal tract due to differing pKa values and time dissociations of the acids (Davidson and Taylor, 2007). Several additional studies have reported that acidifier supplementation decreases the pH of the intestinal tract, resulting in a less suitable environment for pathogenic bacteria (Khan and Iqbal, 2016).

Acidifiers are often combined with essential oils, phytochemicals derived from plants (Liu et al., 2017). Basmacioğlu-Malayoğlu et al. (2016) reported that formic and propionic

organic acids blended with oregano, clove and cumin essential oils resulted in increased apparent digestibility of dry matter and crude protein, and longer villi lengths and shorter crypt depths in broilers. In addition, they reported decrease ileal *Escherichia coli* colonization compared to the control. Cinnamaldehyde is a component of bark extract of cinnamon, known to have antibacterial effects against food-borne pathogens such as *E. coli* and *Salmonella* (Friedman et al., 2002). Kollanoor-Johny et al. (2012) reported that cinnamaldehyde supplementation in broilers decreased *S.* Enteritidis shedding in broilers.

Our current study analyzed the effects of the acidifier product, Biotronic® Top3 (consisting of formic, propionic, and acetic acid in ammonium salt form, and cinnamaldehyde), in laying hens with and without a *Salmonella* challenge. Body weight, weekly hen day egg production (HDEP), cecal microbiota, *Salmonella*-specific IgA and IgG antibodies, cecal tonsil LITAF and IL-10 cytokine mRNA transcription, jejunal villi length, and crypt depth were assessed.

MATERIALS AND METHODS

Birds

Day-old White Leghorn chicks (n = 384 chicks total; Hy-Line North America; Johnstown, OH) were provided ad libitum intake of water and feed and housed in battery cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. The birds were housed in pullet cages (eight birds per cage) for the first 12 wk, after which they were transferred to layer cages (260 sq. in.; two birds

per cage). At wk 18, the light hours were increased gradually from six h to 16 h to stimulate production.

Treatments

The birds were fed a pullet starter diet from 0 to 8 wk of age, a pullet grower diet from 8 to 18 wk of age, and a layer diet from 18 to 28 wk. Day-old layer chicks were randomly distributed to the acidifier supplementation or control groups. Each treatment was replicated in 24 feeders of eight chicks per replication (n = 24). The basal diet was based on corn and soybean meal. For the acidifier treatment groups, acidifier product was added to the feed at a rate of 1 g/kg from day of hatch until the end of the project at 28 wk of age (Biotronic® Top3, Biomin, San Antonio, TX).

At 14 wk of age, the pullets were weighed and 32 pullets per treatment were vaccinated with a *S.* Enteritidis bacterin vaccine, resulting in a 2 (control and acidifier supplementation) X 2 (vaccinated and unvaccinated group) factorial arrangement of treatments. During the pre-*Salmonella* challenge portion of the study, there were 16 replicate chicks for the vaccinated group (n = 16) and 8 replicate chicks for the unvaccinated groups (n = 8). Birds were vaccinated subcutaneously with 0.3 cc of *S.* Enteritidis vaccine (Poulvac® SE, Zoetis, Florham Park, NJ) at 14 wk of age, with a booster dose at 16 wk of age (Luoma et al., 2017). Body weight was measured at 14 to 28 wk of age. Eggs were collected and recorded daily from day of first egg (19 wk of age) until the end of the study.

At 24 wk of age, half of the birds in the vaccinated groups and all the birds that were not vaccinated were challenged with 250 µl of 1 X 10^9 CFU *S.* Enteritidis, resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control, acidifier) factorial arrangement of treatments.

The number of replicates was eight for all the treatment groups post-*Salmonella* infection (n = 8) and each replication had eight hens.

A pure culture of wild-type *S.* Enteritidis was used for the challenge (Luoma et al., 2017).

A pure culture of wild-type *S.* Enteritidis was used for the challenge (Luoma et al., 2017). *S.* Enteritidis was grown in tryptic soy broth for 12 hours. The cells were washed three times with PBS followed by centrifugation (3,000xg), and the concentration of the bacteria in the media was estimated spectrophotometrically at 600 nm until the concentration of the bacteria reached approximately 1 x 10^9 CFU/ml. The concentration of the bacteria was further confirmed by serial dilution plating of the inoculum on Xylose Lysine Tergitol-4 (XLT) agar plates. At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, one bird was randomly chosen from six out of the eight replications for sample collection.

Effect of Acidifier Supplementation on Bacterial Analysis of Cecal Content

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). Cecal contents (0.1 g) were diluted in 1 mL of sterile PBS and centrifuged at 18,000xg. The pellet was resuspended in EDTA and treated with lysozyme (20 mg/ml) for 45 min at 37°C. After incubation, samples were centrifuged at 18,000xg for two min and supernatant discarded. Samples were then treated with lysis buffer and Proteinase K (10 mg/ml) for five min at 80°C. After five min incubation, 6M NaCl and isopropanol were added to the cell lysate and centrifuged at 18,000xg for two min. DNA pellets were then resuspended, washed in 70% ethanol, and then resuspended in about 100 µl of TE buffer. Cecal microflora was analyzed by

real-time PCR (Amit-Romach et al., 2004). The annealing temperature for analyzed cecal microflora primers was 55.0°C. Primers are described in Table 2.

Effect of Acidifier Supplementation on Anti-*Salmonella* IgA and IgG Antibody Titers

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). *Salmonella*-specific IgA and IgG titers in the chicken bile and plasma were analyzed using an enzyme-linked immunosorbent assay (ELISA). Reagent concentrations were established using checkerboard titrations with dilutions of bile, plasma, *Salmonella* antigens, and conjugates. *Salmonella*, grown from a pure culture, was lysed two times by glass beads size 425-600 µm (Sigma, St. Louis, MO) in a TissueLyser LT (Qiagen Hilder, Germany) for five min at 50 1/s. Flat-bottomed 96-well microtitration plates were coated with 80.0 µl of the lysed *Salmonella* (1.093 ug/ml), diluted in 0.1M carbonate buffer, and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (50mM Tris, pH 7.4, containing 150mM sodium chloride and 0.05% Tween 20). Wells were blocked with PBS-Tween 20 – 2.5% nonfat dry milk and incubated for one h at 37°C. For analysis of *Salmonella*-specific IgA, the plasma was diluted to 1:10 and the bile was diluted to 1:200 in PBS-Tween 20-2.5% nonfat dry milk, added to the plates in duplicates (100 µl/well), and incubated for 1 h at room temperature. After washing, HRP-labeled anti-chicken IgA was diluted 1:100,000 in PBS-Tween 20 – 2.5% nonfat dry milk, was added to each well (100 µl/well), and incubated for 1 h at room temperature. For analysis of Salmonella-specific IgG, HRP-labeled anti-chicken IgG was diluted 1:25,000 in PBS-Tween 20 – 2.5% nonfat dry milk, added to each well (100 µl/well), and incubated for 1 h at room temperature. 100 µl of TMB solution (3 M

sodium acetate, TMB, hydrogen peroxide) was added to reveal peroxidase activity. The reaction was stopped by adding 100 µl of 2M sulfuric acid, and the optical density (OD) value was measured at 490 nm in a microplate reader. The baseline OD value was set at 0 for anti-*Salmonella* IgA and 0.02 for anti-*Salmonella* IgG, based on the average OD values from chicken bile and plasma samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge.

Effect of Acidifier Supplementation on LITAF and IL-10 mRNA Transcription in the Cecal Tonsils

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). The RNA was collected from cecal tonsils and reverse transcribed into cDNA (Selvaraj and Klasing, 2006). The mRNA was analyzed for LITAF and IL-10 by real-time PCR (iCycler, BioRad, Hercules, CA) using SyBr green after normalizing for β-actin mRNA housekeeping gene. The housekeeping gene was verified by analyzing the average Ct value for β-actin in each treatment group and using ANOVA to confirm that the P –value for differences between groups was less than 0.05. Primers are described in Table 1. The annealing temperature for IL-10 was 57.5°C. The annealing temperature for LITAF and β-actin was 57°C. Fold change from the reference was calculated using the comparative Ct method. The calculation is described as $2^{(Ct\ Sample - Housekeeping)}/2^{(CtReference - Housekeeping)}$ (Schmittgen and Livak, 2008). The Ct, the threshold cycle, was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background.

Effect of Acidifier Supplementation on Jejunal Villus Length and Crypt Depth

At 5 d pre-*Salmonella* challenge and 3, 8, 10, 17, 22, 24, and 30 d post-*Salmonella* challenge, six birds in each treatment group were randomly chosen for sample collection (n = 6). Six jejunum samples per treatment group were collected by cutting sections beginning from the end of the duodenal loop and ending before the Meckel's diverticulum. Samples were stored in 10% formalin. Using a Leica TP 1020 tissue 45 processor (GMI Inc., Ramsey, MN), jejunum samples were dehydrated at room temperature in a graded series of alcohols: 15 min in 50% ethanol, 15 min in 70% ethanol, 15 min in 96% ethanol, and 30 min in 100% ethanol with one change at 15 min. Samples were then cleared in Pro-par (Anatech, Battle Creek, MI) for 45 min with two changes at 15 and 30 min, and infiltrated with paraffin at 60 °C overnight with one change at 15 min. Paraffin blocks were cut into five μm cross-sections and mounted on frosted slides. Slides were then stained with hematoxylin and eosin as described previously (Velleman et al., 1998). Cross sections were viewed using CellSens Imaging software (Olympus America, Central Valley, PA). Five villi and crypts per section and five sections per sample were measured, resulting in a total of 25 villi and crypts per bird.

Statistical Analysis

A two-way ANOVA was used to examine the interaction effects of vaccination X acidifier on dependent variables collected from 14 to 24 wk of age. A two-way ANOVA was used to examine the interaction effects of vaccination/challenge X acidifier on dependent variables collected from 24 to 28 wk of age. When the interaction effects were not significant ($P > 0.05$), the main effects of acidifier were analyzed. When interaction or main effects were significant (P

≤ 0.05), differences between means were analyzed by Tukey's Honest Significant Difference Test. In addition, a repeated measures ANOVA was used to analyze the effect of time (wk) on weekly HDEP production.

RESULTS

Effect of Acidifier Supplementation on Body Weight Gain

There were no significant interaction effects (P > 0.05) between the acidifier supplementation and treatment groups on body weight in hens at 14, 17, 18, 20, 22, and 23 wk of age. There were significant main effects of acidifier supplementation at wk 14 (P < 0.01), 17 (P < 0.01), 18 (P < 0.01), 20 (P < 0.01), 22 (P < 0.01), and 23 (P = 0.01), in that birds supplemented with acidifier had 9%, 9.1%, 6.9%, 9%, 3.9%, and 5.9% higher body weight compared to the un-supplemented groups, respectively (Fig. 6). There were no significant interaction effects between the acidifier supplementation and treatments post-*Salmonella* challenge. At 3 (24 wk; P = 0.03) and 10 (25 wk; P = 0.03) d post-*Salmonella* challenge, there were significant main effects of acidifier supplementation (Fig. 6). Hens in the acidifier supplemented groups had 6.4% and 5.8% higher body weight compared to the un-supplemented groups, respectively.

Effect of Acidifier Supplementation on Weekly Hen Day Egg Production (HDEP)

The repeated measures ANOVA analysis confirmed that there was a significant (P < 0.01) effect of time between treatment groups. At 19 wk of age, weekly HDEP was approximately 1.6% for acidifier supplemented groups and 0.0% for the un-supplemented groups. There was a significant interaction effect (P = 0.04) in hens at 19 wk of age, in that hens supplemented with acidifier had higher weekly HDEP in the unvaccinated group compared to the unvaccinated group

without acidifier supplementation (Table 6). There were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatment on weekly HDEP in hens between 20 to 28 wk of age (Table 6). There was a significant main effect of acidifier supplementation at 20 ($P < 0.01$), 21 ($P < 0.01$), 22 ($P = 0.02$), and 23 ($P = 0.02$) wk of age, in that hens supplemented with acidifier had 83.9%, 41.0%, 6.8%, and 4.9% higher weekly HDEP compared to the un-supplemented groups, respectively.

Effect of Acidifier Supplementation on Relative Percentage of S. Enteritidis in Cecal Content

S. Enteritidis was identified in the cecal content at 3 and 8 d post-*Salmonella* challenge (Fig. 7). There were no significant interaction effects ($P = 0.24$) between the acidifier supplementation and treatments at 3 d post-*Salmonella* challenge. There was a significant main effect of acidifier supplementation ($P = 0.05$) at 3 d post-*Salmonella* challenge, in that hens supplemented with acidifier product had 84.6% decreased cecal *S.* Enteritidis percentage compared to the un-supplemented groups. At 8 d post-*Salmonella* challenge, there was a significant interaction effect ($P = 0.04$) between acidifier supplementation and treatments. Hens supplemented with acidifier in the challenge group had decreased cecal *S.* Enteritidis percentage compared to the challenge group without acidifier supplementation.

Effect of Acidifier Supplementation on Relative Percentage of *Lactobacillus* and *Bifidobacterium* in Cecal Content

There were no significant interaction effects ($P < 0.05$) between the acidifier supplementation and treatment at 3, 8, and 17 d post-*Salmonella* challenge on cecal

Bifidobacterium percentage (Fig. 8). There was a significant main effect of acidifier supplementation at 3 ($P = 0.03$), 8 ($P < 0.01$), and 17 ($P < 0.01$) d post-*Salmonella* challenge, in that hens supplemented with acidifier had increased cecal *Bifidobacterium* percentage by 98.2%, 99.7%, and 93.2% compared to the un-supplemented groups, respectively. There were no significant interaction effects or main effects ($P < 0.05$) between the acidifier supplementation and treatment on cecal *Lactobacillus* percentage (data not shown).

Effect of Acidifier Supplementation on Bile and Plasma Anti-*Salmonella* IgA Titers and Plasma Anti-*Salmonella* IgG Titers

There were significant interaction effects ($P = 0.02$) between the acidifier supplementation and treatments at 10 d post-*Salmonella* challenge on bile anti-*Salmonella* IgA titers (Table 7). At 10 d post-*Salmonella* challenge, acidifier supplementation decreased bile anti-*Salmonella* IgA in the challenge group by 88.2% compared to the hens in the challenge group without acidifier supplementation. There were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatments at 22 d post-*Salmonella* challenge on bile anti-*Salmonella* IgA titers (Table 7). At 22 d post-*Salmonella* challenge, there was a significant main effect of acidifier supplementation ($P < 0.01$), in that acidifier supplementation increased anti-*Salmonella* IgA titers by 71.6% compared to the un-supplemented groups.

There were significant interaction effects between the acidifier supplementation and treatments at 17 ($P < 0.01$), 22 ($P < 0.01$), 24 ($P < 0.01$), and 30 ($P = 0.03$) d post-*Salmonella* challenge on plasma anti-*Salmonella* IgA titers (Table 7). Birds supplemented with acidifier in

the challenge group had 100.0%, 90.6%, 86.3%, and 76.2% higher plasma anti-*Salmonella* IgA compared to the challenge group without acidifier supplementation, respectively.

There was a significant interaction effect ($P < 0.01$) between the acidifier supplementation and treatments at 24 d post-*Salmonella* challenge (Table 7). At 24 d post-*Salmonella* challenge, acidifier supplementation had lower anti-*Salmonella* IgG titers in the vaccine + challenge group by 42.3% compared to the vaccine + challenge group without acidifier supplementation.

Effect of Acidifier Supplementation on Cecal Tonsil LITAF and IL-10 mRNA Transcription

At 3 and 10 d post-*Salmonella* challenge, there were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatments on cecal tonsil LITAF mRNA transcription. (Fig. 9). There was a significant main effect of acidifier supplementation ($P < 0.01$) at 3 and 10 d post-*Salmonella* challenge, in that acidifier supplementation decreased LITAF mRNA transcription compared to the un-supplemented groups by 84.8% and 98.7%, respectively. At 10 d post-*Salmonella* challenge, there were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatments on IL-10 mRNA transcription (Fig. 10). There was a significant main effect of acidifier supplementation ($P < 0.01$) at 10 d post-*Salmonella* challenge. Hens supplemented with acidifier had 77.9% lower IL-10 mRNA transcription compared to the un-supplemented groups. There was a significant interaction effect ($P = 0.02$) between acidifier supplementation and treatments at 30 d post-*Salmonella* challenge. Birds supplemented with acidifier in the vaccine + challenge group had 97.2% lower IL-10

mRNA transcription compared to the vaccine + challenge group without acidifier supplementation.

Effect of Acidifier Supplementation on Jejunal Villi Length and Crypt Depth

At 17 d post-*Salmonella* challenge, there were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatments on jejunal villi length. There was a significant main effect of acidifier supplementation at 17 ($P = 0.03$) d post-*Salmonella* challenge. Hens supplemented with acidifier had 9.5% longer jejunal villi lengths compared to the un-supplemented groups (Fig. 11). There were no significant interaction effects ($P > 0.05$) between the acidifier supplementation and treatments on jejunal crypt depth at 17 d of age.

DISCUSSION AND CONCLUSION

The results of this experiment demonstrate that acidifier supplementation enhanced laying hen performance, significantly modulated the anti-*Salmonella* immune response, and decreased *Salmonella* infection in laying hens. The effects of acidifier supplementation on increasing body weight in chickens has been shown in several studies (Khan and Iqbal, 2016). Increased body weight may be due to the organic acids' modulation of the intestinal microbiota, which can lead to longer villi within the intestine and, subsequently, in improved absorption of nutrients (Khan and Iqbal, 2016). Bagal et al. (2016) reported that broilers supplemented with 1% citric acid had greater final body weights than the un-supplemented control broilers. Jensen and Chang (1976) reported improved body weights in laying hens supplemented with calcium propionate. Additionally, our results observed an earlier onset of egg production and increased weekly HDEP in the hens supplemented with acidifier. This result is likely due to the increased

body weight observed in birds supplemented with acidifier, since chickens with higher body weights often have an earlier onset of egg production (Dunnington and Siegel, 1984).

Our study analyzed relative percentages of *S.* Enteritidis, *Bifidobacterium*, and *Lactobacillus* in the cecal content. Our results identified *S.* Enteritidis in the cecal content at 3 and 8 d post-*Salmonella* challenge. Acidifier supplementation resulted in significantly decreased relative percentage of *S.* Enteritidis in laying hens compared to the un-supplemented groups. Organic acids have been shown to inhibit pathogenic bacteria such as *Salmonella* by diffusing through the bacterial cell membrane and decreasing cytoplasmic pH, thus interfering with the metabolism of the cell and resulting in bacterial cell death (Hedayati et al., 2014). Our results correlated with similar studies in poultry, which observed decreased *Salmonella* colonization in birds supplemented with acidifiers (Van Immerseel et al., 2005; Fernandez-Rubio et al., 2009; Borsoi et al., 2011).

Furthermore, our results identified that birds supplemented with acidifier had increased relative percentage of *Bifidobacterium* in the cecal content. *Bifidobacteria* are commonly used as probiotics, as they can confer health benefits to the host (Mack, 2005). *Bifidobacteria* can inhibit pathogenic bacteria within the intestine through competitive exclusion and production of bactericidal substances such as lactic acid (Lutful Kabir et al., 2009). Emami et al. (2017) reported increased intestinal beneficial bacteria in broilers that were challenged with *E. coli* and supplemented with formic and propionic acids, compared to un-supplemented, challenged broilers. The increase in beneficial bacteria is likely due to the inhibition of pathogenic bacteria, resulting in increased available resources for alternative bacteria within the intestines. Beneficial

bacteria such as *Lactobacillus* and *Bifidobacteria* are less pH-sensitive than *Salmonella,* and are therefore are more resistant to acidifier supplementation (Kim et al., 2005).

Our analysis of the effects of acidifier supplementation on the anti-*Salmonella* antibody response showed that acidifier supplementation significantly modulated bile and plasma antibody titers. Acidifier supplementation resulted in decreased plasma IgG titers, both increased and decreased bile IgA titers, and increased plasma IgA titers post-*Salmonella* challenge. Plasma anti-*Salmonella* IgA titers were highest in the challenge group supplemented with synbiotics at 17, 22, 24, and 30 d post-challenge. It is surprising that the vaccine group did not have increased anti-*Salmonella* antibodies as observed in previous studies (Tran et al., 2010). However, this may be due to the decreased *Salmonella* infection observed in the vaccinated and challenged hens compared to the challenged hens as early as 8 d post-challenge.

Although many studies have reported that acidifier supplementation can manipulate the intestinal microbiota by killing pathogenic bacteria, many fewer studies have reported the immunological effects of acidifiers in poultry (Lee et al., 2017). It is well known that intestinal microbiota can directly affect the host immune response (Yang et al., 2012). Because acidifiers manipulate the intestinal microbiota, it is hypothesized that acidifiers can indirectly manipulate the humoral immune response through modulation of the intestinal microbiota (Lee et al., 2017).

Research on the effects of acidifier supplementation on antibody titers have had mixed results. Lee et al. (2017) reported decreased levels of serum H9N2-specific IgG titers in broilers that were supplemented with organic acids (citric, formic, and lactic acids) and vaccinated with H9N2 vaccine, compared to vaccinated broilers without acidifier supplementation. Ahmed et al.

(2014) reported that citric acid supplementation increased serum IgG in pigs challenged with *Salmonella* and *Escherichia coli.* Hedayati et al. (2014) found no significant changes in antibody titers of broilers supplemented with a commercial acidifier product containing citric acid, acetic acid, propionic acid, lactic acid, and mannan oligosaccharide, and concluded that because the mode of action of acidifiers is through inhibition of pathogenic bacteria, the lack of significant difference in immune response was likely due to the birds being raised in a nearly aseptic environment.

Our experiment analyzed mRNA transcription of inflammatory and anti-inflammatory cytokines within the cecal tonsils and found that the acidifier supplementation decreased LITAF and IL-10 mRNA transcription in laying hens post-*Salmonella* challenge. *Salmonella* infection activates the innate immune system in chickens, resulting in a proliferation of immune cells such as macrophages, heterophils, and granulocytes (Hurley et al., 2014). As a result, *Salmonella* infection is correlated with an increase in inflammatory cytokines such as LITAF and IL-12 in the ceca (Berndt et al., 2007). Furthermore, *Salmonella* can stimulate IL-10 production as a strategy to manipulate the host defense system to allow for its colonization (Ghebremicael et al., 2008). The decrease in cytokines LITAF and IL-10 seen in the present experiment may be due to the inhibitory effect of the acidifiers on the *Salmonella* infection, thus resulting in a downregulation of these cytokines.

Acidifier supplementation resulted in longer jejunal villi length compared to the control at 17 d post-*Salmonella* challenge. Adil et al. (2010) observed that broilers supplemented with either butyric, formic, or lactic acid had longer villi lengths in the small intestine compared to the

un-supplemented control. Longer villi length observed at 17 d post-*Salmonella* challenge in the present study may be due to decreased *Salmonella* colonization within the intestinal tract. A decrease in pathogenic bacteria within the intestinal tract has previously correlated with longer villi lengths (Borsoi et al., 2011). Basmacioğlu-Malayoğlu et al. (2016) observed longer villi lengths in broilers that were supplemented with formic and propionic acids combined with essential oils compared with the control. Longer villi lengths are associated with increased absorption due to greater surface area of the mucosal layer (Shang et al., 2015).

The combined analysis of antibody levels, cytokine mRNA transcription, cecal microbiota, histology, and performance in laying hens indicate that acidifier supplementation can significantly manipulate immune response post-*Salmonella* challenge.

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Chapter 5: Protective Effects of Polyanhydride Nanoparticle *Salmonella* Vaccine when Administered Orally to Layer Chickens

ABSTRACT

Salmonellosis is a foodborne illness frequently attributed to the consumption of contaminated poultry meat and eggs with *Salmonella.* Reducing *Salmonella* colonization through vaccine use in poultry is one tool that can be used to decrease the overall incidence of salmonellosis. Nanoparticle vaccines consist of a polymer coating that encapsulates the vaccine antigen, thereby protecting the vaccine against chemical, enzymatic or immunological degradation within the gastrointestinal tract. The current study analyzed the immunological effects of an oral *Salmonella* nanoparticle vaccine (OMPs-F-PNPs) containing *Salmonella* outer membrane proteins (OMPs) and flagellin proteins. At 6 wk of age, chicks were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded OMPs-F-PNPs suspension in 1 mL of sterile PBS (OMPs-F-PNPs). The same dose and route of delivery was administered at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. At 12 ($P = 0.05$) and 15 ($P = 0.01$) wk of age, chickens vaccinated with OMPs-F-PNPs had significantly higher OMPs-specific serum IgG titers when compared with the soluble antigen vaccinated group pre-*Salmonella* challenge. At 10 d post-*Salmonella* challenge, chickens that were vaccinated with OMPs-F-PNPs had significantly ($P < 0.01$) higher serum OMPs-specific IgG titers compared to

that from the mock + challenge and the mock treatment groups. At 10 d post-challenge, birds that were vaccinated with OMPs-F-PNPs had significantly ($P < 0.01$) higher bile OMPs-specific IgG titers compared to that from the mock + challenge and the mock treatment groups. Birds vaccinated with OMPs-F-PNPs had significantly ($P < 0.01$) higher TLR-4 mRNA transcription compared with the mock + challenge group at 10 d post-challenge. Lastly, the OMPs-F-PNPs vaccinated birds had decreased cecal *S.* Enteritidis colonization compared wirh the mock + challenge group at 10 d post-challenge. The observed effects suggest that OMPs-F-PNPs vaccination has high potential as an effective oral vaccine against *Salmonella.*

INTRODUCTION

Salmonellosis is a foodborne illness frequently attributed to the consumption of contaminated poultry meat and eggs with *Salmonella* (Denagamage et al., 2017). *Salmonella Enterica* Serotype Enteritidis represents one of the most commonly isolated serovars of *Salmonella* (Tarabees et al., 2017). *S.* Enteritidis is an intracellular gram-negative bacterium that colonizes within the intestine of poultry and resides primarily within the cecum (Ahmer and Gunn, 2011). Despite contributing to illness in humans, *S.* Enteritidis colonization in chickens has few clinical effects in chickens (Andino and Hanning, 2015). The reasons for its minimal clinical effects in chickens is unknown, however, it is hypothesized that *S.* Enteritidis suppresses the host immune response by stimulating the anti-inflammatory cytokine IL-10 (Ghebremicael et al., 2008). Clearance of *Salmonella* in poultry requires strong humoral and cell-mediated immune responses, commonly associated with B and T lymphocyte infiltration at the site of infection (Van Immerseel et al., 2002; Sheela et al., 2003). Reducing *S.* Enteritidis colonization in poultry may effectively decrease the transfer of *S.* Enteritidis to humans, resulting in fewer cases of salmonellosis (Greig and Ravel, 2009).

Salmonella vaccines in poultry are commonly used for decreasing *S.* Enteritidis colonization. *Salmonella* vaccines are available in the form of live attenuated vaccines and killed whole bacteria vaccines (termed bacterins) (Kong et al., 2011; Tran et al., 2010). However, current vaccines are not fully effective in completely eliminating *Salmonella* colonization in chickens (Desin et al., 2013). Bacterins provide only partial protection due to its inability to induce cell-mediated immunity (Barrow et al., 2007). Live attenuated vaccines are often more

effective than bacterins because they produce both a strong humoral and cell-mediated response (Lalsiamthara et al., 2016). However, live attenuated vaccines are associated with safety concerns for consumers, including the risks of becoming more virulent and or spreading to humans (Lauring et al., 2010).

One alternative to both bacterins and live attenuated vaccines are subunit vaccines. Subunit vaccines consist of immunogenic components of the bacteria including lipopolysaccharide, fimbriae, and outer membrane proteins (OMPs) (Ochoa et al., 2007). Khan et al. (2003) demonstrated that chickens subcutaneously vaccinated with *Salmonella* OMPs had lower *Salmonella* colonization when compared to the unvaccinated control. Okamura et al. (2012) later reported higher OMPs-specific IgG titers in chickens vaccinated intramuscularly with an OMPs vaccine compared to the unvaccinated control. Meenakshi et al. (1999) reported that OMPs induced greater protective response against *Salmonella* infection compared to bacterins in layer chickens.

The protective effect of subunit vaccines can be further enhanced with the addition of vaccine adjuvants such as nanoparticles (Ochoa et al., 2007). Nanoparticles are advantageous for use as an oral vaccine, which are easier to administer than injected vaccines and more effective in invoking local, intestinal immune responses (Ochoa et al., 2007). Nanoparticle vaccines consist of a polymer coating that encapsulates the vaccine antigen, thereby by providing several benefits as an oral vaccine including: i) prevention of antigen degradation, ii) increased concentration of antigens within the mucosal tissues, iii) co-delivery of antigens and adjuvants, and iv) receptor-ligand mediated targeted delivery (Zhao et al., 2014). These functions could be

optimized through adjusting controllable properties such as size, surface charge, and antigen loading of the nanoparticles (Bachmann and Jennings, 2010). Furthermore, surface conjugation of ligands to the nanoparticle surface can target the vaccine antigens to specific sites within the gastrointestinal tract (Salman et al., 2009). Conjugation of flagellin proteins to a nanoparticle *Salmonella* vaccine in mice mimics the natural colonization of *S.* Enteritidis in the gastrointestinal tract, resulting in uptake of the antigen by the ileal Peyer's patches (Salman et al., 2009). Peyer's patches are aggregates of immune cells including macrophages, dendritic cells, and T and B lymphocytes, and are located throughout the intestines beneath the epithelial cellular layer (Jung et al., 2010).

The basic colloidal and degradation properties of a nanoparticle vaccine depends on its copolymer composition. Among the different available biodegradable polymers available for oral delivery of vaccines, polyanyhdride (methyl vinyl ether-co-maleic anhydride) is well characterized and has significant potential for biomedical applications (Garland et al., 2011). Copolymerization of polyvinyl methyl ether and maleic acid results in biocompatibility and bioadhesive properties (Arbos et al., 2003). Oral polyanhydride nanoparticle vaccines induce local mucosal immunity (Ulery et al., 2011) and activates toll-like receptors (TLRs) -2 and -4 required for triggering both humoral and cellular immune responses (Reboucas et al., 2012; Tamayo et al., 2010).

Despite studies reporting the immunological effects of polyanhydride vaccines in mice, few studies have assessed the effects of *Salmonella* polyanhydride vaccines in chickens. The current study analyzed the immunological effects of a polyanhydride *Salmonella* vaccine in

laying hens post-*Salmonella* challenge. We hypothesized that the oral delivery of the polyanhydride vaccine, loaded with S. Enteritidis immunogenic antigens (OMPs and flagellin proteins) and surface-conjugated with flagellin proteins, would deliver the vaccine antigens to the peyer's patches (PPs) within the ileum and induce a robust mucosal and cellular immune response which is required to reduce colonization of *Salmonella.*

MATERIALS AND METHODS

Preparation of PNPs *Salmonella* vaccine

Isolation of OMPs. The OMPs from *S.* Enteritidis were isolated using a sequential detergent extraction method as described previously with some modifications (Ochoa et al., 2004). Briefly, the growing stationary phase bacterial culture was washed using PBS and the cells were lysed in a French Press (QIAGEN- TissueLyser LT, MD). The bacterial inner membrane was solubilized by treating with 1% Sarkosyl (Sigma, MO) for 30 min and centrifuged (Optima^{TM} L-100XP Beckman Coulter ultracentrifuge) at 20,000×g for 30 min. The pellet was suspended in 10% sodium dodecyl sulfate (SDS) (in 0.5 M Tris-HCl, pH 6.8) for 30 min, centrifuged at 20,000×g for 30 min, and the supernatant containing soluble OMPs enriched extract was dialyzed against Milli-Q water and freeze-dried with 5% sucrose as a cryoprotectant. The protein concentration was estimated using a micro BCA protein assay kit (Thermo Scientific, MA) as per the manufacturer's instruction. Sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-page) was used to compare molecular weights of isolated OMPs with previously published data (Salman et al., 2009).

Isolation of flagellin proteins. *S.* Enteritidis bacterial culture was grown on XLT4 agar plates overnight, inoculated into brain heart infusion broth, and incubated for 48 h at 37°C without shaking. The cells were washed with PBS and centrifuged at 7000×g for 30 min. The cells' pellet was treated with 3M potassium thiocyanate (Sigma, MO) in PBS for 2 h at room temperature with magnetic stirring. Subsequently, the cell suspension was centrifuged at 35,000×g for 30 min and the supernatant containing the flagellin enriched extract was dialyzed once against PBS, followed by Milli-Q water, and freeze-dried with 5% sucrose as a cryoprotectant. The protein concentration was estimated using micro BCA protein assay kit. SDS-page analysis was used to compare molecular weights of isolated OMPs with previously published data (Salman et al., 2009).

Preparation of OMPs-loaded and flagellin-loaded, flagellin-coated polyanhydride nanoparticle Salmonella vaccine. The OMPs-loaded and flagellin-loaded, flagellin-coated polyanhydride nanoparticle (OMPs-F-PNPs) *Salmonella* vaccine was formulated by a solvent displacement method as described previously with some modifications (Salman et al., 2009). Briefly, 2.5 mg each of both OMPs and flagellin were dispersed in 3 mL of acetone, added into 100 mg of sonicated polyanhydride (Mw ~216,000, Sigma, MO), and dissolved in 2 mL acetone under magnetic stirring. 50 µl of Span® 80 nonionic surfactant (Sigma, MO) were added and the organic solution was magnetically stirred for 1 h at room temperature. The polymer was desolvated by adding 7 mL of absolute ethanol followed by 3 mL deionized water containing 2.5 mg of flagellin proteins, and magnetic stirring was continued for another 1 h to evaporate the organic solvents. The formulated nanoparticle suspension with surface adsorbed flagellin was cross-linked

by incubation with 100 µg 1,3- diaminopropane for 5 min. Nanoparticles were obtained by centrifugation at 27,000×g for 20 min and freeze-dried with 5% sucrose as a cryoprotectant.

OMPs-F-PNPs Vaccination *In Vivo* Experiment

Birds. One-day-old Austra White laying chicks of both sexes (n = 50 birds total) were used in the present study. Chickens were provided *ad libitum* intake of water and feed and housed in cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University.

Treatments. At 6 wk of age, chicks were orally vaccinated with either PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chicks were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Birds were placed as pairs in cages in a completely randomized design.

Salmonella Challenge. A pure culture of nalidixic acid-resistant *S.* Enteritidis pure culture (Phage type 13a) was grown in 10 mL tryptic soy broth (TSB) at 37ºC without shaking. After 8 h of incubation, 100 µL of the bacterial suspension was transferred into 10 mL of fresh TSB and incubated overnight at 37ºC. One mL of the bacterial suspension was transferred into 100 mL of fresh TSB and incubated at 37ºC until the concentration of the bacteria reached approximately 1 x 10^9 CFU/ml as estimated spectrophotometrically at 600 nm. The bacteria were washed three times with PBS, serially diluted, and plated on Xylose Lactose Tergitol™ 4 (XLT4) agar plate to confirm actual concentration by enumeration of *S.* Enteritidis colonies

(CFUs). At 15 wk of age, birds were fasted for 8 h and then challenged using an oral gavage with 1 mL of $1x10^9$ CFU *S.* Enteritidis in PBS. Chickens were necropsied at 10 d post-*Salmonella* challenge for sample collection.

Effect of OMPs-F-PNPs Vaccination on OMPs-specific Antibody Titers. Serum, cloacal, bile, ileal, and tracheal anti-OMPs IgG and IgA antibody titers were analyzed using ELISA. Blood samples were collected at 6, 9, 12, and 15 wk of age pre-*Salmonella* challenge and at 10 d post-*Salmonella* challenge. Blood samples were centrifuged at 3000xg for 10 min and the serum-containing supernatants were collected and stored at -80^{o}C until use. Cloacal swabs were collected in 2 ml PBS, vortexed, centrifuged at 3000xg for 10 min, and supernatants were collected and stored at -80^{o}C until use. Ileum samples (0.01 m segments) were collected from the end of the duodenal loop and before the Meckel's diverticulum, homegenized with PBS, vortexed, centrifuged at 3000xg for 10 min, and supernatants were collected and stored at -80^{o}C until use. Trachea samples (0.01 m segments) were collected in PBS, homogenized with PBS, vortexed, centrifuged at 3000×g for 10 min, and supernatants were collected and stored at -80^{o}C until use. Bile samples were collected from the gallbladder using an insulin syringe and aliquots were directly stored at -80^{o}C until use.

Flat bottom, high binding 96-well plates (Greiner Bio-one, NC) were coated with pre-titrated amount of OMPs (2 µg/mL or 7.5 µg/mL for IgG or IgA ELISA, respectively) in 0.05 M sodium carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4^{o}C. Plates were washed three times and blocked with 5% skim milk powder in PBS Tween-20 (0.05%) (PBST) for 1 h at room temperature. Plates were then washed again in PBST three times. For analysis of serum

and bile, samples were diluted in 2.5% skim milk, and 50 µl of each sample were added in duplicate to the wells. For analysis of cloaca, ileum, and trachea, 50 µl of each undiluted sample were added in duplicate to the wells. Samples were then incubated for 2 h at room temperature. Plates were washed three times, and 50 µL/well of goat anti-chicken IgG conjugated HRP (Southern Biotech, AL) (1:10,000 in 2.5% skim milk powder in PBST) or goat anti-chicken IgA conjugated HRP (Gallus immunotech, NC) (1: 3000 in 2.5% skim milk powder in PBST) secondary antibodies were added. Plates were incubated for 2 h at room temperature, washed three times, and then 50 µl/well of TMB peroxidase substrate (1:1 mixture of TMB peroxidase substrate and TMB peroxidase substrate solution B) (KPL, MD) were added to each well. The reaction was stopped after 10 to 20 min by adding 1 M phosphoric acid. The OD was measured at 450 nm using the ELISA plate reader. The corrected OD was obtained by subtracting the treatment group OD from blank control OD.

Effect of OMPs-F-PNPs Vaccination on Lymphocyte Proliferation. At 10 d post-*Salmonella* challenge, blood and spleen samples were collected for isolation of peripheral blood mononuclear cells (PBMC). Blood samples were collected in sterile EDTA tubes and spleen samples were collected in 2 mL RPMI medium (GE Healthcare Life Sciences, UT). Samples were enriched with 10% E-RPMI (fetal bovine serum (Sigma), antibiotic-antimycotic (Gibco), sodium pyruvate, 1M HEPES, MEM NEAA). PBMC were isolated by using Ficoll-paque plus (GE Healthcare, PA) as per the manufacture's protocol with slight modifications. Briefly, blood samples were diluted in PBS (1:1 ratio), and an equal volume of Ficoll-paque plus solution was added and centrifuged at 450 xg for 25 min at 20^{o}C with breaks on. Lymphocytes at the cells

interface were collected, washed two times in PBS, and suspended in E-RPMI medium. Splenocytes were isolated by teasing spleen cells through a cell strainer using PBS. An equal volume of Ficoll-paque plus solution was added, and the suspension was centrifuged at 450 xg for 30 min at 4ºC with breaks on. Splenocytes at the interface were collected, washed two times with PBS, and cells were suspended in E-RPMI medium.

For the lymphocyte proliferation assay, PBMC and splenocytes were suspended in E-RPMI medium in triplicate wells and seeded in a 96-well flat bottom plate (Greiner bio-one, NC) at $2x10^6$ cells/well in 100 µL volume. Cells were re-stimulated with OMPs (5 µg/mL) in 100 µL E-RPMI medium and incubated for 72 h at 39ºC in a 5% CO_2 incubator. After incubation, 100 µL of supernatant was collected and 20 µL of MTS+PMS solution was added into cells and incubated for 4 h at 37ºC in a 5% CO_2 incubator. The OD was taken at 490 nm on an ELISA plate reader. Stimulation index (SI) was calculated by dividing OD of stimulated cells by OD of unstimulated control cells from the same chicken.

Effect of OMPs-F-PNPs Vaccination on Cecal Tonsil Toll-like Receptors and Cytokines. Cecal tonsil samples were collected at 10 d post-*Salmonella* challenge. Total RNA were extracted by using TRIzol reagent (Invitrogen). The isolated RNA were dissolved in Tris-EDTA (pH 7.5) buffer, and the concentration was determined using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The cDNA synthesis was achieved with 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions. The mRNA transcription of TLR-2, TLR-4, cytokines IFN-γ and IL-4, and the house keeping gene β-actin were analyzed by real-time PCR using the iQ™ SYBR®

Green Supermix (Bio-Rad, CA). Target gene transcription were normalized to the β-actin housekeeping gene. The housekeeping gene was verified by analyzing the average Ct value for β-actin in each treatment group and using ANOVA to confirm that the P –value for differences between groups was less than 0.05. Fold change from the reference was calculated using the $2^{(Ct\ Sample - Housekeeping)}/2^{(CtReference - Housekeeping)}$ comparative Ct method, where Ct is the threshold cycle (Schmittgen and Livak, 2008). The Ct was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background. Primers are described in Table 1.

Effect of OMPs-F-PNPs Vaccination on Cecal S. Enteritidis Colonization. At 10 d post-*Salmonella* challenge, whole ceca were collected. The ceca were weighed individually, homogenized in 2x concentration of peptone water growth medium, and incubated for 12 h at 37^{o}C for initial enrichment of the bacteria (Kallapura et al., 2014). Subsequently, samples were streaked on naladixic acid-resistant XLT plates and incubated for 24 h at 37^{o}C. The black *S.* Enteritidis colonies present on the plates were qualitatively confirmed as *Salmonella* as indicated in previous literature (Zheng et al., 2013).

Statistical Analysis. The data is represented as the mean ± standard error of mean (SEM) of 8 to 10 chickens (n = 8 to 10). Data were analyzed using a one-way ANOVA. When results were significant ($P < 0.05$), differences between means were analyzed using Tukey's Honest Significant Difference Test. Cecal *Salmonella* colonization was analyzed for presence or absence and was compared using the chi-squared test of independence, testing all possible group combinations to determine significance ($P < 0.05$).

RESULTS

Effect of OMPs-F-PNPs vaccination on OMPs-specific antibody titers pre-Salmonella challenge

There were significant differences among treatments at 12 and 15 wk of age (P = 0.05, P = 0.01, respectively). Birds vaccinated with OMPs-F-PNPs had significantly higher OMPs-specific serum IgG titers compared to those in the soluble antigen vaccinated group pre-*Salmonella* challenge by 48% and 49%, respectively (Fig. 12). There were no significant differences in OMPs-specific serum IgA titers among treatment groups pre-*Salmonella* challenge (9 wk, P = 0.53; 12 wk, P = 0.71, 15 wk, P = 0.90) (Fig. 13).

Effect of OMPs-F-PNPs vaccination on OMPs-specific IgG titers post-Salmonella Challenge

There were significant (P < 0.01) differences in serum IgG titers among treatments at 10 d post-*Salmonella* challenge (Fig. 14). Birds that were vaccinated with OMPs-F-PNPs had significantly higher serum OMPs-specific IgG titers compared to those from the mock + challenge and the mock treatment groups by 19% and 85%, respectively. There were significant (P < 0.01) differences in bile IgG titers among treatments at 10 d post-*Salmonella* challenge (Fig. 15). Birds that were vaccinated with OMPs-F-PNPs had significantly higher bile OMPs-specific IgG titers compared to those from the mock + challenge and the mock treatment groups by 42% and 98%, respectively.

Effect of OMPs-F-PNPs vaccination on OMPs-specific IgA titers post-Salmonella Challenge

There were significant (P < 0.01) differences in bile IgA titers among treatments at 10 d post-*Salmonella* challenge (Fig. 16). Birds that were vaccinated with OMPs-F-PNPs had

significantly higher bile OMPs-specific IgA titers compared to those from the mock treatment group. At 10 d post-*Salmonella* challenge, there were significant ($P < 0.01$) differences in cloacal IgA titers among treatments (Fig. 17). Birds that were vaccinated with OMPs-F-PNPs had significantly higher cloacal OMPs-specific IgA titers compared to those from the mock treatment group by 96%. There were significant ($P < 0.01$) differences in intestinal IgA titers among treatments at 10 d post-*Salmonella* challenge (Fig. 18). Birds that were vaccinated with OMPs-F-PNPs had significantly higher intestinal OMPs-specific IgA titers compared to those from the mock treatment group by 92%. There were significant ($P < 0.01$) differences in tracheal IgA titers among treatments at 10 d post-*Salmonella* challenge (Fig. 19). Birds that were vaccinated with OMPs-F-PNPs had significantly higher tracheal OMPs-specific IgA titers compared to those from the mock treatment groups.

Effect of OMPs-F-PNPs vaccination on PBMC and splenocyte proliferation

There were no significant ($P > 0.05$) effects of OMPs-F-PNPs vaccination on PBMC and splenocyte proliferation, respectively (Fig. 20 and 21).

Effect of OMPs-F-PNPs vaccination on mRNA transcription of TLR-2, TLR-4, IFN-γ, and IL-4 in the cecal tonsils at 10 d post-Salmonella challenge

Birds vaccinated with OMPs-F-PNPs had increased ($P < 0.01$) TLR-4 mRNA transcription compared to that from the mock + challenge group by 44% (Fig. 22). There were no significant ($P > 0.05$) differences in TLR-2 mRNA transcription among treatment groups at 10 d post-*Salmonella* challenge (Fig. 23). There were no significant ($P > 0.05$) differences among the

treatment groups on IFN-γ mRNA transcription and IL-4 mRNA transcription (Fig. 24 and 25, respectively).

Effect of OMPs-F-PNPs vaccination on cecal Salmonella colonization

At 10 d post-*Salmonella* challenge, the OMPs-F-PNPs vaccinated birds had significantly fewer positive samples with cecal *S.* Enteritidis compared to that of the mock + challenge group by 33% (Table 8).

DISCUSSION AND CONCLUSION

Both humoral and cellular immune responses were assessed to study the effects of OMPs-F-PNPs vaccination on the anti-*Salmonella* immune response in layer chickens. At 10 d post-challenge, the OMPs-F-PNPs vaccinated birds had significantly higher OMPs-specific IgG antibody titers in the serum and bile compared to the mock + challenge treatment. The OMPs-F-PNPs vaccinated birds had significantly higher OMPs-specific IgA titers compared to the mock treatment in the bile, cloacal, ileal, and tracheal samples. There were no significant differences among treatments in PBMC and splenocyte proliferation. The OMPs-F-PNPs vaccinated birds had significantly higher TLR-4 transcription compared to the mock + challenge treatment. Lastly, the analysis of *S.* Enteritidis colonization in the ceca demonstrated that the OMPs-F-PNPs vaccination significantly decreased *S.* Enteritidis colonization by 33%.

The co-polymer coating of the OMPs-F-PNPs vaccine prevents degradation of the nanoparticle from acidity, resulting in increased survivability of the antigen while transiting through the gastrointestinal tract to the site of *Salmonella* infection within the small intestine (Ochoa et al., 2007). The conjugation of surface-ligands, such as flagellin proteins, to the

nanoparticle vaccine enhances uptake through the peyer's patches by mimicking the pathway of natural *Salmonella* bacteria (Salman et al., 2005). The M cells of peyer's patches sample and process mucosal foreign antigens, thus activating mucosal immune responses (Miller et al., 2007). The goal of oral vaccination is to target antigens to mucosal M cells, thereby activating antigen presenting immune cells within the peyer's patches, trying to imitate the natural process of initiating immunity against enteric pathogens (Azizi et al., 2010).

The analysis of OMPs-F-PNPs vaccination in layer chickens challenged with *Salmonella* showed a significantly higher OMPs-specific IgG response and numerically higher OMPs-specific IgA response when compared to the mock + challenge group. The OMPs are major immunodominant proteins and have previously elicited high antibody titers in chickens administered with OMPs vaccines (Meenakshi et al., 1999). A possible explanation for significantly higher OMPs-specific IgA compared to only the mock group, as opposed to the mock + challenge group, is that secretory IgA antibodies adhere to *Salmonella* to prevent *Salmonella* colonization (Shroff et al., 2995) and can therefore be difficult to accurately measure during a challenge. An additional explanation for the significant OMPs-specific IgG response compared to the OMPs-specific IgA response may be due to the low vaccine dose. Therefore the observed systemic response may have been induced by the polyanhydride coating as opposed to the vaccine dose (Gregory et al., 2013).

Our current experiment utilized real-time PCR analysis and identified that OMPs-F-PNPs vaccination significantly increased cecal tonsil TLR-4 mRNA transcription. This is likely due to the protective polyanhydride coating of the nanoparticles used in this study. Polyanhydrides are

recognized by immune cells as damage associated molecular patterns, resulting in an initiation of inflammatory response and upregulation of toll-like receptors (Seong and Matzinger, 2004). TLR-4 is positively correlated with activation of the NF-kB signaling pathway and production of inflammatory cytokines (Keestra and van Putten, 2008). Higher TLR-4 mRNA transcription may indicate that OMPs-F-PNPs vaccination had significant positive effects on the cell-mediated immune response.

Tamayo et al. (2010) demonstrated that polyanhydride nanoparticles stimulated both TLR-2 and TLR-4 in human cell lines. In addition, Tamayo et al. (2010) observed that polyanhydride nanoparticle vaccination in mice resulted in significantly higher levels of Th1 and Th2 cytokines IFN-γ and IL-4 in splenic cells, respectively. In the current study, we did not observe significant differences in cecal TLR-2 or IFN-γ. We also did not observe differences in cecal IL-4 transcription, despite observing a significant OMPs-specific humoral immune response. It is important to note that *S.* Enteritidis colonization in mice causes a systemic, typhoid-like infection, whereas colonization in adult chickens rarely results in clinical effects (Silva et al., 2012). Consequently, inducing significant immune responses in chickens challenged with *Salmonella* may require alternative vaccine approaches compared to mice.

To further analyze the effects of OMPs-F-PNPs vaccination on the cell-mediated immune response, a lymphocyte proliferation assay was utilized for PBMC and splenocytes. However, there were no significant differences in lymphocyte proliferation in our experiment. Sood et al. (2005) reported a higher proliferative response in splenocytes from mice that were vaccinated intraperitoneally with OMPs compared to the control. This is likely a result of the antigens

entering the peyer's patches and stimulating a cell-mediated immune response (Sood et al., 2005). It is possible that we did not see significant differences due to our sampling time at 10 d post-*Salmonella* challenge. Sood et al. (2005) reported significant increases in splenocyte proliferation at 3 d post-*Salmonella* challenge followed by a gradual decrease by 7 d. These authors also reported that the decrease in proliferation may have been due to decreased antigen expression of the antigen presenting cells at 7 d post-challenge.

Our study demonstrated that OMPs-F-PNPs vaccination increased OMPs-specific antibody responses and cecal TLR-4 transcription, suggesting the potential for reducing *Salmonella* colonization. The results also showed that OMPs-F-PNPs vaccination significantly decreased *S.* Enteritidis colonization compared to the mock + challenge group by 33%. Complete protection against *Salmonella (*100% decrease in colonization) may require increasing the vaccine dosage or including additional, secondary vaccine adjuvants (Meenakshi et al., 1999; Sood et al., 2005). It is plausible that the high bacterial challenge dose of 1 x 10^9 CFU of *S.* Enteritidis hindered the vaccine's effectiveness. Fasting the birds overnight may have further increased bacterial cecal colonization (Holt et al., 2006).

Both humoral and cell-mediated immune responses are necessary for complete protection against *S.* Enteritidis infection in chickens (Sheela et al., 2003). The observed significant increase in anti-*Salmonella* humoral response, upregulation of TLR-4, and decrease in cecal *S.* Enteritidis colonization in the birds vaccinated with OMPs-F-PNPs suggests the potential for an effective oral vaccine against *Salmonella.* However, additional studies are needed in OMPs-F-PNPs vaccination for enhancement of the anti-*Salmonella* cell-mediated response, a more

balanced Th1/Th2 immune response, and greater protection against *Salmonella* colonization in chickens.

Chapter 6: Conclusion

Due to the decrease in sub-therapeutic antibiotic use in chickens, it is essential to study alternative strategies for mitigating pathogenic intestinal infection. Laying hens raised in cage-free environments further increases risk of bacterial contamination in eggs, as eggs are more likely to come in contact with feces (Whiley and Ross, 2015). *Salmonella* is an intracellular bacterium that can cause the zoonotic infection, salmonellosis, in humans (Pires et al. 2014). Symptoms of salmonellosis include stomach irritation accompanied by vomiting, diarrhea, and high fever (Crum-Cianflone, 2008). These symptoms are extremely disruptive and can last for several days. Salmonellosis can often lead to death in humans that are immunocompromised, especially those who are very young or old (Crum-Cianflone, 2008). There are over a million of cases of salmonellosis that occur annually in the United States (Fabrega and Vila, 2013). *Salmonella* frequently colonizes within the intestines of chickens; subsequently, salmonellosis is most often attributed to consumption of contaminated poultry meat and eggs (Braden, 2006). Vaccines are commonly administered in poultry to decrease *Salmonella* colonization, but do not completely decrease *Salmonella* in many poultry farm settings (Davies and Breslin, 2003). Consequently, feed additives with bacteriostatic and bactericidal properties are commonly supplemented in conjunction with *Salmonella* vaccines (Patterson and Burkholder, 2003).

Chapter 3 analyzed the effects of drinking water synbiotic supplementation in laying hens with and without a *Salmonella* challenge. Synbiotic supplementation decreased cecal *S.* Enteritidis colonization. Three out of four of the supplemented synbiotics, *L. reuteri, B. animalis,*

and *P. acidilactici* were identified within the cecal contents of supplemented birds. Synbiotic supplementation increased *Salmonella*-specific IgA titers and decreased *Salmonella*-specific IgG titers. Synbiotic supplemention decreased IL-10 and LITAF cytokine transcription compared to unsupplemented birds. At 3 d post-challenge, birds supplemented with synbiotics had longer villi lengths in the jejunum compared to unsupplemented birds. Synbiotics can decrease pathogenic bacteria within the intestinal tract through several mechanisms, including competitive exclusion, stimulation of the immune system, and production of antibacterial substances such as bacteriocins or lactic acid (Van Der Wielen et al., 2000; Yang et al., 2012; Lawley and Walker, 2013). The results of this experiment express that synbiotic supplementation can positively affect the performance and anti-*Salmonella* immune response of laying hens.

Chapter 4 analyzed the effects of acidifier supplementation in laying hens with and without a *Salmonella* challenge. Acidifier supplementation decreased relative percentage of *S.* Enteritidis and increased relative percentage of *Bifidobacteria* within the ceca. Acidifier supplementation decreased IL-10 and LITAF cytokines mRNA transcription compared to unsupplemented birds. The effects of acidifier supplementation on the anti-*Salmonella* immune response decreased plasma IgG titers at 24 d post-challenge, decreased bile IgA titers at 10 d post-challenge, and increased bile IgA titers at 22 d post- challenge. Additionally, acidifier supplementation increased plasma IgA titers in challenged birds at 17, 22, 24, and 30 d post-*Salmonella* challenge. Acidifier supplementation increased body weight and hen day egg production compared to unsupplemented birds. Acidifiers supplementation has previously been associated with lower pathogenic colonization and enhanced performance (Khan and Iqbal,

2016). Organic acids can penetrate the membrane of gram-negative bacteria, resulting in bacteriostatic or bactericidal effects (Hedayati et al., 2014). Additionally, the decrease in pH may result in a less suitable environment for pathogens (Murry et al., 2004). Organic acids are also important sources of energy for intestinal epithelial cells and can stimulate intestinal enterocyte growth and proliferations (Blottiere et al., 2003). The results of the acidifier experiment demonstrate that acidifier supplementation in laying hens can decrease *Salmonella* infection, modulate the cecal bacteria population, and improve body weight and hen day egg production.

In Chapter 5, the effects of an oral polyanhydride *Salmonella* nanoparticle vaccine were analyzed in chickens challenged with *S.* Enteritidis. The OMPs-F-PNPs vaccinated birds had significantly increased serum and bile IgG antibody titers compared to the mock + challenge group. The OMPs-F-PNPs vaccinated birds had significantly higher cecal TLR-4 mRNA transcription compared to the mock + challenge group. The OMPs-F-PNPs vaccinated birds had significantly decreased *S.* Enteritidis colonization by 33% compared to the mock + challenge treatment. The co-polymer coating of polyanhydride nanoparticles increases survivability of the vaccine antigens by preventing degradation of the antigens from acidity and enzymes within the gastrointestinal tract (Ochoa et al., 2007). Furthermore, oral polyanhydride nanoparticle vaccines induce local mucosal immunity (Ulery et al., 2011) and activate toll-like receptors (TLRs) -2 and -4 (Reboucas et al., 2012; Tamayo et al., 2010). The conjugation of flagellin proteins attached to nanoparticle vaccines enhances uptake through the PPs by mimicking the pathway of natural *Salmonella* bacteria (Salman et al., 2005). Our results identified that OMPs-F-PNPs vaccinated birds had increased anti-*Salmonella* immune response and decreased *Salmonella* colonization.

Overall, we found decreased cecal *Salmonella* colonization in birds supplemented with either drinking water synbiotics, in-feed acidifiers, or the polyanhydride nanoparticle vaccine. Additionally, treatment with either synbiotics, acidifiers, or the polyanhydride *Salmonella* nanoparticle vaccine each resulted in enhanced anti-*Salmonella* immune responses. Future studies may be beneficial for optimizing the dosage and design of the polyanhydride nanoparticle vaccine to increase protection against *Salmonella* colonization in chickens. *Salmonella* clearance within the gastrointestinal tract of poultry requires a complex, long lasting immune response (Sheela et al., 2003). Therefore, additional studies would also be beneficial for analyzing the combined effects of synbiotic and acidifier supplementation in conjunction with administration of the OMPs-F-PNPs vaccine. The results of the present studies express the benefit of drinking water synbiotics, in-feed acidifiers, and the OMPs-F-PNPs vaccine in decreasing *Salmonella* infection. These implications are especially important in the interest of decreased antibiotic-use within the poultry industry.

Appendix A: Tables

Table 1. Real-time PCR primers for cytokine and TLR analysis.

Target	Primer	Sequence (5' – 3')	Reference
β-actin	F	ACCGGACTGTTACCAACACC	Selvaraj et al., 2010
	R	GACTGCTGCTGACACCTTCA	
LITAF	F	ATCCTCACCCCTACCCTGTC	Shanmugasundaram and
	R	GGCGGTCATAGAACAGCACT	Selvaraj, 2010
IL-10	F	GAGGAGCAAAGCCATCAAGC	Shanmugasundaram and
	R	CTCCTCATCAGCAGGTACTCC	Selvaraj, 2010
IFN-γ	F	GTGAAGAAGGTGAAAGATATCATGGA	Shanmugasundaram
	R	GCTTTGCGCTGGATTCTCA	et al., 2013
TLR-2	F	GTCAACAGCTTCTCCAAGG	Shanmugasundaram and
	R	CCACCAGGATGAGGATGAAC	Selvaraj, 2011
TLR-4	F	ACCTACCCATCGGACACTTG	Shanmugasundaram and
	R	TGCCTGAGAGGTCAGGTT	Selvaraj, 2011

Table 2. Real-time PCR primers for bacterial analysis.

Target	Primer	Sequence (5' – 3')	Reference
16S	F	AGAGTTTGATCCTGGCTCAG	Amit-Romach et al., 2004
	R	GACTACCAGGGTATCTAATC	
S. enterica	F	GCAGCGGTTACTATTGCAGC	De Medici et al., 2003
	R	CTGTGACAGGGACATTTAGCG	
E. faecium	F	GCAAGGCTTCTTAGAGA	Dutka-Malen et al., 1995
	R	CATCGTGTAAGCTAACTTC	
L. reuteri	F	CAGGATCGGTAATTGATG	Sattler et al., 2014
	R	TGGATATGGAAGTTCGTC	
B. animalis	F	GTGGAGACACGGTTTCCC	Ventura and Zink, 2002
	R	CACACCACACAATCCAATAC	
P. acidilacti	F	GGACTTGATAACGTACCCGC	Mora et al., 1997
	R	GTTCCGTCTTGCATTTGACC	
Bifidobacterium	F	GGGTGGTAATGCCGGATG	Amit-Romach et al., 2004
	R	CCACCGTTACACCGGGAA	
Lactobacillus	F	CATCCAGTGCAAACCTAAGAG	Amit-Romach et al., 2004
	R	CCACCGTTACACCGGGAA	

Table 3. Effect of synbiotic supplementation on relative percentage of *L. reuteri*, *B. animalis* and *P. acidilactici* in cecal content post-*Salmonella* challenge[1]

		Control			Synbiotic						
Cecal Bacteria	Days Post-Challenge	V	C	V+C	V	C	V+C	Standard Error	Trt P Value	Syn P Value	Trt X Syn P Value
L. reuteri	3	0^{b}	0^{b}	0^{b}	0.02^{ab}	0.06^{a}	0.01^{b}	0.01	0.05	0.01	0.05
L. reuteri	8	0	0	0	0.01	0.01	0.01	0.01	0.19	<0.01	0.17
B. animalis	10^{b}	0^{b}	0^{b}	0^{b}	0.01^{b}	0.01^{b}	0.05^{a}	0.01	<0.01	<0.01	<0.01
P. acidilacti	3	0	0	0	0.05	0.13	0.18	0.07	0.61	0.04	0.06
P. acidilacti	8	0	0	0	0.01	0.01	0.01	0.01	0.36	0.01	0.36
P. acidilacti	10	0^{b}	0^{b}	0^{b}	0.02^{b}	0.01^{b}	0.13^{a}	0.02	0.03	0.02	0.03
P. acidilacti	24	0	0	0	0.03	0.13	0.23	0.04	0.10	<0.01	0.10

[1]Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^{9} CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Cecal content samples were collect at 3, 8, 10, and 24 d post-*Salmonella* challenge and the relative percentage of cecal bacteria was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The percentage of cecal bacteria is presented where the total of the examined bacteria was set at 100%. Relative percentages with no common superscript differ ($P \leq 0.05$).

Table 4. Effect of synbiotic supplementation on plasma IgA titers post-*Salmonella* challenge[1]

Days Post-Challenge	Control			Synbiotic			Standard Error	Treatment P Value	Synbiotic P Value	Treatment* Synbiotic P Value
	Vaccine	Challenge	Vaccine+ Challenge	Vaccine	Challenge	Vaccine+ Challenge				
8	0.04^{b}	0.05^{b}	0^{b}	0.65^{a}	0.90^{a}	0.04^{b}	0.09	<0.01	<0.01	<0.01
10	0.01^{d}	0.06^{cd}	0^{d}	0.54^{b}	1.06^{a}	0.30^{bc}	0.07	<0.01	<0.01	<0.01
24	0.01^{b}	0.05^{b}	0.01^{b}	0.04^{b}	0.19^{a}	0.04^{b}	0.02	<0.01	<0.01	<0.01
30	0.01^{b}	0.05^{b}	0.01^{b}	0.06^{b}	0.26^{a}	0.07^{b}	0.02	<0.01	<0.01	<0.01

[1]Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 109 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Plasma samples were collected at 8, 10, 24, and 30 d post-*Salmonella* challenge and analyzed for *Salmonella*-specific IgA titers through ELISA and results are reported as average optical density (OD) values. The baseline OD value was set at 0 for anti-*Salmonella* IgA, based on the average OD values from chicken plasma samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge. The OD values with no common superscript differ ($P \leq 0.05$).

Table 5. Effect of synbiotic supplementation on plasma IgG titers post-*Salmonella* challenge[1]

Days Post-Challenge	Control			Synbiotic			Standard Error	Treatment P Value	Synbiotic P Value	Treatment* Synbiotic P Value
	Vaccine	Challenge	Vaccine+ Challenge	Vaccine	Challenge	Vaccine+ Challenge				
22	0.51	0.45	0.40	0.56	0.56	0.58	0.02	0.04	<0.01	0.10
30	0.39	0.42	0.47	0.56	0.56	0.52	0.03	<0.01	<0.01	0.10

[1]Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Plasma samples were collected at 8, 10, 24, and 30 d post-*Salmonella* challenge and analyzed for *Salmonella*-specific IgG titers through ELISA. Results are reported as average optical density (OD) values. The baseline OD value was set at 0.02 for anti-*Salmonella* IgG, based on the average OD values from chicken plasma samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge. The OD values with no common superscript differ ($P \leq 0.05$).

Table 6. Effect of acidifier supplementation on weekly HDEP (percentage) pre-*Salmonella* challenge[1]

	Control		Acidifier					
Age (wks)	Vaccine	No Vaccine	Vaccine	No Vaccine	Standard Error	Treatment P Value	Acidifier P Value	Treatment X Acidifier P Value
19	0.00[b]	0.00[b]	0.52[b]	2.60[a]	0.49	0.04	<0.01	0.04
20	2.08	4.76	20.83	21.73	2.75	0.53	<0.01	0.75
21	34.08	45.24	69.05	65.48	3.72	0.32	<0.01	0.06
22	78.72	85.12	89.14	86.61	2.29	0.41	0.02	0.06
23	91.98	91.05	96.14	96.30	1.95	0.85	0.02	0.79

[1]Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 23 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age, resulting in a 2 (vaccinated and unvaccinated) X 2 (control and acidifier) factorial arrangement of treatments. Weekly HDEP (percentage) were measured at 19, 20, 21, 22, and 23 wk of age. Weekly HDEP with no common superscript differ ($P \leq 0.05$).

Table 7. Effect of acidifier supplementation on plasma IgA, bile IgA, and plasma IgG titers post-*Salmonella* challenge[1]

Sample Type	Days Post-Challenge	Control			Acidifier			Standard Error	Trt P Value	Acidifier P Value	Trt X Acidifier P Value
		V	C	V+C	V	C	V+C				
Plasma IgA	17	0^{b}	0.01^{b}	0^{b}	0^{b}	0.06^{a}	0^{b}	0.01	<0.01	0.20	<0.01
Plasma IgA	22	0.01^{b}	0.03^{b}	0.02^{b}	0.03^{b}	0.32^{a}	0.03^{b}	0.04	<0.01	<0.01	<0.01
Plasma IgA	24	0^{b}	0.03^{b}	0.01^{b}	0.04^{b}	0.22^{a}	0.07^{b}	0.02	<0.01	<0.01	<0.01
Plasma IgA	30	0.01^{b}	0.05^{b}	0.01^{b}	0.05^{b}	0.20^{a}	0.05^{b}	0.02	<0.01	<0.01	0.03
Bile IgA	10	0.10^{ab}	0.34^{a}	0.08^{ab}	0.09^{ab}	0.03^{b}	0.11^{ab}	0.06	0.25	0.07	0.02
Bile IgA	22	0.12	0.11	0.11	0.24	0.68	0.28	0.06	0.09	<0.01	0.08
Plasma IgG	24	0.70^{a}	0.72^{a}	0.78^{a}	0.70^{a}	0.61^{ab}	0.45^{b}	0.04	0.13	<0.01	<0.01

[1]Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^{9} CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. Plasma and bile samples were collected at 17, 22, 24, and 30 d post-*Salmonella* challenge, and analyzed for *Salmonella*-specific IgA and IgG titers through ELISA. Results are reported as average optical density (OD) values. The baseline OD value was set at 0 for anti-*Salmonella* IgA and 0.02 for anti-*Salmonella* IgG, based on the average OD values from chicken plasma and bile samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge. The OD values with no common superscript differ ($P \leq 0.05$)

Table 8. Effect of PNPs on cecal *S.* Entertidis colonization at 10 d post-*Salmonella* challenge.[1]

	Mock	Mock + challenge	Antigens	PNPs	P-value
Positive Birds/Total Birds	0/9 (0%)[c]	10/10 (100%)[a]	9/10 (90%)[ab]	6/9 (67%)[b]	P < 0.01

[1]At 6 wk of age, birds were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, birds were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Whole ceca were collected at 10 d post-*Salmonella* challenge, enriched for 12 h at 37°C, streaked on naladixic acid-resistant XLT plates, and incubated for 24 h at 37°C. The black *S.* Enteritidis colonies present on the plates were qualitatively confirmed as *Salmonella.* Percentage of *S.* Enteritidis positive samples with no common superscript differ ($P \leq 0.05$).

Appendix B: Figures

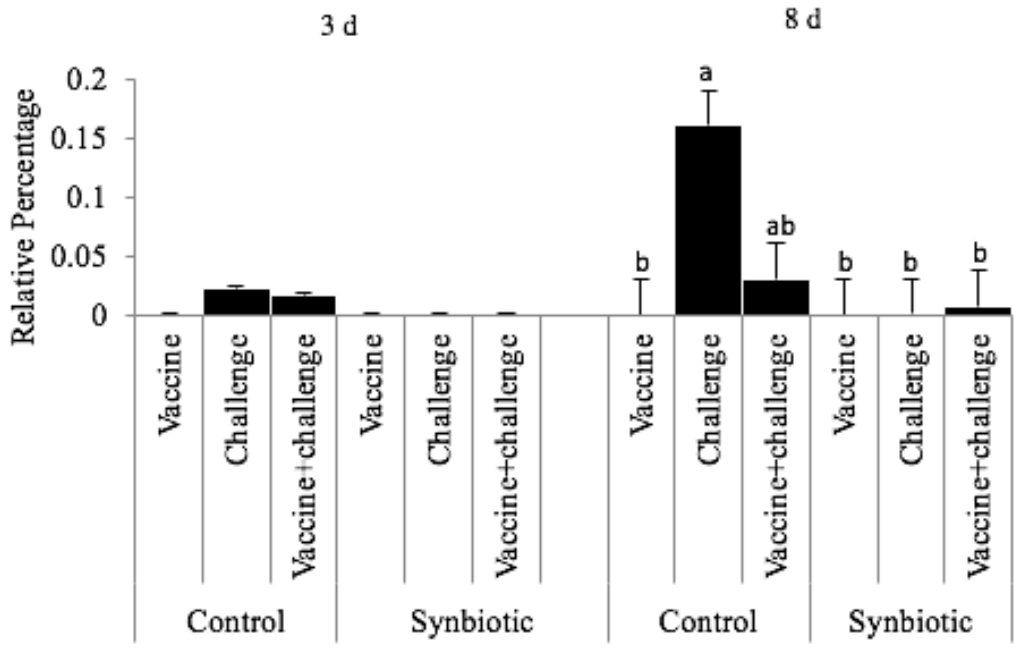


Figure 1. Effect of drinking water synbiotic supplementation on relative percentage of *S.* Enteritidis compared to total bacteria in cecal content at 3 and 8 d post-*Salmonella* challenge. Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. Cecal content samples were collected at 3 and 8 d post-*Salmonella* challenge and the relative percentage of *S.* Enteritidis in the cecal content was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The percentage of *S.* Enteritidis is presented where the total of the examined bacteria was set at 100%. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Day 3, Synbiotic P = 0.27, Treatment P = 0.03, Synbiotic X Treatment P = 0.28. Day 8, Synbiotic P = 0.05, Treatment P = 0.04, Synbiotic X Treatment P = 0.04.

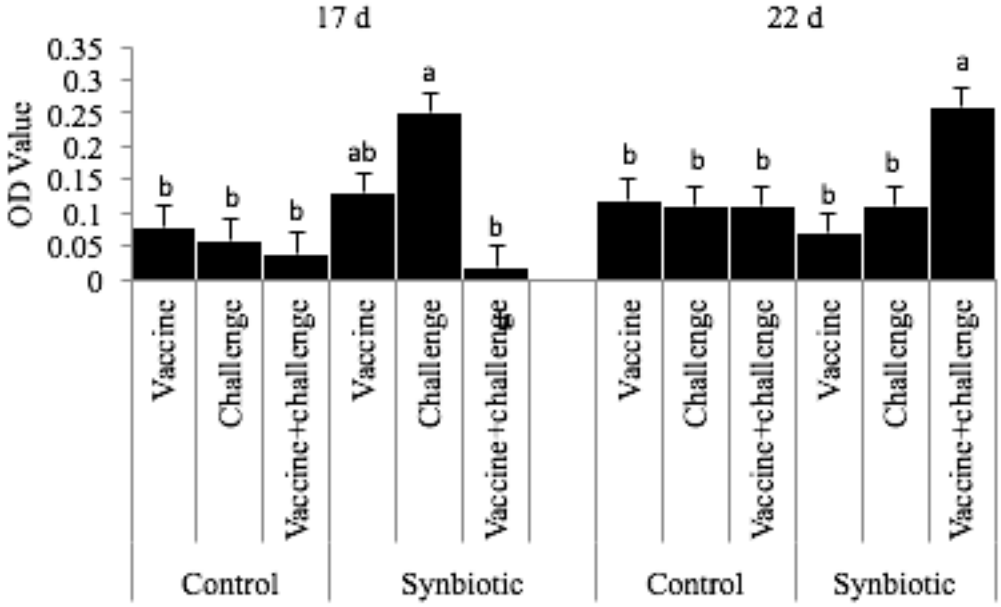


Figure 2. Effect of drinking water synbiotic supplementation on bile anti-*Salmonella* IgA titers at 17 and 22 d post-*Salmonella* challenge. Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. Bile samples were collected at 17 and 22 d post-*Salmonella* challenge and analyzed for *Salmonella*-specific IgA titers through ELISA and results are reported as average optical density (OD) values. The baseline OD value was set at 0 for anti-*Salmonella* IgA, based on the average OD values from chicken bile samples (n = 6) pre-*Salmonella* vaccination and pre-*Salmonella* challenge. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Day 17, Synbiotic P = 0.84, Treatment P < 0.01, Synbiotic X Treatment P = 0.01. Day 22, Synbiotic P = 0.18, Treatment P = 0.01, Synbiotic X Treatment P < 0.01.

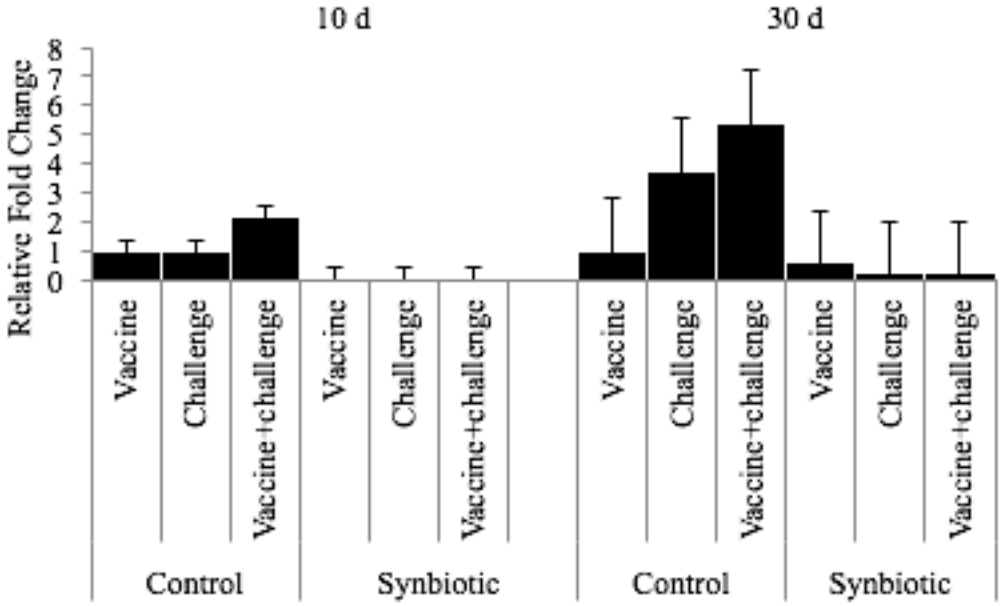


Figure 3. Effect of drinking water synbiotic supplementation on cecal tonsil LITAF mRNA transcription at 10 and 30 d post-*Salmonella* challenge. Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. At 10 and 30 d post-*Salmonella* challenge, cecal tonsils were collected and analyzed for LITAF mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine control group, so all bars represent fold change compared to the vaccine group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Day 10, Synbiotic $P < 0.01$, Treatment $P = 0.24$, Synbiotic X Treatment $P = 0.24$. Day 30, Synbiotic $P = 0.05$, Treatment $P = 0.40$, Synbiotic X Treatment $P = 0.54$.

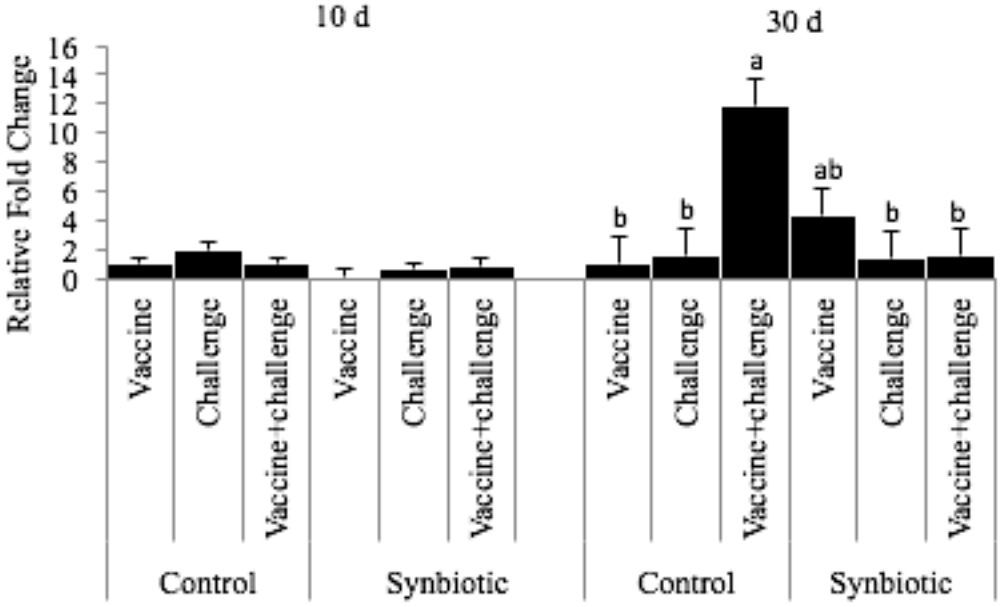


Figure 4. Effect of drinking water synbiotic supplementation on cecal tonsil IL-10 mRNA transcription at 10 and 30 d post-*Salmonella* challenge. Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. At 10 and 30 d post-*Salmonella* challenge, cecal tonsils were collected and analyzed for IL-10 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine control group, so all bars represent fold change compared to the vaccine group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Day 10, Synbiotic P = 0.04, Treatment P = 0.26, Synbiotic X Treatment P = 0.32. Day 30, Synbiotic P = 0.15, Treatment P = 0.04, Synbiotic X Treatment P < 0.01.

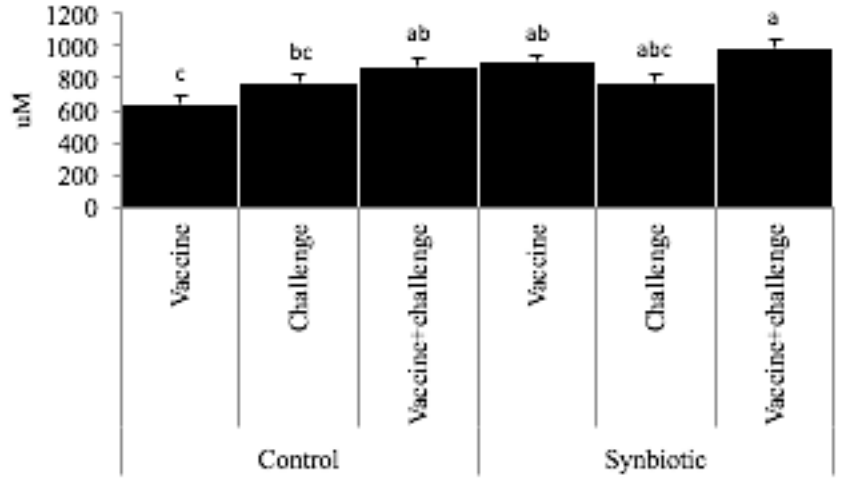


Figure 5. Effect of drinking water synbiotic supplementation on jejunal villi length at 3 d post-*Salmonella* challenge. Birds were fed basal diet and supplemented or not supplemented (control) with drinking water synbiotic product from 0 to 3 days of age and for the 3 days directly following each feed change and the *Salmonella* vaccine and challenge. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial arrangement. At 3 d post-*Salmonella* challenge, jejunum samples were collected and villi length was measured. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Day 3, Synbiotic $P < 0.01$, Treatment $P < 0.01$, Synbiotic X Treatment $P = 0.05$.

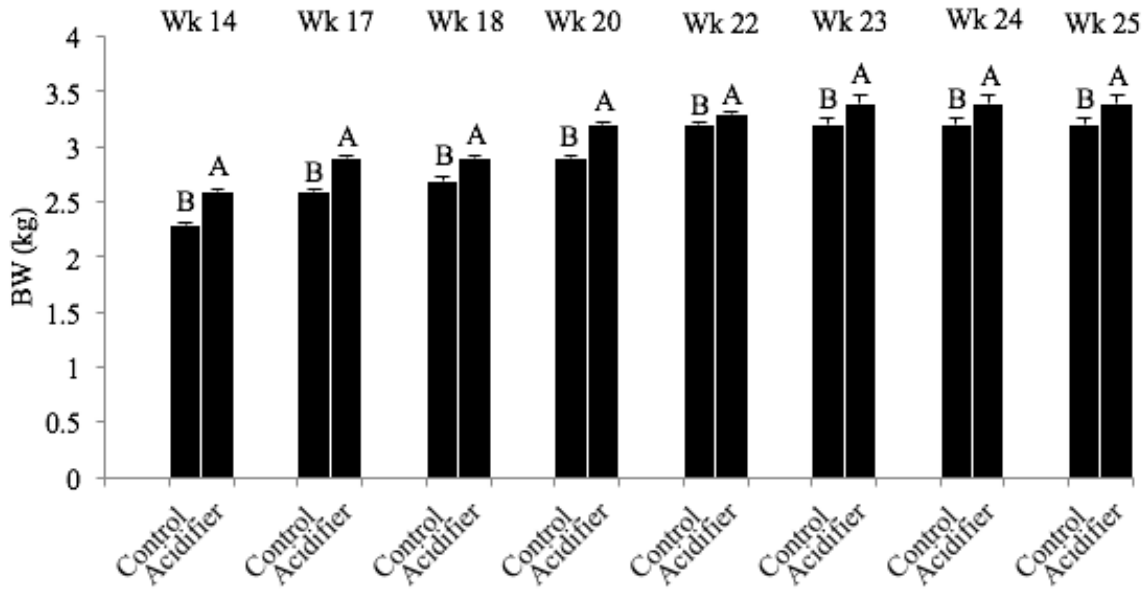


Figure 6. Effect of acidifier supplementation on body weight (kg) pre- and post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age, resulting in a 2 (vaccinated and unvaccinated) X 2 (control and acidifier) factorial arrangement of treatments. Body weights (kg) were measured at 14, 17, 18, 20, 22, and 23 wk of age. At 24 wk of age, birds were challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. Body weights (kg) were measured at 24 and 25 wk of age (3 and 10 d post-*Salmonella* challenge). Because the interaction effects between acidifier supplementation and treatments were not significant ($P > 0.05$), main effects of acidifier were analyzed. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: Bars (+ SE) with no common superscript differ ($P \leq 0.05$). Wk 14, $P < 0.01$; wk 17, $P < 0.01$; wk 18, $P < 0.01$; wk 20, $P < 0.01$; wk 22 $P < 0.01$; wk 24 $P = 0.03$; wk 25, $P = 0.03$.

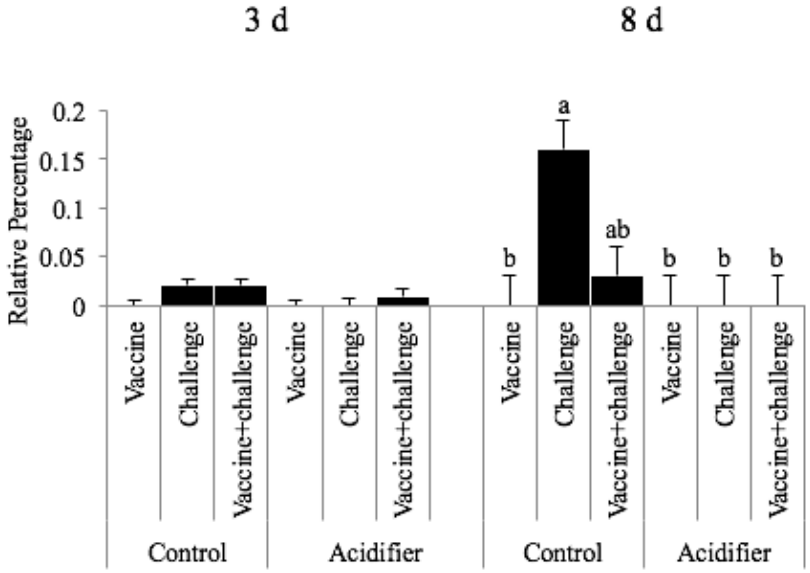


Figure 7. Effect of acidifier supplementation on relative percentage of *S.* Enteritidis compared to total bacteria in cecal content at 3 and 8 d post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. Cecal content samples were collected at 3 and 8 d post-*Salmonella* challenge and the relative percentage of *S.* Enteritidis in the cecal content was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The percentage of *S.* Enteritidis is presented where the total of the examined bacteria was set at 100%. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: D 3, Acidifier P = 0.05, Treatment P = 0.13, Acidifier X Treatment P = 0.24. D 8, Acidifier P = 0.02, Treatment P = 0.04, Acidifier X Treatment P = 0.04.

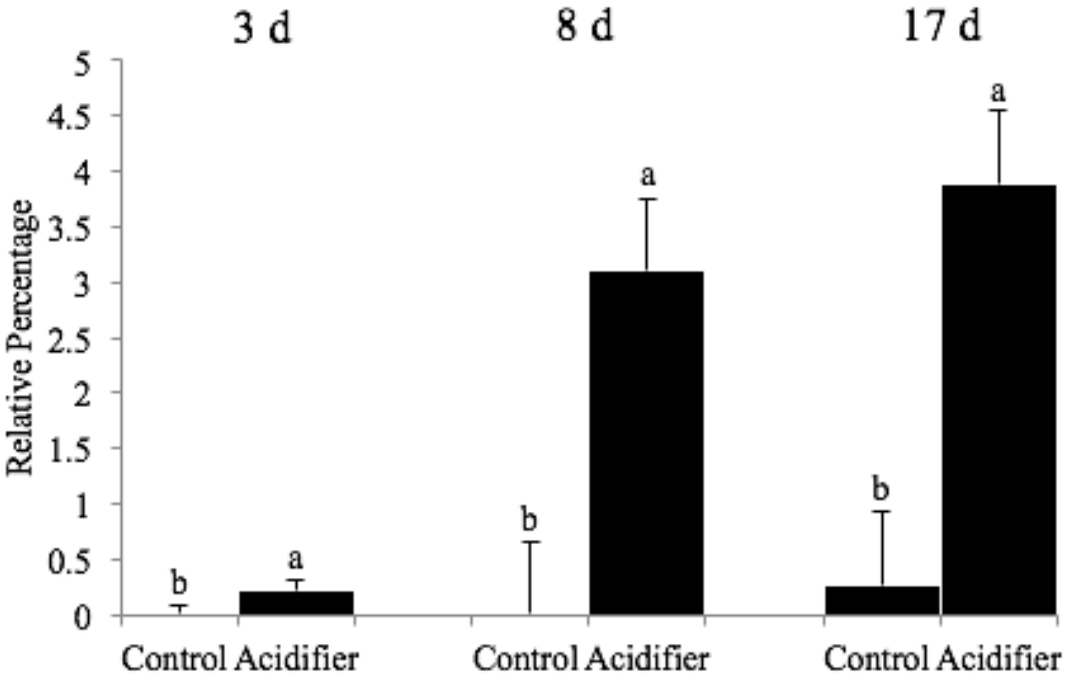


Figure 8. Effect of acidifier supplementation on relative percentage of *Bifidobacterium* compared to total bacteria in cecal content at 3, 8, and 17 d post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. Cecal content samples were collected at 3, 8, and 17 d post-*Salmonella* challenge and the relative percentage of *Bifidobacterium* in the cecal content was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The percentage of *Bifidobacterium* is presented where the total of the examined bacteria was set at 100%. Because the interaction effects between acidifier supplementation and treatments were not significant ($P > 0.05$), main effects of acidifier were analyzed. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: D 3, $P = 0.03$; d 8, $P < 0.01$; d 17, $P < 0.01$.

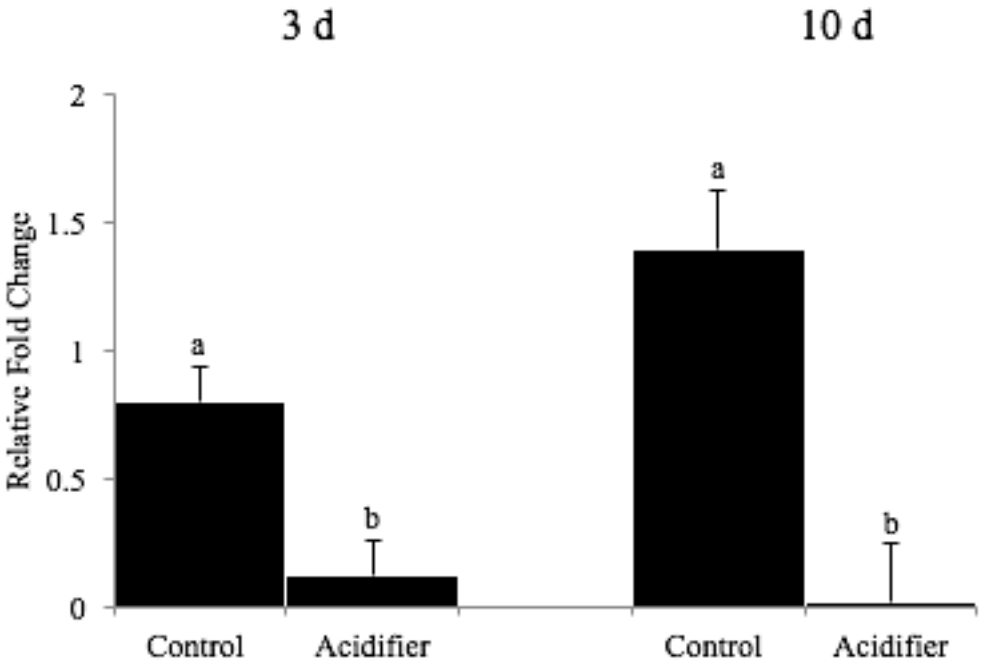


Figure 9. Effect of acidifier supplementation on cecal tonsil LITAF mRNA transcription at 3 and 10 d post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. At 3 and 10 d post-*Salmonella* challenge, cecal tonsils were collected and analyzed for LITAF mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine control group. Because the interaction effects between acidifier supplementation and treatments were not significant ($P > 0.05$), main effects of acidifier were analyzed. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: D 3, $P < 0.01$; d 10, $P < 0.01$.

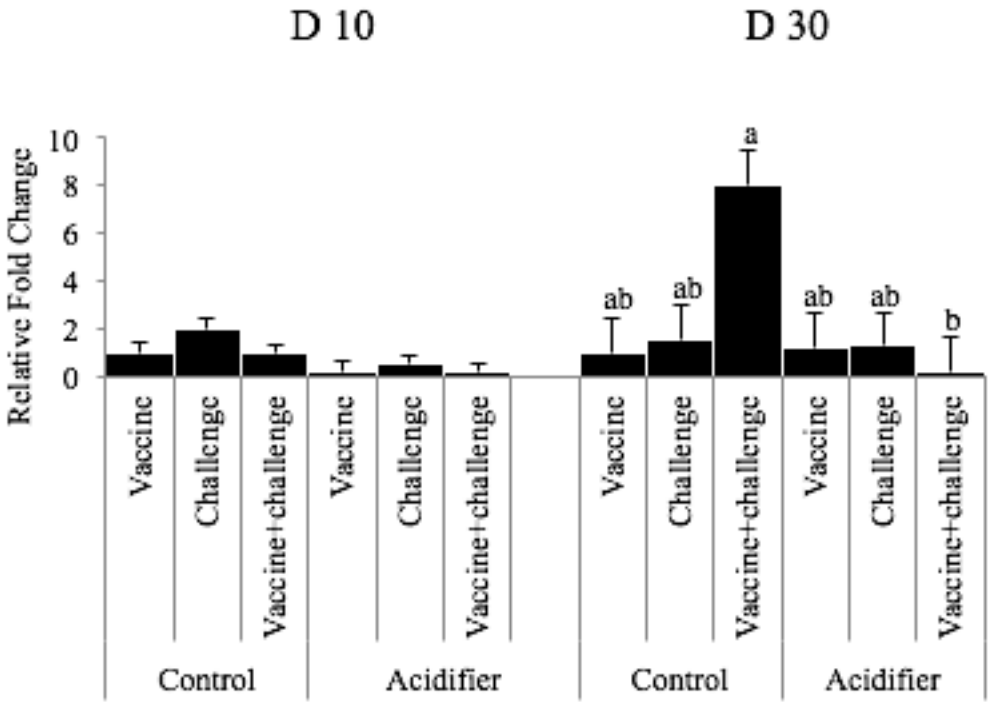


Figure 10. Effect of acidifier supplementation on cecal tonsil IL-10 mRNA transcription at 10 and 30 d post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement of treatments. At 10 and 30 d post-*Salmonella* challenge, cecal tonsils were collected and analyzed for IL-10 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine control group, so all bars represent fold change compared to the vaccine group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: D 10, Acidifier $P < 0.01$, Treatment $P = 0.20$, Acidifier X Treatment $P = 0.62$. D 30, Acidifier $P = 0.04$, Treatment $P = 0.11$, Acidifier X Treatment $P = 0.02$.

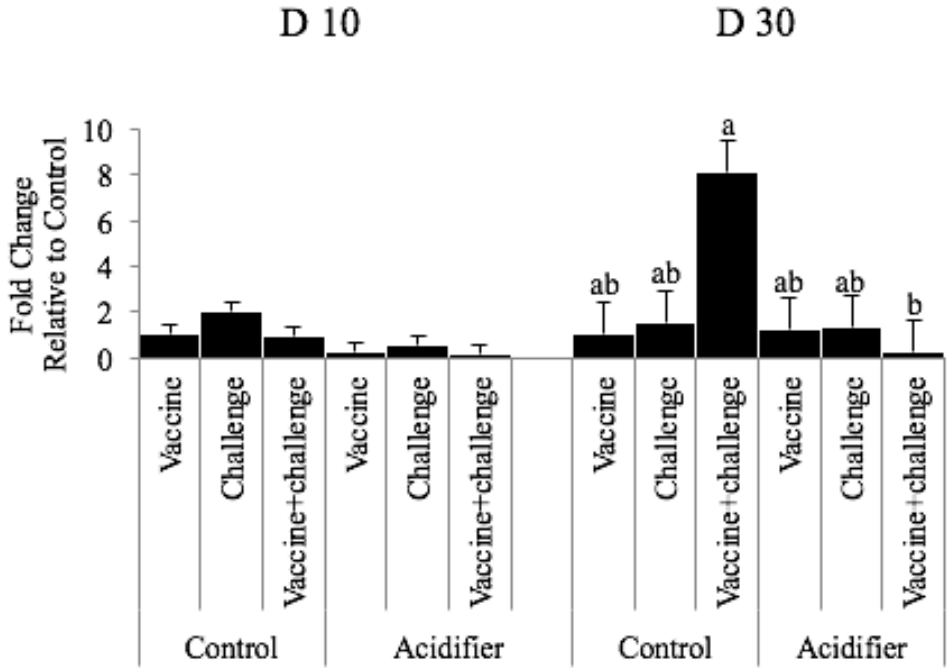


Figure 11. Effect of acidifier supplementation on jejunal villi length at 17 d post-*Salmonella* challenge. Birds were fed basal diet (control) or supplemented with acidifier product from day of hatch through 28 wk of age. Birds were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of *S.* Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and acidifier) factorial arrangement. At 17 d post-*Salmonella* challenge, jejunal samples were collected and villi lengths were measured. Because the interaction effects between acidifier supplementation and treatments were not significant ($P > 0.05$), main effects of acidifier were analyzed. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: D 17, $P = 0.03$.

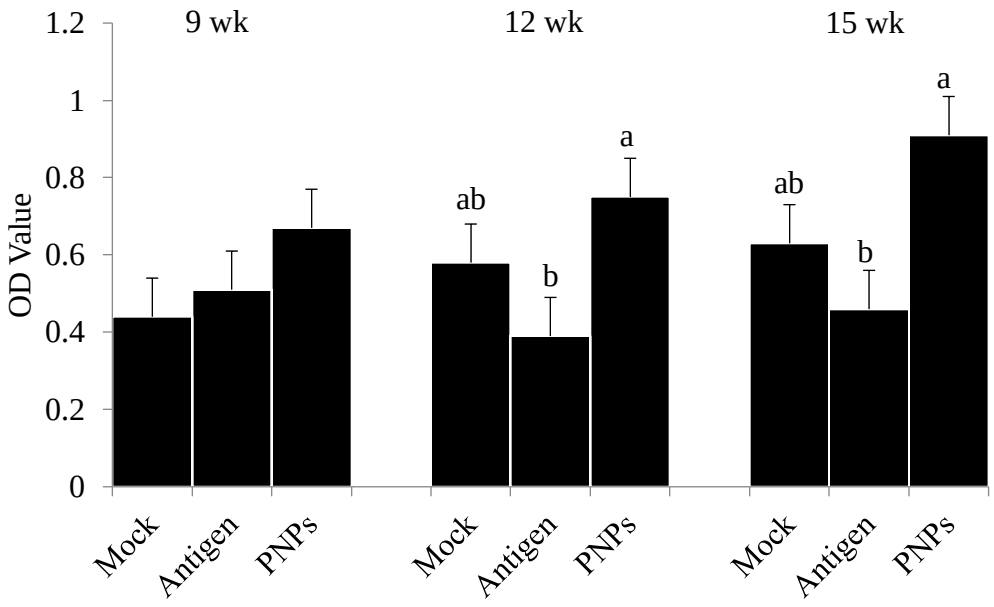


Figure 12. Effect of OMPs-F-PNPs vaccination on serum anti-OMPs IgG titers pre-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. Blood samples were collected at 9, 12, and 15 wk of age and analyzed for OMPs-specific IgG titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: 9 wk, P = 0.31; 12 wk, P = 0.05, 15 wk, P = 0.01.

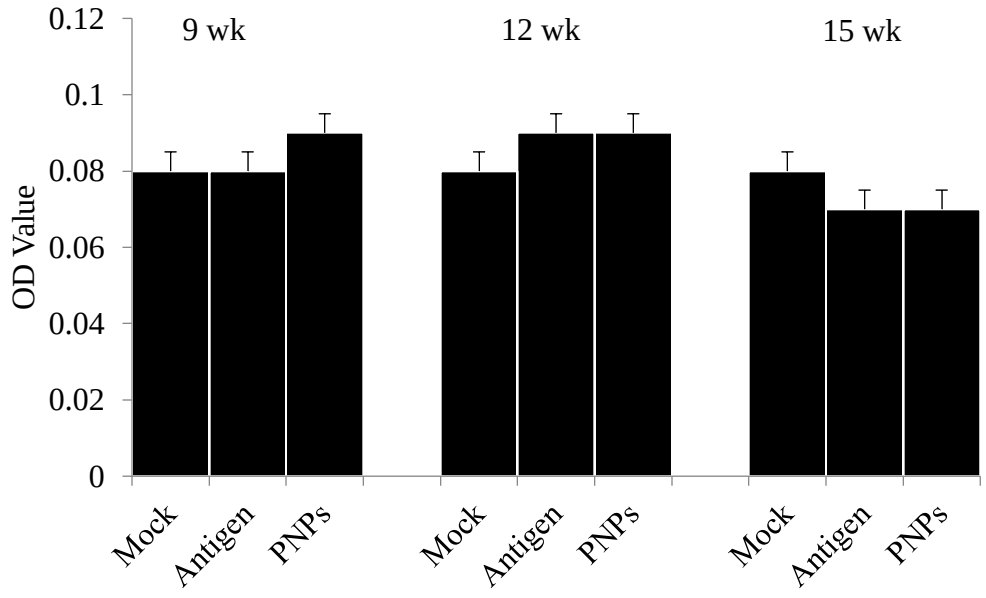


Figure 13. Effect of OMPs-F-PNPs vaccination on serum anti-OMP IgA titers pre-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. Blood samples were collected at 9, 12, and 15 wk of age and analyzed for OMPs-specific IgA titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: 9 wk, $P = 0.53$; 12 wk, $P = 0.71$, 15 wk, $P = 0.90$.

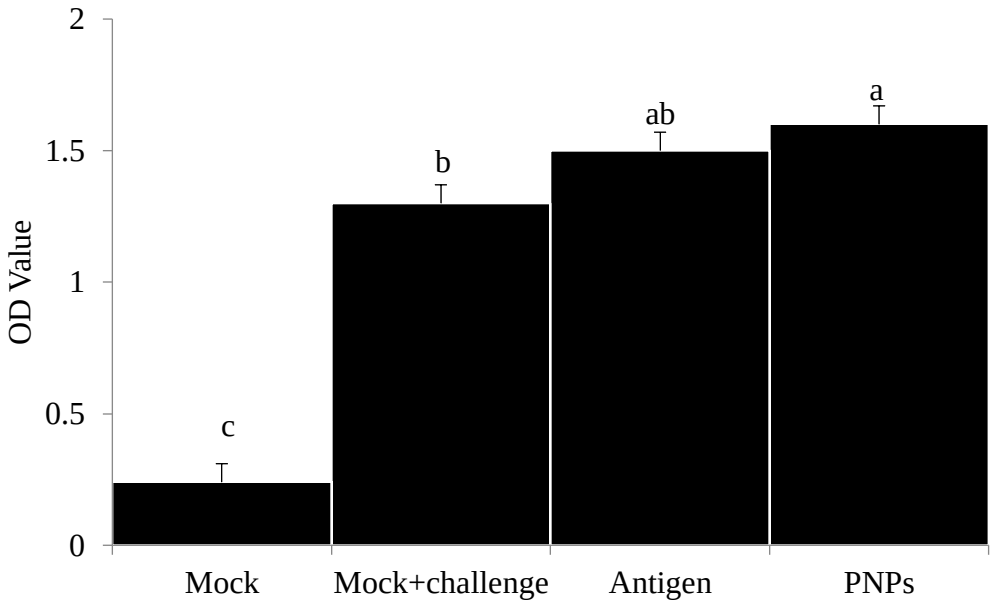


Figure 14. Effect of OMPs-F-PNPs vaccination on serum anti-OMP IgG titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Serum samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgG titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

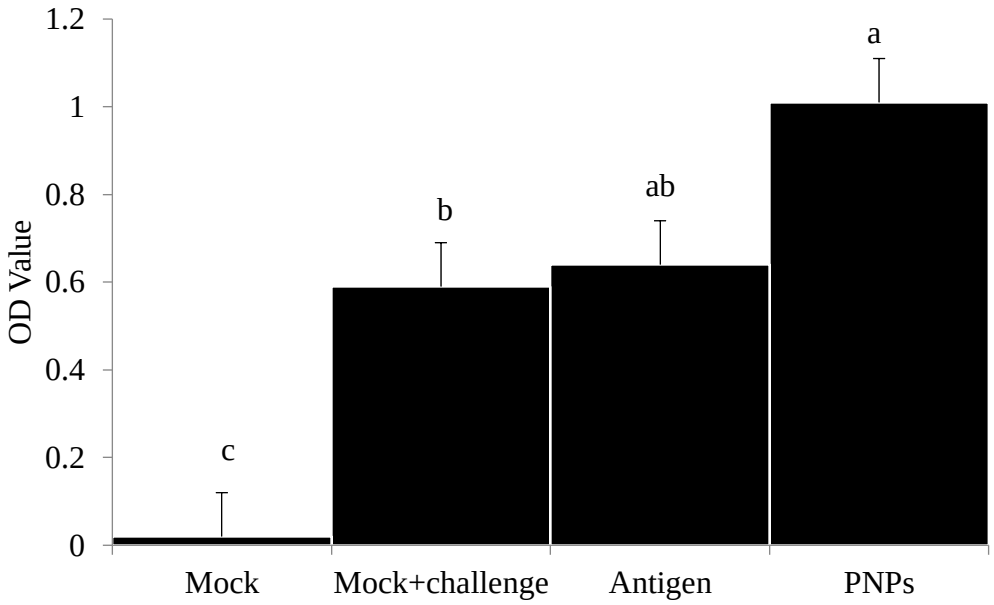


Figure 15. Effect of OMPs-F-PNPs vaccination on bile anti-OMP IgG titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Bile samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgG titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

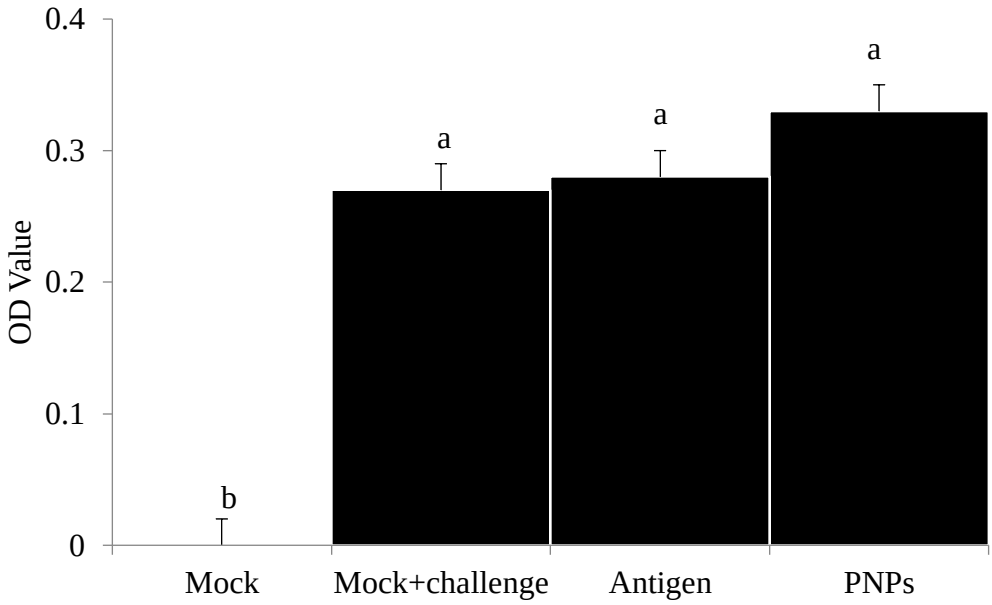


Figure 16. Effect of OMPs-F-PNPs vaccination on bile anti-OMP IgA titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Bile samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgA titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: $P < 0.01$.

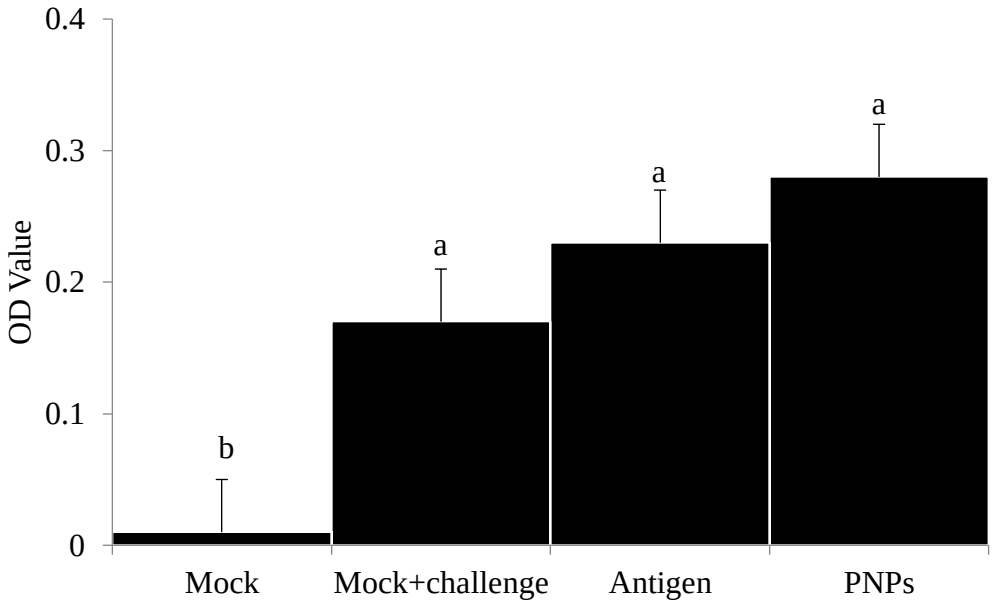


Figure 17. Effect of OMPs-F-PNPs vaccination on cloacal anti-OMP IgA titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Cloacal swab samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgA titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

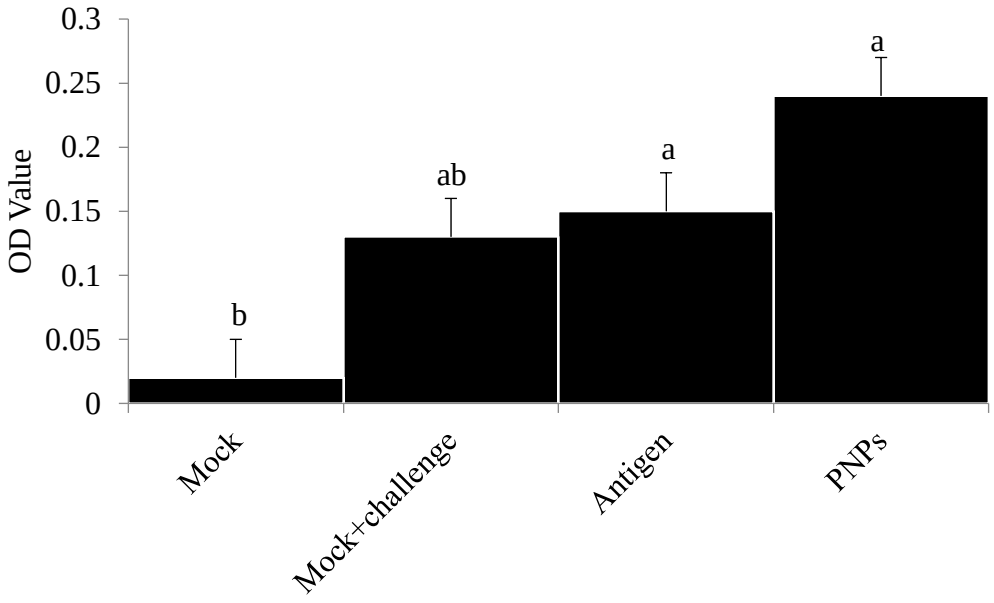


Figure 18. Effect of OMPs-F-PNPs vaccination on intestinal anti-OMP IgA titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Intestinal samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgA titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

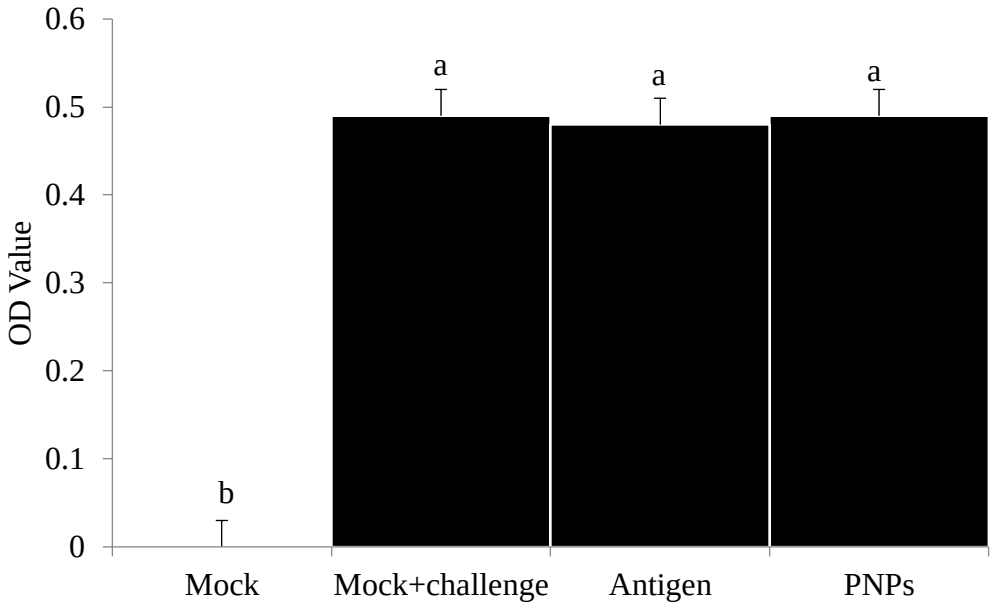


Figure 19. Effect of OMPs-F-PNPs vaccination on tracheal anti-OMP IgA titers at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Tracheal samples were collected at 10 d post-*Salmonella* challenge and analyzed for OMPs-specific IgA titers using ELISA. Results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

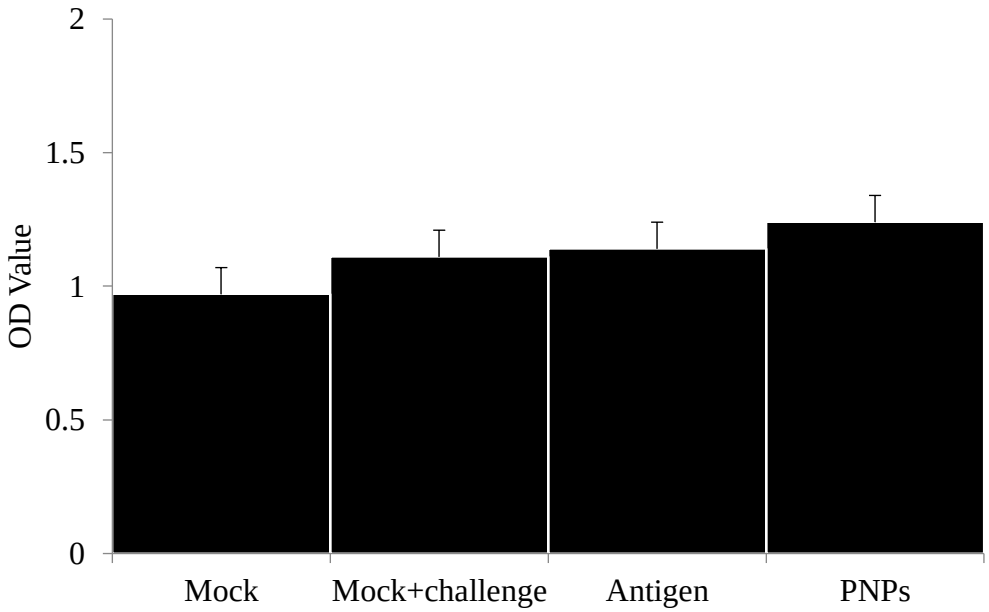


Figure 20. Effect of OMPs-F-PNPs vaccination on PBMC proliferation at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Plasma samples were collected at 10 d post-*Salmonella* challenge and analyzed for PBMC proliferation using a lymphocyte proliferation assay. Results are reported as stimulation index (SI) values, which were calculated by dividing OD of stimulated cells by OD of unstimulated control cells of the same sample. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P = 0.51$.

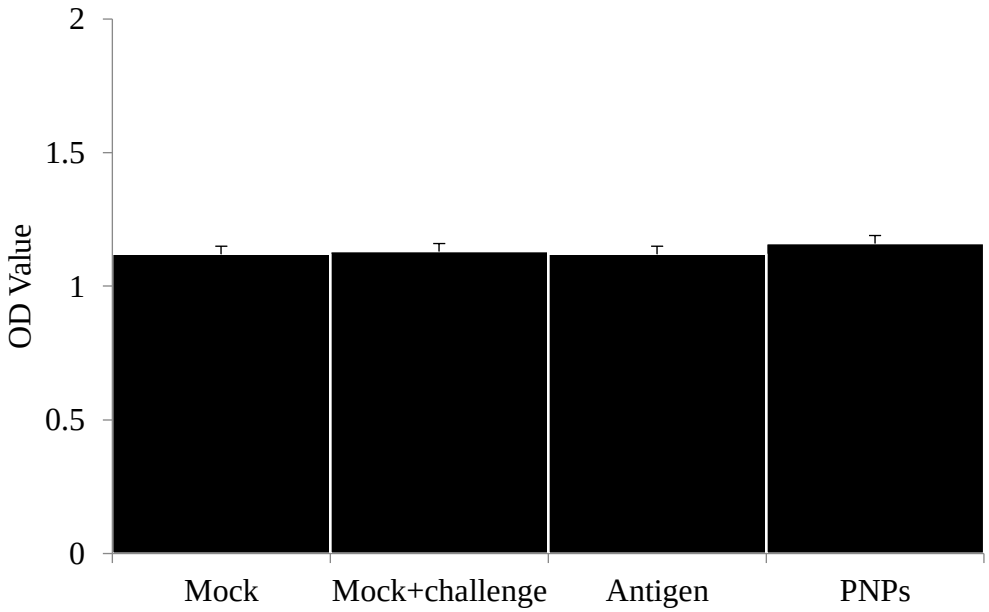


Figure 21. Effect of OMPs-F-PNPs vaccination on splenocytes proliferation at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Spleen samples were collected at 10 d post-*Salmonella* challenge and analyzed for splenocytes proliferation using a lymphocyte proliferation assay. Results are reported as stimulation index (SI) values, which were calculated by dividing OD of stimulated cells by OD of unstimulated control cells of the same sample. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: P = 0.67.

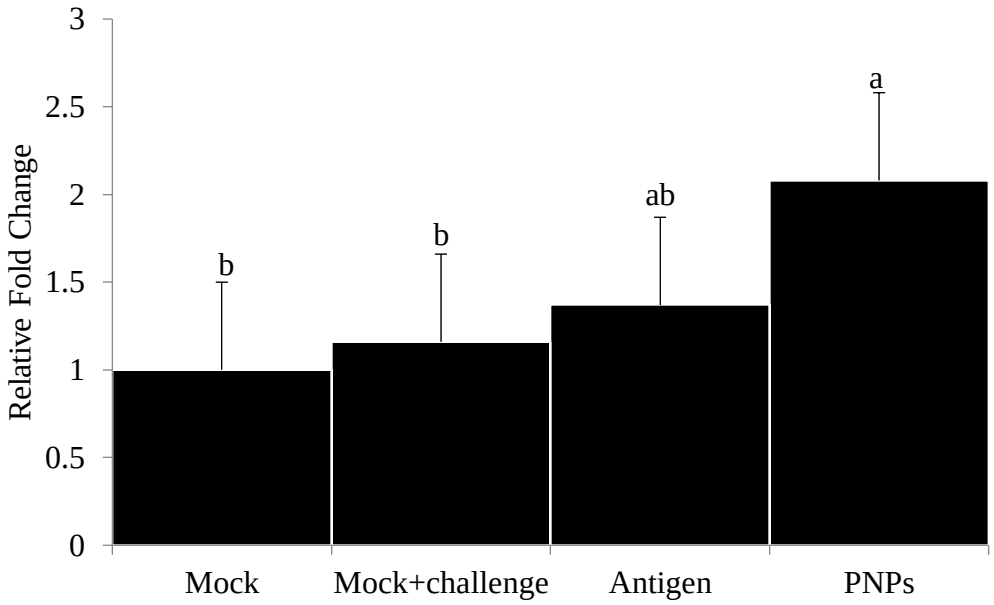


Figure 22. Effect of OMPs-F-PNPs on TLR-4 mRNA transcription at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Cecal tonsil samples were collected at 10 d post-*Salmonella* challenge and analyzed for TLR-4 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the mock group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P < 0.01$.

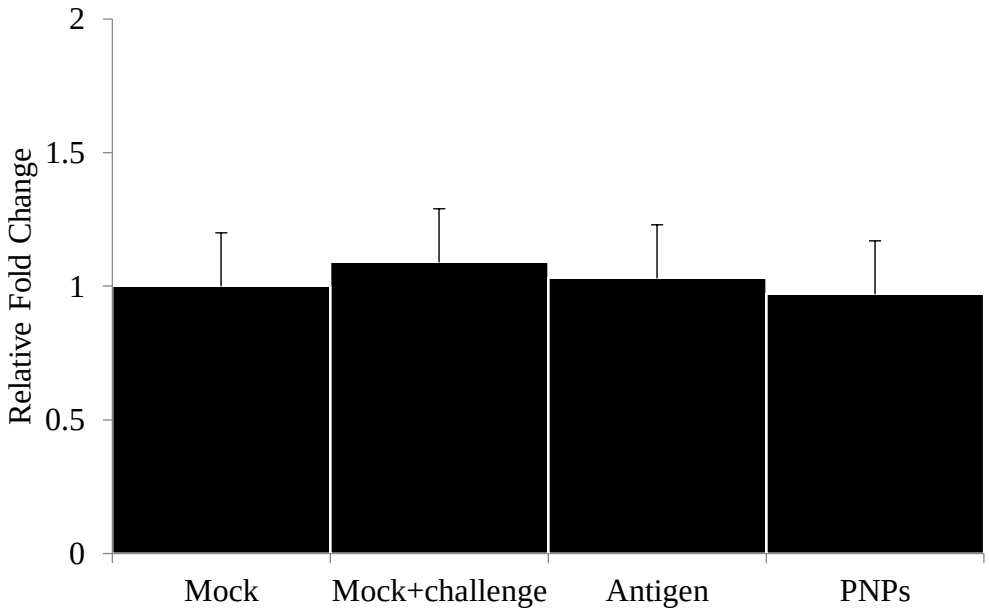


Figure 23. Effect of OMPs-F-PNPs on TLR-2 mRNA transcription at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Cecal tonsil samples were collected at 10 d post-*Salmonella* challenge and analyzed for TLR-2 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the mock group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: P = 0.98.

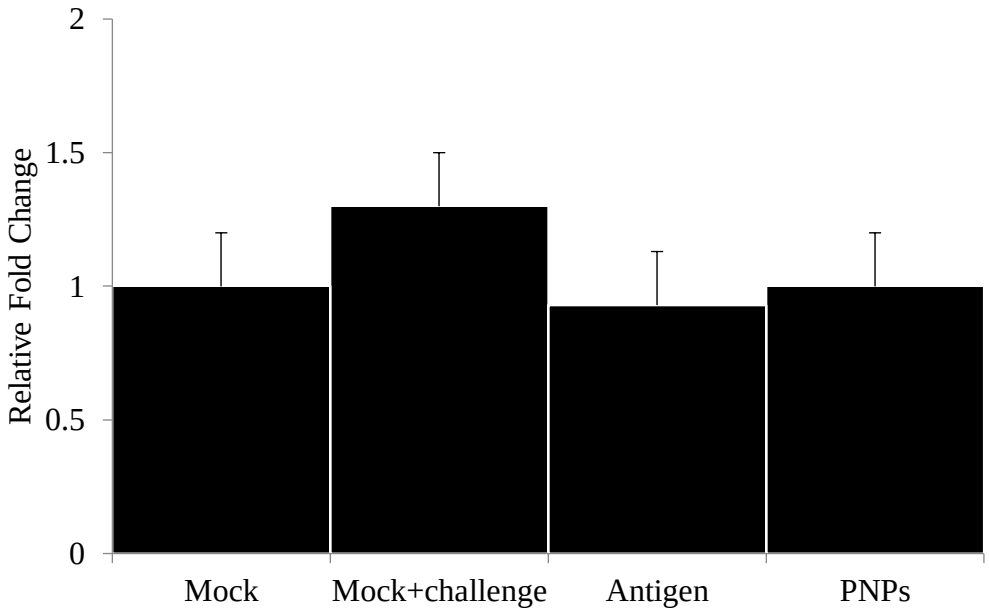


Figure 24. Effect of OMPs-F-PNPs on IFN-γ mRNA transcription at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Cecal tonsil samples were collected at 10 d post-*Salmonella* challenge and analyzed for IFN-γ mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the mock group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: P = 0.32.

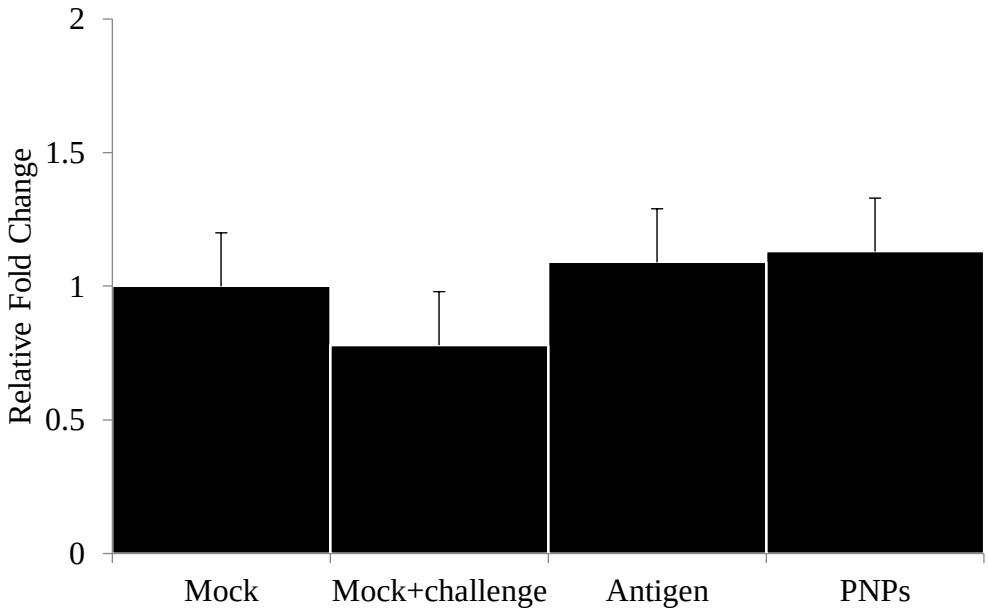
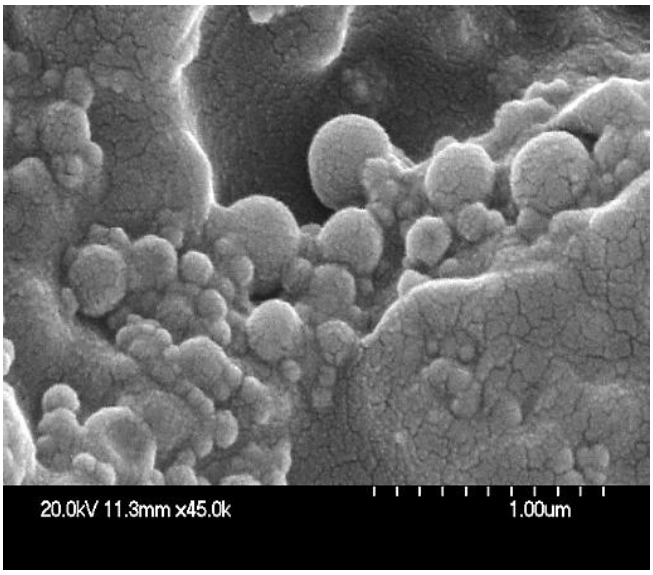


Figure 25. Effect of OMPs-F-PNPs on IL-4 mRNA transcription at 10 d post-*Salmonella* challenge. At 6 wk of age, chickens were either orally vaccinated with PBS (Mock), soluble OMPs (0.05 mg) + flagellin (0.05 mg) in PBS (Antigen), or OMPs (0.05 mg) + flagellin (0.05 mg) loaded in OMPs-F-PNPs in 1 mL of PBS (OMPs-F-PNPs). The same dose and route of delivery was repeated at 9 and 12 wk of age. At 15 wk of age, chickens were challenged with 1 x 10^9 CFU of live *S.* Enteritidis. Cecal tonsil samples were collected at 10 d post-*Salmonella* challenge and analyzed for IL-4 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the mock group. Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P value: $P = 0.6$.

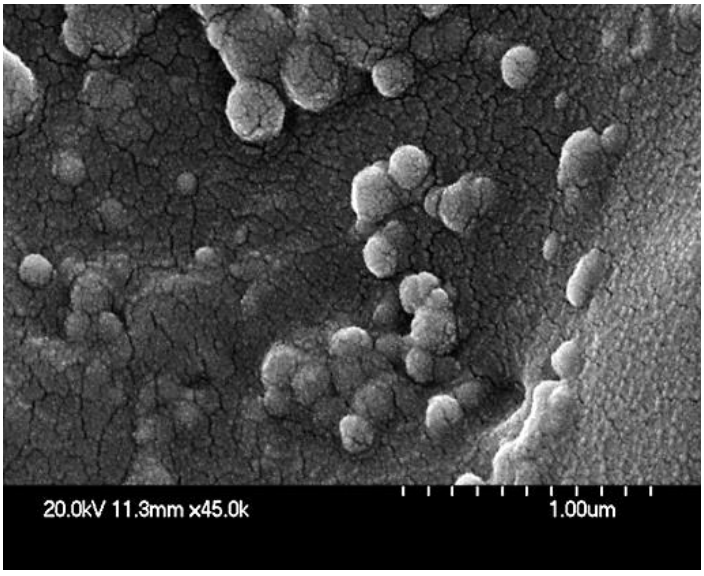
Appendix C: Additional Data

Scanning electron microscope images of A.) unloaded, empty nanoparticles. B.) OMPs loaded and flagellin-coated nanoparticles (OMPs-F-PNPs).

(A)



(B)



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