MUCOSAL IMMUNITY OF THE BROILER CHICKEN VACCINATED WITH NANOPARTICLE VACCINE AND CHALLENGED WITH *SALMONELLA ENTERICA* ENTERITIDIS

By

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ABSTRACT

Non-typhoidal *Salmonella* originating from chicken is a leading cause of gastroenteritis in humans. A commercial vaccine against *Salmonella enterica* Enteritidis in broilers has not be successful due to various reasons. An oral subunit vaccine has the potential to induce immunity in the gut of broilers, the primary site of colonization. An oral nanoparticle vaccine consisting of flagella and OMP A antigens extracted from *S.* Enteritidis and encapsulated within polyanhydride was tested in two studies. The first study looked at determining the optimum dosage of the vaccine and the second study aimed at determining the efficacy of the vaccine following a *S.* Enteritidis challenge. A dose of 250 μg with one booster induced humoral immunity in birds through production of anti-*Salmonella* IgA antibodies ($P < 0.05$). This vaccine showed potential for inducing long-lasting mucosal immunity in broilers.

INDEX WORDS: *Salmonella enterica*, Enteritidis, mucosal immunity, vaccine, polyanhydride

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CHAPTER 1

INTRODUCTION

Salmonellosis, caused by *Salmonella enterica* serovars, is the most prevalent poultry-related causes of foodborne illnesses (Srikumar et al., 2015; Flockhart et al., 2017). *S. enterica* are a facultative and intracellular bacteria, which allows the bacteria to thrive inside the gastrointestinal (GI) tract of animals. Infection of *Salmonella* in poultry is typically asymptomatic for months, even when the bacteria is shed in the feces. (Zhang-Barber et al., 1999; Flockhart et al., 2017; Kogut and Arsenault, 2017). The bacteria is primarily transmitted horizontally through the fecal to oral route in poultry ; but ovary to egg route in cases of vertical transmission has been observed (Zhang-Barber et al., 1999; Foley et al., 2011). In humans, *Salmonella* infections result in gastroenteritis (Ramos-Morales, 2012). In 2006, *S.* Enteritidis was the second most common cause of infection in humans and was related to 19.2% of all *Salmonella* infections in 2009 (Foley et al., 2011). In 2013, *Salmonella* caused over 20% of foodborne-related illnesses, which cost about US$14.6 billion (Kogut and Arsenault, 2017). Within the species of *S. enterica*, there are two common types of the subtype known to infect humans: typhoidal and non-typhoidal. In humans, typhoidal serovars cause systemic infection while non-typhoidal serovars cause gastroenteritis. Typhoidal serovars of *S. enterica* include Typhi and Paratyphi (Gal-Mor et al., 2014; Johnson et al., 2018). These serovars are adapted well to the human GI tract and cause enteric fever in infected humans(Gal-Mor et al., 2014). The non-typhoidal serovars of *S. enterica* are more opportunistic and can adapt well to most food animals. These serovars cause up to 680,000 deaths annually in the world (Srikumar et al., 2015). Over 2,500 serovars of *S. enterica* have been isolated, with the

majority being of the non-typhoidal type. Non-typhoidal serovars include Enteritidis, Heidelberg, Dublin, Kentucky, and Newport. Enteritidis, consists of up to 30% of human isolates; while Heidelberg is the third most common isolate (Joshi et al., 2009; Foley et al., 2011; Ramos-Morales, 2012; Flockhart et al., 2017; Kogut and Arsenault, 2017). Enteritidis and Typhimurium are the primary cause of systemic infection in poultry (Withanage et al., 2005; Kogut and Arsenault, 2017).

Over 40 billion birds are raised in the US poultry industry annually. The use of litter in poultry houses allows chicks to gain microbial diversity to protect the chicks from infections through seeder populations (Oakley et al., 2014). Vaccine administration prior to chick placement in broiler houses may reduce infections. The current methods against *Salmonella* in the broiler industry focus on detection instead of prevention. There is no completely successful method to eliminate the bacteria from being found in commercial broiler operations (Joshi et al., 2009; Humphrey et al., 2014). Although the industry focuses on detection methods for *Salmonella* and other bacteria causing food-borne illnesses, there is ongoing research in intervention methods as preventative measures. Interventions to reduce Salmonellosis and other foodborne-illnesses cost over $500 million annually to poultry producers (Kogut and Arsenault, 2017). The cage-free housing system of broiler chickens, housing upwards of 60,000 birds at a time and over 9 billion broilers per year (Foley et al., 2011), allows close contact of the chickens and encourages horizontal transmission of *Salmonella.* Antimicrobial treatments and vaccines are used to reduce *Salmonella* infections (Wigley, 2013). Antibiotic use in animal production has contributed to the development of antibiotic resistant Salmonella strains (Barrow, 2007). Vaccines, such as killed, subunit, and live, are currently used as preventative measures to reduce *Salmonella* in layers and broiler breeder chickens. Intramuscular (IM) vaccines can decrease carcass quality of chickens;

therefore, it is not used in broiler chickens. In addition, intranasal vaccines are not ideal because *Salmonella* is transmitted in the fecal-oral route and colonization occurs in the GI tract. Therefore, an oral *Salmonella* vaccine that is not degraded in the foregut and allows colonization in the small intestine is the best method for route of administration.

CHAPTER 2

LITERATURE REVIEW

Salmonella in the poultry industry

Salmonella is one of the major causes of food poisoning in humans and can cause localized and systemic infections in humans and chickens (Peng et al., 2011). *Salmonella enterica* serovars are categorized into typhoidal and non-typhoidal *Salmonella*. Typhoidal serovars are host-adapted to humans and spread through contaminated food sources(Dougan and Baker, 2014; Gal-Mor et al., 2014). The non-typhoidal serovars are not host adapted and cause asymptomatic infections in birds but may also transmit the bacteria to humans through contaminated poultry products. Vaccinating against non-typhoidal *Salmonella* serovars is not as successful as vaccinating against typhoidal strains. Non-typhoidal strains are host-specific and are not as pathogenic to chickens as the typhoidal serovars (Barrow, 2007).

Salmonella first colonizes the intestine of hatchlings and increases the pro-inflammatory response of the chicks (Kogut and Arsenault, 2017). The bacteria are transmitted through the fecal-oral route from one chick to another. After the bacteria are ingested by the chicks, the bacteria travel through the GI tract to the small intestine and finally the ceca; where the bacteria colonize and proliferate (Barrow, 2007; Kogut and Arsenault, 2017). After colonization, the chicks become a *Salmonella* carrier and shed the bacteria in the feces to the environment and other animals. Commercial broilers can remain asymptomatic from hatch to processing, which allows the bacteria to exponentially increase in the intestine of the bird (Kogut and Arsenault, 2017).

Salmonella is a public health risk when the poultry products are contaminated during poultry processing. (Simonsen et al., 1987; Barrow, 2007). The monitoring strategy is the current method in preventing *Salmonella* induced food-borne illnesses caused by poultry products (Hogue et al., 1998). Because of the implementation of Hazard Analysis Critical Control Point (HACCP), *Salmonella* contamination of chicken carcasses were reduced; thereby reduced the incidence of salmonellosis in humans (Simonsen et al., 1987). *Salmonella* contamination is monitored immediately following the chilling tank in processing plants and is an effective method in preventing a majority of *Salmonella* induced food-borne illnesses caused by poultry products (Hogue et al., 1998).

Avian gastrointestinal tract

Birds, with their inability to masticate, have unique features such as the crop, proventriculus, and gizzard/ventriculus in the GI tract to compensate for this. The crop is located after the esophagus and serves as a storage compartment for feed. While the crop does not have a direct role in digestion of food particles, it adds moisture to help digestion in the proventriculus (Svihus, 2014). Prolonged storage in the crop leads to fermentation of feed, resulting in an increase in acidity to a pH about 4.6. This is primarily a concern when birds are on a set schedule for feeding rather than fed *ad libitum* (Svihus, 2014).

Chemical digestion begins in the proventriculus. The proventriculus is a glandular organ that allows secretion of digestive enzymes for chemical digestion prior to physical digestion in the gizzard. The pH of the proventriculus is typically low, ranging from 2 to 5 (Svihus, 2014). Aside from digestive enzymes, such as pepsinogen, produced in the proventriculus, hydrochloric acid and mucous are secreted to chemically degrade the digesta (Svihus, 2014; Alshamy et al., 2018).

The gizzard, located after the proventriculus, is a muscular organ that physically digests the digesta through grinding motions.

After the mechanical digestion, the digesta travels from the gizzard to the duodenal loop by peristaltic contractions (Svihus, 2014). In this part of the GI tract, the acidic digestive contents mix with bile and pancreatic fluids. This causes an increase in pH, and further digests the contents received from the gizzard. Following the duodenal loop are Meckel's diverticulum and the jejunum (Svihus, 2014). The former is the remnants of the yolk sac from before the chick was hatched. The yolk sac remains in the chick for several days post hatch, continuing to provide nutrients to the chick (Bhanja et al., 2009). In the broiler industry, the chicks can remain in the incubator for up to two days before being transported to poultry houses (Juul-Madsen et al., 2004). Although the yolk sac can provide nutrients to the chicks after hatching, prolonged feed withdraw can decrease growth and immune development (Juul-Madsen et al., 2004; Bhanja et al., 2009). The jejunum further digests and begins absorption of the contents passed through the GI tract. The duodenum and jejunum are the primary areas of nutrient absorption. Following the jejunum, the ileum is the primary site of water absorption in birds (Svihus, 2014). Absorption may change depending on the gut health of the bird. Throughout the lamina propria (LP) of the small intestine are clusters of lymphoid tissue called Peyer's Patches. The Peyer's Patches contain mucosal associated lymphoid tissues (MALT) located throughout the body (Lillehoj and Trout, 1996; Vaughn et al., 2006).

At the end of the ileum is the ileo-ceco-colic junction. The ceca are blind ended sacs connected to this junction. The cecal length varies depending on the diet and age of bird (Svihus, 2014). Connecting the ileo-ceco-colic junction to the ceca are the cecal tonsils consisting of lymphoid tissue, with germinal centers for lymphocyte maturation along with other immune cells (Lillehoj and Trout, 1996). The large intestine and cloaca are located after this junction.

Immunity of the broiler chicken

The chicken immune system consists of innate and adaptive immunity against pathogens. However, due to generations of selective breeding for growth, the immune system of the broilers has suffered at the cost of improved growth performance (Humphrey et al., 2014). The chicken's first line of defense in the GI tract consists of physical and chemical barriers to prevent infections. The physical and chemical layers of the small intestine include mucus, junction proteins, commensal microbes, and antimicrobial peptides (Oakley et al., 2014). Mucus from intestinal goblet cells, is the first layer of defense against pathogenic microbes (Smirnov et al., 2005). The mucus provides a physical barrier between pathogens and the enterocyte, as well as containing antimicrobial peptides. The small intestine contains specialized immune cells such as M cells and dendritic cells found within the Peyer's Patches, MALT, and gut-associated lymphoid tissue (GALT). The specialized cells sample antigens in the small intestine and activate immune responses. The M cells are adept at antigen uptake and transportation of the antigens to the Peyer's Patches for processing. Because of the close proximity of the M cells and the Peyer's Patches, which contain dendritic cells, B and T lymphocytes, the antigens are presented by antigen presenting cells such as macrophages and dendritic cells to the adaptive immune cells causing the adaptive immune cells to activate into effectors (Neutra et al., 1996; Guimarães et al., 2011; Helft et al., 2015).

An often-forgotten component of the immune system of intestinal immunity is the regulatory T cell (T-regs). T-regs are essential in the function of commensal bacteria. T-regs promote tolerance in immunity. Without T-regs, the immune system would indiscriminately target antigens including those of commensal bacteria and cause excess inflammation (Shanmugasundaram and Selvaraj, 2012). T-regs are increased in chickens by *S.* Enteritidis to

prevent clearance of the bacteria by the immune system (Shanmugasundaram et al., 2015). However, persistant *Salmonella* infections can overcome the down-regulation of the immune system by T-regs.

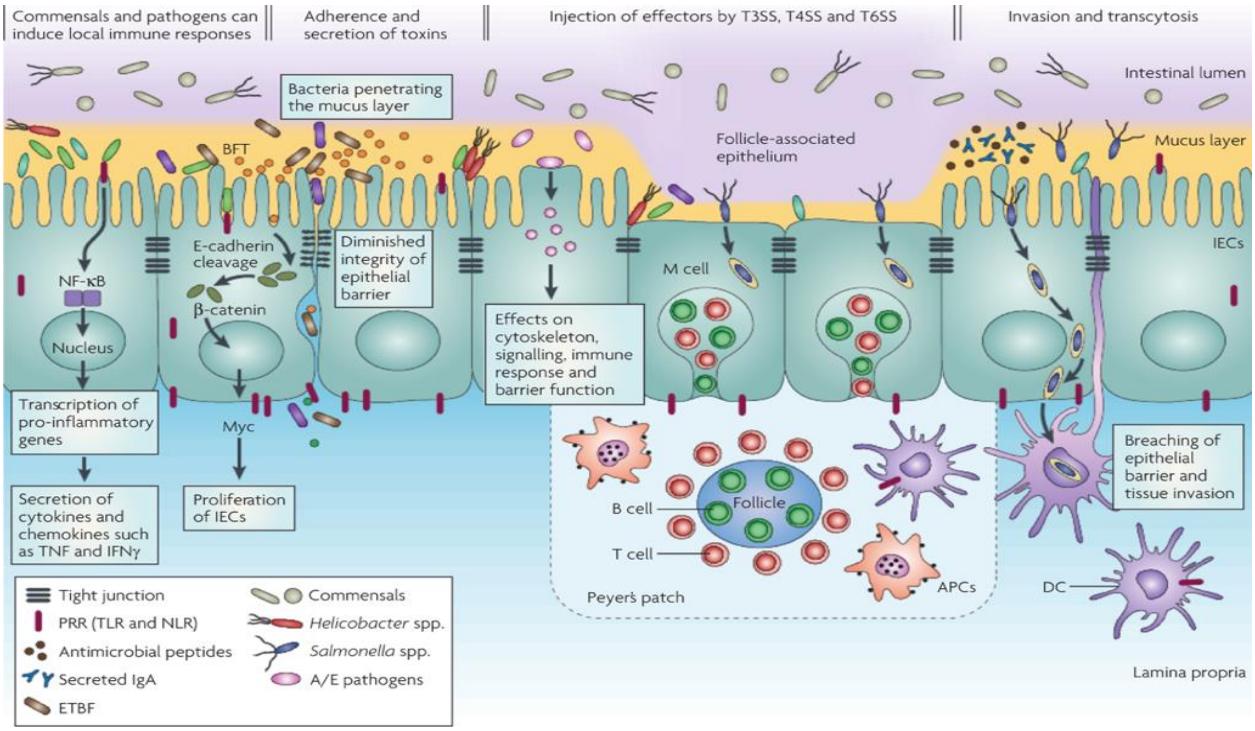

Figure 2.1: Diagram of the M cell and leukocytes of the small intestine (Neutra et al., 1996)

Pathology of Salmonella in poultry and the poultry immune response

The susceptibility of a bird to *Salmonella* infections is contributed to many factors including stress, overcrowding, and density of the bacteria in the environment. Climate affects susceptibility of the chickens to *Salmonella* infections. Heat stress increases the prevalence of *Salmonella* infections (Van Goor et al., 2016). Younger birds with an underdeveloped immune system are more susceptible, while older birds develop resistance to the bacteria as they age (Yokoyama et al., 1998; Smirnov et al., 2005).

Of the two types of *Salmonella* causing illnesses in humans, the non-typhoidal serovars are the current focus in the broiler industry. *Salmonella enteria* serovars co-evolved with poultry

resulting in disease tolerance in chickens. This allows the bacteria to colonize chickens without eliciting any disease symptoms (Barrow, 2007; Kogut and Arsenault, 2017). When chicken carcasses are contaminated in the slaughter plants from punctured or torn intestines during evisceration in processing, *Salmonella* can cause food-borne illnesses in humans. To reduce *Salmonella* contamination to poultry products, *Salmonella* shedding by live birds is a critical control point (Withanage et al., 2005). Shedding can cause infection in other poultry through vertical or horizontal transmission. Vertical transmission occurs from the hen's ovary becoming infected by the bacteria as a result of systemic infection and passed to the chicks. Horizontal transmission occurs from contaminated litter of previous flocks or other chickens in the same flock (Foley et al., 2011).

Salmonella infection occurs in three stages in broiler chickens: resistance, tolerance and homeostasis (Kogut and Arsenault, 2017). Resistance is marked by a pro-inflammatory response through innate immune cells and pro-inflammatory cytokines after the infection and lasting up to four days. Resistance is then followed by tolerance. Tolerance is marked by an abundance of anti-inflammatory cytokines initiated by T-regs. The tolerance stage is short, lasting between four to five days post infection. Tolerance is followed by homeostasis. Homeostasis occurs when the bacteria are no longer recognized as a threat to the immune system after five days post-infection.

In order for *Salmonella* to infect a host, the bacteria must survive the harsh environment factors in the GI tract such as the acidity of the stomach and antimicrobials proteins on the mucus membrane (Jones et al., 2007; Wigley, 2013; Srikumar et al., 2015). *Salmonella* has adapted to survive the innate immunity and move through the mucosal layer of the small intestine. The intestinal M cells within the Peyer's Patches in the ileum survey the intestine for pathogens (Cirillo et al., 1998; Yokoyama et al., 1998; Srikumar et al., 2015). The circulating macrophages near the

M cells are one of the initiators of innate immunity. Once the antigen is recognized by the macrophages, the antigen is phagocytosed and processed (Cirillo et al., 1998; Guimarães et al., 2011). Like macrophages, dendritic cells recognize and target the outer-membrane proteins (OMPs) and lipopolysaccharides (LPS) of *Salmonella*. Macrophages and dendritic cells activate the humoral and cell-mediated responses against the bacteria (Yokoyama et al., 1998). If the immune system cannot control the *Salmonella* invasion, the bacterial facilitate apoptosis of M cells which allows for uncontrolled proliferation in the Peyer's Patches (Gayet et al., 2017). Dendritic cells are professional antigen presenting cells that monitor pathogens in the intestine and then present the antigens to naïve T cells (Howard et al., 2004; Van Goor et al., 2016).

To evade antigen presentation, *Salmonella* utilize the Type 3 Secretion System (T3SS) by altering the ability of dendritic cells and macrophages to process the antigens (Ramos-Morales, 2012; Srikumar et al., 2015). Three T3SS mechanisms lead to the cell death of antigen presenting cells through epithelial cell apoptosis, rapid T3SS1 depended macrophage pyroptosis, and delayed T3SS2 dependent macrophage pyroptosis. Pyroptosis differs from the more common apoptosis in that it requires capase-1 activation to proceed. This allows for a swift lysis of the cell and release of pro-inflammatory contents (Ramos-Morales, 2012). Some strains, like *S.* Enteritidis stimulate anti-inflammatory responses from T-regs through IL-10 (Shanmugasundaram et al., 2015). There are also less well-known pathways that vary between serovars such as bypassing the immune cells and altering the tight junctions of the intestinal cell wall through T3SS effectors (Foley et al., 2011; Ramos-Morales, 2012). The T3SS weakens the intestinal cell wall and the tight junctions found in the ileum by decreasing transmembrane resistance and increasing permeability. The use of effectors by the *Salmonella* T3SS can also alter host cell functions including decreased signal transduction, induced apoptosis or incomplete phagolysosome formation (Hautefort et al., 2008;

Van Goor et al., 2016). The increase in *Salmonella* infection associated with heat stress is attributed to increased permeability through the effector systems (Van Goor et al., 2016). Proteins, such as OMPs and flagella, in T3SS are highly conserved between *Salmonella* serovars. Both major types of *Salmonella*, typhoidal and non-typhoidal, express these proteins that contribute to their virulence. The proteins are recognized by toll-like receptors (TLRs) of innate immune cells. After recognition, the pro-inflammatory response is activated through the NF-κB signaling pathway and leads to the production of pro-inflammatory cytokines such as IFN-γ (Wigley, 2013; Gayet et al., 2017). The flagella of *Salmonella* are responsible for the motility of the bacteria. The OMPs are recognized by B cells to produce antibodies against the bacteria; therefore, the OMPs are targeted in vaccine development (Yokoyama et al., 1998).

The *Salmonella* pathogenicity islands (SPI) genetically influence these proteins and the virulence of the bacteria itself (Kubori et al., 2000; Hautefort et al., 2008; Velge et al., 2012). Influenced by SPI, the bacteria uses the T3SS proceed to infect macrophages and other monocytic cells and when the infection becomes systemic, going on to the liver, spleen, and ovary (Jones et al., 2001; Hautefort et al., 2008; Foley et al., 2011; Ramos-Morales, 2012; Velge et al., 2012). There are two types of SPI, 1 and 2, of which different serovars can use one or both to facilitate the spread of infection though the T3SS. SPI1 induces a strong pro-inflammatory response through the release of cytokines like IL-1β and activation of pathways including JNK (Hautefort et al., 2008). *Salmonella* serovars Gallinarum, Pullorum, and Typhimurium specifically rely on SPI2 in the T3SS to prevent release of enzymes in phagocytes and cause systemic infection in poultry, while SPI1 in Pullorum can also contribute to systemic disease (Cirillo et al., 1998; Jones et al., 2001, 2007; Hautefort et al., 2008). Typhimurium mutants lacking the SPI2 gene are unable to migrate past the Peyer's patches. The bacteria use SPI to facilitate the creation of safe harbors

within macrophages, known as *Salmonella* containing vacuoles (SCV), causing a downregulation of the creation of phagolysosomes and antimicrobial products such as nitric oxide (Jones et al., 2001; Hautefort et al., 2008). These SCVs provide an area to replicate further and promote systemic infection (Srikumar et al., 2015). This vacuole can occur in numerous immune cells excluding dendritic cells (Ramos-Morales, 2012).

As *Salmonella* persists in the bird, the humoral and cell-mediated responses of the adaptive immune response produce an antigen specific response. T-helper 1 (Th1) cells use IFN-γ and IL-12 cytokines to aid in the immune response. Th1 cells plays a more prominent role in *Salmonella* immunity than other helper T cells (Withanage et al., 2005; Barrow, 2007). Helper T cells signal humoral immunity through cytokine signaling. IgG, IgM, and IgA antibodies increase days after the initial infection when B cells are activated by antigen presenting cells (Withanage et al., 2005; Barrow, 2007). After five to seven days following the initial infection, the adaptive immune responses are typically detected in the bird (Wigley, 2013). IFN- γ, from Th1 cells stimulate IgG1 antibody production by B cells. IL-4 from Th2 cells stimulate IgG2a antibody production. IgA, is the predominant mucosal antibody and provides the majority of antibody defense against *Salmonella* infection (Barrow, 2007).

Pro-inflammatory cytokines, such as IL-1, are produced by Th1 and Th17 cells during the resistance stage of an infection (Kogut and Arsenault, 2017). In cellular immunity, IFN-γ has a prominent role in immunity against *Salmonella* in chickens during the initial pro-inflammatory response (Withanage et al., 2005). Anti-inflammatory cytokines such as IL-10 are produced by T-regs during the 'disease tolerance' and 'homeostasis' stages of an infection (Kogut and Arsenault, 2017). TGF-β cytokines are increased during the later stage of *Salmonella* infection as the immune system begins to reduce inflammation (Withanage et al., 2005).

Inducing immunity against Salmonella

In oral vaccination, the vaccine antigen(s) are transcytosed by the mucosal membrane of the small intestine enterocytes. M cells, located near the Peyer's Patches and MALTs of the small intestine, uptake and transport the vaccine antigen to nearby antigen presenting cells within the Peyer's Patches (Lillehoj and Trout, 1996; Salman et al., 2009). Antigen uptake by M cells is facilitated by the lack of a mucin producing gene, MUC2. Flagella antigens of a *Salmonella* bacteria promotes differentiation of M cells in the gut and initiation of the innate immune response by TLR5. The use of flagella in a *Salmonella* vaccine would initiate the proinflammatory response in the intestinal cells (Barrow, 2007; Gayet et al., 2017). The antigen presenting cells are drained to mesenteric lymph nodes in the abdominal cavity where naïve lymphocytes congregate to become activated (Gayet et al., 2017). The antigen presenting cells then present the processed antigens with the Major Histocompatibility Complex (MHC) to the T and B cells. The naïve lymphocytes of the adaptive immunity are activated by the APCs into effector lymphocytes. The effector B cells produce antibodies against the Salmonella antigens. The effector T cells differentiate into cytotoxic T cells (CTLs) and T helper (Th) cells. Gut-homing B1 cells depend on Th cells to promote class switching to the IgG and IgA isotypes needed to promote clearance of the bacteria and create memory and plasma B cells. The IgA isotype, known is primarily found in mucosal tissues, promote microbiota homeostasis and prevents bacteria entry into the epithelial cells of the gut (Corthsy, 2009). IgA antibodies are primarily associated with a Salmonella immune response in the mucosal layer and IgG and IgM are associated to the bacterial immune response in the blood (Gayet et al., 2017).

Salmonella vaccines in the broiler industry

Several *Salmonella* vaccines are produced for broiler chickens in the poultry industry. These vaccines protect chickens from Typhimurium, Enteritidis, and Heidelberg serovars. The

Kentucky serovar is currently found predominantly in production animals such as poultry, beef, and pork. A vaccine against the Kentucky serovar may be needed in the future when the isolate becomes more prominent in humans (Flockhart et al., 2017). Live, attenuated vaccines are more virulent and produce a strong immune response, developing long term immunity. The killed and subunit vaccines are safer but produce a weaker humoral and cell-mediated response compared to the live vaccines (Zhang-Barber et al., 1999; Peng et al., 2011; Jawale and Lee, 2014). The more virulent live vaccines have a higher chance of developing resistance compared to attenuated, killed vaccines (Withanage et al., 2005). According to manufacturers, Elanco and Zoetis, the live vaccines are not to be administered within 21 days of slaughter. The mandatory withdrawal period along with consumer perception of live vaccines prevent the broiler industry from using these commercially available live oral vaccines.

The live, attenuated vaccines result in an increased in Th1 cytokines, including IL-12 and IFN-γ; signaling a strong cell-mediated response (Neutra et al., 1996; Peng et al., 2011). The vaccine antigen can be attenuated by different methods. For example, Salmonella serovar Gallinarum is attenuated by genes encoding to delete or inactivate SPI2 (Jones et al., 2001). This results in the inability of the bacteria to infect local lymphoid tissue in the intestine. Although the response lacks memory that allows adaptive immune cells to respond against pathogens, the killed vaccines elicit a strong non-specific innate immune response towards the pathogen (Hajam et al., 2017). The killed vaccines are not strong enough alone. Therefore, adjuvants are used to augment the immunogenicity (Barrow, 2007). In addition, multiple doses are used to bolster the protection (Jawale and Lee, 2014). Despite the lower immunogenicity compared to live vaccines, attenuated vaccines are favored due to the lower risk in infection (Jawale and Lee, 2014; Hajam et al., 2017). Subunit vaccines are produced by alteration of the antigen through serial culturing or selective

gene editing (Hajam et al., 2017). Commonly, a subunit vaccine combines a selected antigen or two, and paired with an adjuvant to increase the immunogenicity (Desin et al., 2013). Subunit vaccines decrease bacteria shedding which may prevent a bacterial outbreak (Barrow, 2007). The subunit vaccines are normally directed towards a specific serovar such as *S.* Enteritidis; therefore cross-protection is less likely. In addition, by targeting only a specific serovar, the subunit vaccines may allow other serovars to populate the intestine (Foley et al., 2011).

Intramuscular (IM) administration can results in lesions on the muscles of broiler chickens and decrease carcass quality (Jawale and Lee, 2014). Therefore, vaccination against *Salmonella* by IM injects is not used in broiler chickens. Since *Salmonella* is transmitted through the fecal oral route, the vaccine is best administered orally. Oral vaccines should be designed to induce mucosal immunity and have protective features to allow survival through the harsh environment of the GI tract, such as low pH and enzymatic degradation, before reaching the site of absorption in the small intestine (Desin et al., 2013). Oral vaccines are ideal for broiler chickens because of the noninvasive delivery to the primary site of infection. However, the oral vaccines must survive the hostile and acidic environment of the stomach that leads to degradation of the vaccine before reaching the target (Salman et al., 2009; Iglesias et al., 2017). Oral vaccinations have been shown to increase mucosal IgA antibodies as well as produce a systemic immune response (Freytag and Clements, 2005; Salman et al., 2009). However, multiple and high doses of an oral vaccine are need to produce an effective response (Salman et al., 2009). In practice, the oral vaccines are aerosolized and ingested by the chicks. Fluorescent colors are also used to encourage the chicks to ingest the vaccine. Adjuvants including cholera toxin (CT) derived from Vibrio cholerae, thermolabile toxin (LT) derived from Escherichia coli, synthetic oligodeoxynucleotides with unmethylated CpG dinucleotides (CpG ODN), and mono-phosphoryl lipid A (MPL) can be used

in poultry vaccines to increase immunogenicity (Freytag and Clements, 2005; Thiam et al., 2015; Batista et al., 2017). However, some of these adjuvants are not easy to implement. The A subunit of both CT and LT causes enterotoxicity in some cases; while the B subunit is not effective when administered orally (Thiam et al., 2015).

Table 2.1: Current *Salmonella* vaccines available for layers and broilers.

Vaccine	Route	Bacteria	Type	Age for dose
Layermune	sc.	ST	Inactivated	W5, W9
Corymune	im	ST	Inactivated	W5, W9
Salenvac	im	ST	Inactivated	D1, W4
SE guard	sc	SE phage types 4, 8, 13a	Inactivated	10W and up
PoulVac ST	Spray or water	SE, ST, SH	Modified live	D3, W3, M3
PoulVac SE	sc	SE Phage types 4, 8, 13a	inactivated	W12, W20
PoulVac SE-ND-IB	sc	SE	killed	
AviPro Megan Vac 1	oral	SE, ST, SH	Live	D1, W2, W5

Use of nanoparticles to aid in vaccine delivery and efficacy

The toxic adjuvants such as CT and LT were believed to be needed to produce a robust vaccine response (Muller et al., 1989). The use of nanoparticles as adjuvants help protect the vaccine antigens from degradation in the GI tract, In addition, the efficient release of the antigen and the low toxicity of nanoparticle vaccines may prove to be safer and more effective than using CT and LT adjuvants (Salman et al., 2009; Mahapatro and Singh, 2011). Polymeric and polyanhydride nanoparticles show the most promise in terms of being a carrier molecule for antigens. Polymeric nanoparticles are classified as natural or polyester. While polyanhydride is

similar to the polyester nanoparticles, they are also more diverse and more easily manipulated (Smith et al., 2015).

The use of anti-OMP antibodies incorporated with LPS are shown effective in inducing immunity in mice (Yokoyama et al., 1998). LPS proteins, particularly for *S.* Enteritidis, target toll-like receptor-4 (TLR-4) located on the gut epithelium (Mahapatro and Singh, 2011). Inclusion of OMPs in chicken *Salmonella* vaccines facilitates M cell uptake and promotes antibody production by B cells. Nanoparticle vaccine uptake is increased by labeling the surface of nanoparticles with flagella in nanoparticle vaccines, by targeting the M cells using flagella(Forbes et al., 2012). Moreover, it has been shown that the flagellar surface labeling of a nanoparticle offers a long lasting immune response through an increase in antibody titers (Sharma et al., 2015). The nanoparticles are absorbed and degraded by the phagosome and phagolysosome of APCs. In infection models of *S.* Typhimurium, the OMP antigen was the primary target of sIgA antibodies (Forbes et al., 2012). Through the incorporation of flagella and OMP antigens, the potential for an effective vaccine against *Salmonella enterica* serovars could mitigate the issues with vaccination of broilers in industry settings. The nanoparticles aid in antigen delivery by protecting the antigens through the GI tract and gradually release the antigens for effective absorption.

The physiochemical properties, such as particle size and shape, also allow for more effective vaccine delivery (Vartak and Sucheck, 2016). The small size of the nanoparticle promotes absorption by allowing higher binding by the intestinal cells, such as M cells. The hydrophobicity of nanoparticles increase detection by APCs and uptake by dendritic cells (Salman et al., 2009; Petersen et al., 2011). The nanoparticles can alter the release of the vaccine by either extending or delaying the release of the vaccine (des Rieux et al., 2006). Therefore, antigen affinity is maximized. Nanoparticle vaccines induce strong humoral and cell mediated immune responses, *in*

vivo (Chattopadhyay et al., 2017). Because of the absence of live pathogens in nanoparticle vaccines, it is less pathogenic; therefore, safer than traditional live vaccines. The ability to include multiple antigens in the nanoparticle vaccines, allows protection against different serovars of pathogens (Chattopadhyay et al., 2017). Some nanoparticle vaccines can be altered to target a specific immune response including the improvement of antigen uptake and antigen cross-presentation by antigen presenting cells such as dendritic cells and macrophages (des Rieux et al., 2006; Gamazo et al., 2015; Kashyap et al., 2015).

Current development of nanoparticles poses technical difficulties. For example, a higher dose is needed to induce a sufficient immune response for long term protection with chitosan and poly-(dl-lactide-co-glycolide) based nanoparticles (Mahapatro and Singh, 2011). The polyanhydride nanoparticle has been proven to provide adequate immune stimulation without the need of additional adjuvants (Vela Ramirez et al., 2017). A single dose of a polyanhydride based vaccine was sufficient to induce adequate immunity when administered intranasally (Delgado et al., 1999; Hori et al., 2005; Ulery et al., 2011). In mice, a single dose of a *Shigella* polyanhydride vaccine orally administered, has shown to induce a Th1 response along with increase secretory IgG antibodies(Camacho et al., 2013).

Polyanhydride, a synthetic polymer, is biodegradable. The adhesion properties are altered depends on the target and has an extended release of the encapsulated antigen (Iglesias et al., 2017). This nature of the nanoparticle allows for its surface to be modified with ease. Therefore, it has high bioadhesive properties, which allows the vaccine to persist in the intestine and gradually release the antigens (Iglesias et al., 2017). The low solubility in an acidic solution but high solubility in a basic solution allow polyanhydride to be an ideal candidate for an oral *Salmonella* vaccine (Calleja et al., 2015). The nanoparticle vaccines were successful in activating immune

cells *in vitro* by increasing activation of dendritic cells due to the extended release of antigens while increasing Th and CTL activation through antigen presentation with MHC (Torres et al., 2011; Kim et al., 2014). The nature of this process proves that polyanhydride nanoparticles serve as effective adjuvants. In order to determine the efficacy of this nanoparticle vaccine, we performed *in vivo* studies to characterize the protective properties against *Salmonella* infection in broiler chickens.

CHAPTER 3

DOSAGE DETERMINATION OF A POLYANHYDRIDE NANOPARTICLE VACCINE[1]

[1] Lester, Bailey. To be submitted to *Poultry Science*

ABSTRACT

This study aimed at determining the optimum dose and booster series for a polyanhydride nanoparticle vaccine for *Salmonella enterica* Enteritidis. Birds were vaccinated with different dosages and series of booster vaccinations to determine the efficacy of a nanoparticle encapsulated *S.* Enteritidis vaccine along with a PBS solution serving as the negative control. A total of 210 birds were vaccinated with PBS (treatment 1), 250 μg (treatment 2 and 4) or 500 μg (treatment 3 and 5) of the vaccine dissolved in 100 μL of PBS and administered through oral gavage on d 0 and d 6. On d 12 and d18, birds from treatment 4 and 5 received two additional boosters of the vaccine. When vaccinated with a single dose of the 250 μg vaccine, birds had increased bile IgA antibodies 6 days after the administration ($P = 0.02$), while birds vaccinated with 500 μg and a booster had increased bile IgA antibodies on d 12 ($P = 0.03$). There was no difference in birds who received the vaccine and a single booster versus three boosters of the same concentration vaccine through day 20, two days after the third booster was administered ($P = 0.0008$). This study determined that vaccinating at d 0 and administering a booster at d 6 with doses of either 250 μg or 500 μg of a polyanhydride nanoparticle vaccine was sufficient to induce *S* Enteritidis immunity in broiler chicks.

INTRODUCTION

Vaccination against human diseases began in the late 18th century with the smallpox vaccine developed by Edward Jenner (Stern and Markel, 2005). It was over a century later when the first measures for immunization in poultry were developed for a viral vaccine against fowl pox (Espeseth and Lasher, 2010). Throughout the early development of poultry vaccines, the industry encountered multiple issues including reduced immunogenicity and bacterial contamination

(Espeseth and Lasher, 2010). Later, in the 1950s, the first practical vaccines for a *Salmonella enterica* serovar were developed, specifically for *Salmonella enterica* Gallinarum (Smith, 1956). While these initial vaccines were successful, they only produced a serovar-specific response against *S.* Gallinarum, a poultry-specific serovar. Developing a non-host specific *Salmonella* vaccine has not been as universally successful (Barrow, 2007).

Vaccination of birds against diseases is common among both broiler and layer sectors of the poultry industry. According to PennVet and Merck Veterinary Manual, broiler chicks are vaccinated against Marek's Disease, Newcastle disease, and Infectious Bronchitis on day 1, with boosters for both Newcastle and Infectious Bronchitis between day 14 and 21 with an initial vaccination of Infectious Bursal Disease also administered. PennVet also recommends laryngotracheitis in endemic areas, such is the case in the southeast USA.

Salmonella is not included in the vaccine protocols for broiler chicks as the history of delivering a killed vaccine has not provided long term immunity but does reduce colonization while live vaccines succeed in eliciting an immune response and do not persist in the gut (Dórea et al., 2010; Desin et al., 2013). The main issue with live vaccines in the commercial industry is that they cannot be given within 21 days of slaughter according to the manufacturers Elanco and Zoetis. Initially, vaccine control of *Salmonella* was aimed to reduce the bacteria in breeder stock. By reducing the *Salmonella* load in broiler breeders, vertical transmission to broiler chicks was reduced (Dórea et al., 2010). The next task in the broiler industry is to reduce the horizontal transmission of *Salmonella* between flocks in the broiler houses.

The vaccine to be evaluated in this study is a subunit vaccine consisting of two prominent antigens on the surface of *S.* Enteritidis: flagella and outer membrane protein A. Considerations such as pH resistance and nanoparticle size need to be taken into account when designing an orally

administered vaccine (Desin et al., 2013). The characteristics of the subunit nanoparticle vaccine tested in this study are listed in Table 3.1 below. An important characteristic of nanoparticle vaccines shared with subunit vaccines in general is their pH stability. As these vaccines are commonly administered orally, how well they maintain their structure as the pH decreases as well as protecting the entrapped antigens is an important factor to consider. Entrapment and surface labeling efficiency is a unique feature to nanoparticle vaccines (Pati et al., 2018). The former determines how much of antigen the nanoparticle is able to encapsulate, providing protection during the digestive process while the latter allows antigen presenting cells to recognize the nanoparticle and initiate the signaling pathway that fronts the immune response (Pati et al., 2018). The objective of this study is to evaluate the efficacy of the described vaccine and its ability to induce a *S.* Enteritidis specific response in broiler chicks.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Georgia. A total of 210 day-old male broilers (n=210) were distributed equally into 30 battery cages. Birds were provided *ad libitum* water and a starter diet.

Treatments

All birds were orally inoculated via gavage needle with 0 (treatment 1), 250 (treatments 2 and 4) or 500 μg (treatments 3 and 5) per bird in 100 μL of PBS. All chicks were vaccinated on d 0 with a booster on d 6 for all treatments. Treatment 1 received 100 μL of PBS, treatments 2 and 4 received 250 μg in 100 μL of PBS, treatments 3 and 5 received 500 μg in 100 μL of PBS. On d 12 and d 18, treatments 4 and 5 received two additional boosters of 250 and 500 μg in 100 μL of PBS respectively. Bile and serum samples were collected on day 6, 12, 18, and 20 and analyzed for IgA and IgG concentration by ELISA. Optical density was measured at 450 nM to determine

antibody concentrations. Bile was collected using either a 1ml or 3 ml syringe with a 21g needle. Samples were then placed in a 1.5 mL Eppendorf tube and placed in -80° C until tested. Whole blood was collected using a 3 mL syringe using a 21g needle. Samples were then placed in a 3 mL Eppendorf tube and centrifuged at 3000 rpm for 10 minutes. Serum was removed and collected in a 1.5 mL Eppendorf tube then placed in -80° C until tested.

Effect of Salmonella antigen coated nanoparticle vaccine on bile IgA and serum IgG antibodies

At d 6, 12, 18, and 20, 6 birds per treatment were chosen for sample collection of blood serum and bile (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 100 μL of the antigen (10 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) (200 μL/well). To prevent non-specific binding, wells were blocked with PBS-Tween 20 – 8% nonfat dry milk (200 μL/well) incubated for 90 m at room temperature on orbital shaker. Plates were washed three times using PBS- Tween 20 (200 μL/well). For IgA analysis, the bile was diluted 1:200 in PBS-Tween 20 – 5% nonfat dry milk and added to the plates (100 μL/well) in duplicates (two wells per sample) and incubated for 90 m at room temperature on an orbital shaker. Plates were washed three times using PBS- Tween 20 (200 μL/well). After washing, HRP-conjugated anti-chicken IgA diluted 1:100,000 in PBS-Tween 20 – 5% nonfat dry milk was added to each well (100 μL/well) and incubated for 1 h at room

temperature. For IgG analysis, the serum was diluted 1:10 and the HRP-conjugated anti-chicken IgG was diluted 1:8,000 in PBS-Tween 20 – 5% nonfat dry milk, added to each well (100 μL/well), and incubated for 30 m at room temperature. Plates were washed three times using PBS- Tween 20 (200 μL/well) then 100 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 20 m by adding 1 N hydrochloric 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.

Statistical Analysis

A one-way ANOVA (JMP®, Version 14. SAS Institute Inc., Cary, NC, 1989-2019) was used to examine the effects of vaccination on antibody levels, cytokine gene expression, and *Salmonella* shedding in ceca from d 7 to d 25 of age. When the effects were significant ($P < 0.05$), differences between means were analyzed by Tukey's HSD test.

RESULTS

Effect of Salmonella antigen coated nanoparticle vaccine on bile IgA antibodies

Bile IgA antibodies increased on d 6, 12 and 20 compared to the unvaccinated control. Bile IgA antibodies increased by 86% and 49% for treatments 2 and 3 on d 6, following their initial dose 6 days prior ($P = 0.02$). Bile IgA antibodies increased by 60% and 114% for treatments 2 and 3 on d 12, 6 days after receiving their booster vaccine ($P = 0.03$). Bile IgA antibodies decreased by 24%, and 4% for treatments 2 and 4 and increased by 23% and 11% for treatments 3 and 5 on d

18.and 114% on d 18 (P = 0.057). On d 20, bile IgA antibodies increased by at least 90% for all vaccinated treatments, compared to the unvaccinated treatment (P = 0.0008).

Effect of Salmonella antigen coated nanoparticle vaccine on serum IgG antibodies

Compared to treatment 1, serum IgG antibodies were increased by 13% and decreased by 5% for treatments 2 and 3, on d 6 (P = 0.21). Compared to treatment 1, serum IgG antibodies increased by 9% and decreased by 23% for treatments 2 and 3, on d 12 (P = 0.04). Compared to treatment 1, serum IgG antibodies were decreased by 41% for treatment 2, 10% for treatment 3, 13% for treatment 4, and 49% for treatment 5 on d 18, (P = 0.04). Compared to treatment 1, serum IgG antibodies were increased by 40% for treatment 2, 3% for treatment 3, 23% for treatment 4, and 34% for treatment 5 on d 20, (P = 0.29).

DISCUSSION

Previously there hasn't been an oral subunit vaccine for *Salmonella enterica* Enteritidis in broilers (Desin et al., 2013). The need for an oral vaccine for *Salmonella* in broilers is due to the bacteria's enteric nature (Broz et al., 2012). Vaccination as a preventative intervention in breeder stock is a more common method to decrease *Salmonella* load in carcasses (Dórea et al., 2010). Vaccines for broiler breeders and layers, including AviPro Megan Vac1 by Elanco and PoulVac ST and SE by Zoetis respectively, have been proven successful in overall reduction of *Salmonella* infection. The issue with these vaccines is that they are designed for and marketed towards layers and broiler breeders, not broilers. The vaccines are designed for administration over longer periods of time. For example, PoulVac ST is a modified-live vaccine designed to be administered at 3 days, 3 weeks and 3 months and require a 21-day withdrawal period prior to slaughter. Broilers are raised up until 5 to 7 weeks, which would prevent the 2nd and 3rd boosters of this vaccine to not be administered (Desin et al., 2013). The vaccination of broiler breeders does not factor in

horizontal transmission that occurs in the commercial broiler houses, which can cause rampant *Salmonella* colonization.

In this study, IgA antibodies were increased in the bile of all vaccinated birds compared to the unvaccinated control starting 6 days post vaccination. Serum IgG antibodies did not have a significant difference in the treatments compared to the nonvaccinated control. *Salmonella* is a primarily enteric pathogen and our results reflect that showing a significant increase in the mucosal antibody, IgA. To determine whether this antibody is present at the site of infection, future studies should also collect mucosal scrapings of the intestinal linings to test for ani-*Salmonella* IgA via ELISA. We determined that a two series vaccine was able to induce the production of anti-*Salmonella* antibodies in the vaccinated bird. Less boosters for this vaccine would help decrease the overall cost to vaccinate and time devoted to preparing an oral vaccine.

Further work in administration methods should be performed to evaluate the ability of the vaccine to elicit an immune response beyond the oral gavage method used in this study. In ovo, spray, and drinking water methods for vaccine administration have all been proven to be successful in vaccines for *Salmonella* and other poultry diseases such as Marek's disease and Newcastle Disease (Desin et al., 2013).

TABLES AND FIGURES

Table 3.1 Polyanhydride nanoparticle vaccine characteristics

Character	Polyanhydride
Average size	208 nm
Size range	80-380 nm
Entrapment efficiency	78%
Surface labeling efficiency	25%
Hemolysis	0%
pH stability	
at 3.5 pH	7.1%

at 4.5 pH	2%
at 5.5 pH	5.7%
at 6.5 pH	0%
at 8 pH	0%

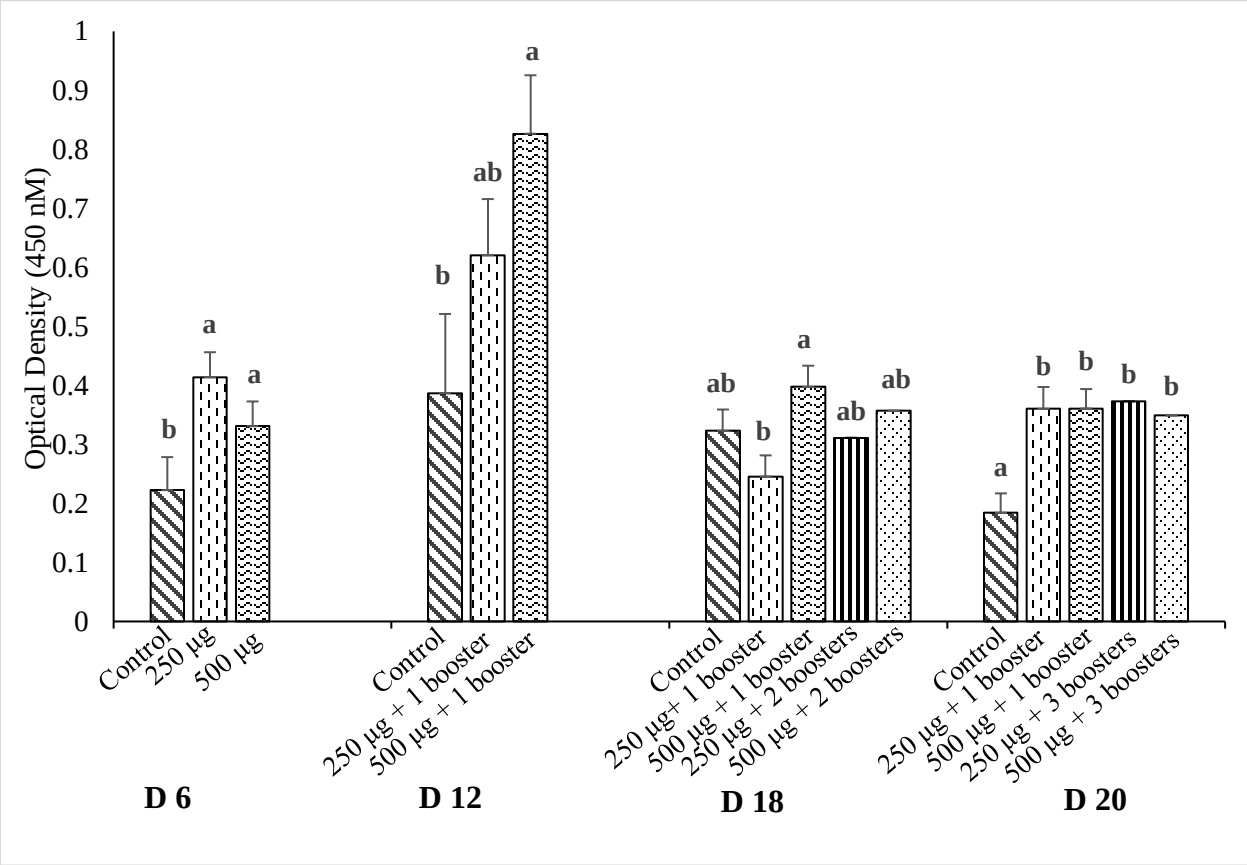

Figure 3.1: Effect of *Salmonella* antigen coated nanoparticle vaccine on bile IgA antibodies

Broiler birds were inoculated orally with either 0 (treatment 1), initial dose plus 1 booster of 250 µg (treatment 2), initial dose plus 1 booster of 500 µg (treatment 3), initial dose plus 3 booster of 250 µg (treatment 4), or initial dose plus 3 booster of 500 µg (treatment 5) per bird in 100 µL of PBS on d 0 and 6 for all treatments and d 12 and 18 for treatments 4 and 5. Serum samples were collected on day 0, 6, 12, 18, 20, and 22 and analyzed for IgA content by ELISA. OD values at 450 nM are reported. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 6.

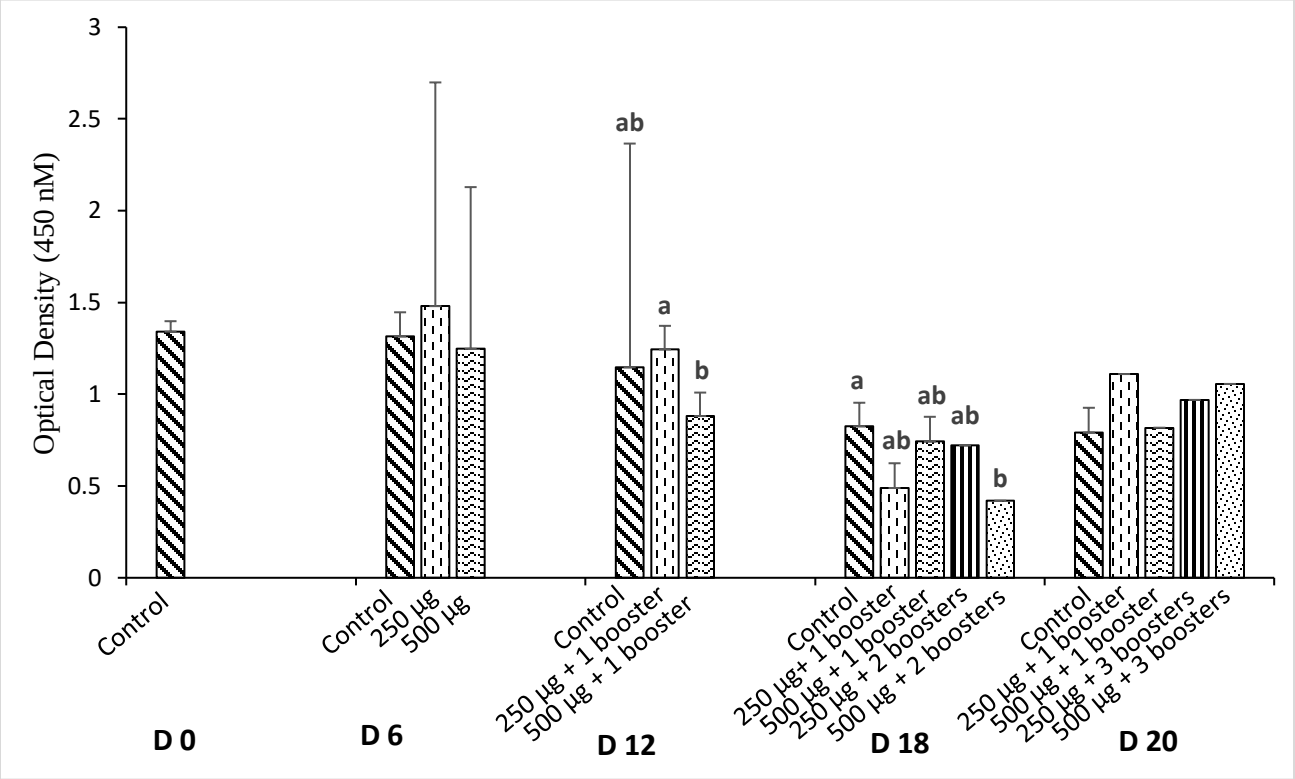

Figure 3.2: Effect of *Salmonella* antigen coated nanoparticle vaccine on serum IgG antibodies

Broiler birds were inoculated orally with either 0 (treatment 1), initial dose plus 1 booster of 250 µg (treatment 2), initial dose plus 1 booster of 500 µg (treatment 3), initial dose plus 3 booster of 250 µg (treatment 4), or initial dose plus 3 booster of 500 µg (treatment 5) per bird in 100 µL of PBS on d 0 and 6 for all treatments and d 12 and 18 for treatments 4 and 5. Bile samples were collected on day 6, 12, 18, 20, and 22 and analyzed for IgG content by ELISA. OD values at 450 nM are reported. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 6.

CHAPTER 4

DOSAGE AND EFFICACY OF A POLYANHYDRIDE NANOPARTICLE VACCINE IN BROILERS CHALLENGED WITH *SALMONELLA* ENTERITIDIS[2]

[2] Lester, Bailey. To be submitted to *Poultry Science*

ABSTRACT

Birds were vaccinated with a nanoparticle vaccine with two doses of either 250 μg, 500 μg, or 1000 μg per dose on d 0 and d 7. All birds were then challenged with 2.4 x 10^6 CFU of a wild-type strain of *Salmonella enterica* Enteritidis. Vaccine efficacy was determined through an antibody response curve, bacterial load analysis and cytokine gene expression of the cecal tonsil via PCR. Birds vaccinated with 250 had a 73% decrease of bile IgA antibodies 11 days post infection compared to the unvaccinated group ($P < 0.05$). Birds that received 1000 μg of the vaccine had a .6% decrease in serum IgG antibodies 9 days post infection compared to the unvaccinated group ($P = 0.024$). The bacterial load of the cecal contents and the cytokine gene expression in the cecal tonsil did not report significant difference in the treatments compared to the control indicating clearance of the bacteria from the ceca of the birds.

INTRODUCTION

The immune response of broilers to non-typhoidal *Salmonella enterica* has previously been broken down into three stages: resistance, tolerance, and homeostasis (Kogut and Arsenault, 2017). Resistance is marked by innate immune cells infiltrating the mucosal associated lymphoid tissue of the small intestine and driving an inflammatory response to the bacteria while activating adaptive immune cells to achieve bacterial clearance, activating *Salmonella* specific B and T cells. Roughly three days post-infection, the pro-inflammatory response begins to wane and the rise of the anti-inflammatory response, marked by regulatory T cell, begins as the bacteria colonize in the gut followed quickly by homeostasis in the gut a few days later. On the other side, if the bird was able to mount a sufficient immune to the infection and achieve clearance of the bacteria, the immune cells driving this are pro-inflammatory. IFN-γ is one of the more prominent cytokines feature in the response (Withanage et al., 2005).

Non-typhoidal serovars of *Salmonella* contribute to the onset of foodborne illnesses worldwide along with *E. coli* and *Campylobacter* serovars (Zhao et al., 2001). These bacteria invade through horizontal and vertical transmission then proceed to colonize in the lower GI tract of the birds. Among *Salmonella enterica* serovars are the host adapted and non-host adapted serovars. Host adapted serovars, including Pullorum and Gallinarum, causing diseases specifically in poultry while non-host adapted serovars, such as Enteritidis and Heidelberg, infect other species such as humans but are mainly asymptomatic in poultry (Desin et al., 2013). The host-specific serovars were discussed briefly in Chapter 3. Currently the serovars of interest are the non-host adapted serovars. While serovars like *S.* Enteritidis may not directly have an impact on poultry, the infection of poultry carcasses contaminated with the bacteria are an issue with the general human population when they consume the contaminated carcasses (Barrow and Methner, 2013).

Creating an effective vaccine that induces a Th1 driven immune response paired with high production of cross-reactive antibodies would potentially allow for protection among the multitude of *Salmonella* serovars in broilers, decreasing the incidence of salmonellosis as a public health concern worldwide.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Georgia. One-day-old Cobb 500 male broilers (n=14) were distributed equally into 14 battery cages. Birds were provided *ad libitum* water and a starter diet.

Treatments

All birds were orally inoculated via gavage needle with 0 (control), 250, 500 or 100 μg per bird in 100 μL of PBS on d 0 and 7 with a *S.* Enteritidis encapsulated nanoparticle vaccine. On d 14, all birds were challenged with 100 μL of 2.4 X 10^6 CFU *S.* Enteritidis. The *S.* Enteritidis

bacteria were grown from a pure culture wild-type strain. The bacteria were grown overnight for 8 hours in Tryptic Soy Broth. Before challenging the birds, the CFU was tested using a spectrophotometer read at 600 nm and confirmed using serial dilution of the bacteria on Tryptic Soy Agar. Serum samples were collected on d 0 pre-*Salmonella* challenge and 8 h, d 3, d 6, d 9, and d 11 post-*Salmonella* and analyzed for IgG concentration by ELISA. Bile samples were collected on d 11 post-*Salmonella* challenge and analyzed for IgA concentration by ELISA. Optical density was measured at 450 nM to determine antibody concentrations.

Bile was collected using either a 1ml or 3 ml syringe with a 21g needle. Samples were then placed in a 1.5 mL Eppendorf tube and placed in -80 C until tested. Whole blood was collected using a 3 mL syringe using a 21g needle. Samples were then placed in a 3 mL Eppendorf tube and centrifuged at 3000 rpm for 10 minutes. Serum was removed and collected in a 1.5 mL Eppendorf tube then placed in -80° C until tested. Cecal contents were collected and placed in a 1.5 mL Eppendorf tube then placed in -80° C until tested. Cecal tonsils were collected and placed in a 2 ml cryovial and frozen in liquid nitrogen. Vials were then transferred to -80° C.

Effect of Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on serum IgG and bile IgA antibodies

At d 0 pre-*Salmonella* challenge and 8 h, d 3, d 6, d 9, and d 11 post-*Salmonella* challenge, 3 birds per treatment were chosen for sample collection of blood serum (n = 3). At d 11 post-*Salmonella* challenge, 3 birds per treatment were chosen for sample collection of bile (n = 3). *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 100 µL of the antigen (10 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). Plates were washed three times using PBS- Tween 20 (200 µL/well). To prevent non-specific binding, wells were blocked with PBS-Tween 20 – 8% nonfat dry milk (200 µL/well) incubated for 90 m at room temperature on orbital shaker. Plates were washed three times using PBS- Tween 20 (200 µL/well). For IgA analysis, the bile was diluted 1:200 in PBS-Tween 20 – 5% nonfat dry milk and added to the plates (100 µL/well) in duplicates (two wells per sample) and incubated for 90 m at room temperature on an orbital shaker. Plates were washed three times using PBS- Tween 20 (200 µL/well). After washing, HRP-conjugated anti-chicken IgA diluted 1:100,000 in PBS-Tween 20 – 5% nonfat dry milk was added to each well (100 µL/well) and incubated for 1 h at room temperature. For IgG analysis, the serum was diluted 1:10 and the HRP-conjugated anti-chicken IgG was diluted 1:8,000 in PBS-Tween 20 – 5% nonfat dry milk, added to each well (100 µL/well), and incubated for 30 m at room temperature. Plates were washed three times using PBS- Tween 20 (200 µL/well) then 100 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 20 m by adding 1 N hydrochloric 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 Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on pro and anti-inflammatory cytokines

At d 11 post-*Salmonella* challenge, 3 birds per treatment were chosen for sample collection of cecal tonsils (n = 3). The RNA collected from cecal tonsils was reverse transcribed into cDNA as described by Selvaraj and Klasing, (2006). The mRNA was analyzed for IFN- γ, IL-1β, IL-4 and IL-10 by real-time PCR (iCycler, BioRad, Hercules, CA) using SyBr green after normalizing for RPS-13 mRNA. Primers are shown in Table 4.1. Annealing temperatures were 57.5°C for all primers. The CT was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background. The housekeeping gene was verified by analyzing the average Ct value for RPS-13 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 vaccine treatment was calculated using the $2^{(\text{CT Sample} - \text{Housekeeping})}/2^{(\text{CT reference} - \text{Housekeeping})}$ comparative CT method where CT is the threshold cycle (Selvaraj et al., 2010).

Effect of Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on bacterial load of cecal contents

At d 11 post-*Salmonella* challenge, three birds per treatment were selected for sample collection of cecal contents (n = 3). Cecal content samples (0.10 g) were diluted individually in 1 mL of sterile PBS and centrifuged at 11,000 x g for two minutes to remove debris. The pellet was washed two more times. The pellet was resuspended in 50 mM EDTA (50 mM) and lysozyme (20 mg/ml) then incubated for 45 minutes at 37°C. After incubation, samples were re-centrifuged at 11,000 x g for two minutes and the supernatant discarded. Samples were resuspended with lysis buffer and Proteinase K (10 mg/ml) then incubated for ten minutes at 80°C. RNAse was then added to the solution then mixed by inversion followed by incubation for 30 minutes at 37°C. NaCl (6M)

was added to the cell lysate then placed on ice for 5 minutes. Samples were then centrifuged at 11,000 x g for 5 minutes and the supernatant was transferred to 1.5 mL microtubes. Isopropanol was added to the supernatant mixed by inversion. The DNA thread was transferred to filter columns, then centrifuged for 1 m 30 s at 12,000 x g. The supernatant was discarded, and the DNA was subsequently washed twice with 70% ethanol with centrifuge after each wash for 1 m 30 s at 11,000 x g. The filter column was transferred to 1.5 mL microtubes then TE buffer was added to the DNA in the column and incubated for 2 minutes at room temperature. The samples were spun for 3 minutes at 14,000 x g then incubated for 60 minutes at 60°C. DNA samples were stored at 4°C. *Salmonella* load was analyzed by real-time PCR (Amit-Romach et al., 2004) using the primer shown in Table X. The annealing temperature for *Salmonella enterica* primers was 60.5°C. The CT was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background. CFU analysis was then performed as described by Shanmugasundaram et al., (2019) using the equation $CFU = \frac{DNA\ quantity}{(mean\ mass\ x\ 6.023x\ 10^{23})}$. Mean mass was obtained using the equation $mean\ mass = ((number\ of\ base\ pairs\ x\ 607.4) + 157.9$. The number of base pairs for the *S. enterica* primers was 310.

Statistical Analysis

A one-way ANOVA (JMP®, Version 14. SAS Institute Inc., Cary, NC, 1989-2019) was used to examine the effects of vaccination on antibody levels, cytokine gene expression, and *Salmonella* shedding in ceca from d 7 to d 25 of age. When the effects was significant ($P < 0.05$), differences between means were analyzed by Tukey's HSD test.

RESULTS

Effect of Salmonella nanoparticle vaccine on bile IgA antibodies in birds challenged with Salmonella Enteritidis

Vaccines were administered with either 0 (control), 250, 500, 1000 μg per bird in 100 μL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Bile IgA samples were collected on day 25 and analyzed for antibody concentration via ELISA. Birds vaccinated with 250 μg had a 73% decrease in bile IgA antibodies compared to the unvaccinated challenged control 11 days following challenge ($P < 0.05$). Broiler birds that were not vaccinated had higher ($P = 0.054$) bile IgA content compared to the vaccinated birds. Compared to the control birds, birds inoculated with 250 μg, 500 μg, and 1000 μg had 73%, 40% and 11% decreased bile IgA content, respectively.

Effect of Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on serum IgG antibodies

Vaccines were administered with either 0 (control), 250, 500, 1000 μg per bird in 100 μL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Serum IgG samples were collected on d 7, 14, 17, 20, 23, and 25 and analyzed for antibody concentration via ELISA. There was an increase in serum antibodies following challenge from d 7 to d 14. On d 17, there was a decrease in serum IgG antibodies, 3 days post infection, in all groups, maintaining the level through d 20. On d 23, serum IgG antibodies increased from previous days but did not reach levels like that of immediately post challenge serum IgG levels. The 1000 μg vaccinated group had a significant difference ($P = 0.024$) compared to the unvaccinated birds. All treatments had decreased serum IgG antibodies compared to the control group. 250 μg birds had a 11% decrease, 500 μg birds had a 24% decrease and 1000 μg birds has a .6% decrease in serum IgG antibodies ($P = 0.015$).

Effect of Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on bacterial load of cecal contents

Vaccines were administered with either 0 (control), 250, 500, 1000 μg per bird in 100 μL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal content samples were collected on d 25 and analyzed for *Salmonella* Enteritidis. All groups had low *Salmonella* load in the cecal contents samples. The 250 μg vaccinated treatment had a 37% decrease in CFU/g compared to the challenged control while the 500 and 1000 μg vaccinated groups had a 600% increase in CFU/g ($P > 0.05$).

Effect of Salmonella antigen coated nanoparticle vaccine and Salmonella enteria Enteritidis challenge on pro and anti-inflammatory cytokines

Vaccines were administered with either 0 (control), 250, 500, 1000 μg per bird in 100 μL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal tonsil samples were collected on day 25 and analyzed for pro and anti-inflammatory cytokine expression levels. Vaccinated birds in the 500 μg and 1000 μg treatments had 63% and 15% decreased IFN- γ expression levels, respectively, compared to the unvaccinated treatment ($P = 0.38$). All vaccinated birds showed increased expression of IL-1β compared to the unvaccinated treatment, with 16%, 1%, and 23% in the treatments 250, 500, and 1000 μg, respectively ($P = 0.96$). Vaccinated birds in the 500 μg and 1000 μg treatments had 48% and 20% decreased expression of IL-4 cytokine gene expression compared to the unvaccinated treatment ($P = 0.8$). All vaccinated treatments had decreased expression of IL-10 cytokine expression compared to the unvaccinated treatment with the 500 μg group reporting 48% reduced levels ($P = 0.47$).

DISCUSSION

During this study, we saw a significant decrease in bile IgA antibodies of the 250 μg dose treatment compared to the unvaccinated treatment. Despite there being a low CFU count in the cecal content 11 days post-challenge, which the low challenge dose is a contributing factor, this

group had a lower antibody count. The significant decrease in antibodies may be due to a more rapid clearance of the bacteria compared to the other treatments and the control. A decrease in bacterial IgA has been shown in previous studies following bacteria clearance (Cawthraw et al., 2002). With the anti-*Salmonella* IgG analysis, there was a spike in antibodies 8 hours post-challenge compared to the serum IgG antibodies from the week before. Compared to the control group, all treatments saw an increase in antibodies, indicating possible memory B cells secreting a higher level of antibodies compared to naïve B cells activating and subsequently beginning production of antibodies in response to the infection. The spike in antibodies in all treatments indicates that there was an immune response to the challenge. In previous studies, live vaccines had higher IgG antibody responses compared to killed vaccines but not compared to unvaccinated challenged controls (Harrison et al., 1997). While there was a discernable change in the antibodies of vaccinated birds, there was no significance in differential expression of cytokines in the cecal tonsil of the birds 11 days following challenge. In previous studies, the immune system may become unresponsive to the bacterial challenge, indicating a return to homeostasis prior to the *Salmonella* challenge (Kogut and Arsenault, 2017). In this experiment, it appears that the birds achieved clearance of *Salmonella* and were able to prevent infection.

As there isn't just one problematic serovar of *Salmonella*, a vaccine that creates cross-reactive antibodies towards other serovars would aid in the further reduction of food-borne illnesses caused by *Salmonella.* There are vaccines for layers and broiler breeders that aid in the reduction of *Salmonella* serovars Enteritidis, Typhimurium, and Heidelberg but as mentioned in Chapter 3, these vaccines are not a good option for administration in broiler chicks. With the use of a nanoparticle vaccine, we can incorporate antigens of these serovars into the subunit vaccine. Another way to increase the cross-reactivity of antibodies is to incorporate antigens conserved

among problematic serovars, allowing for a higher density of specific antigens instead of a broad variety of antigens. This was the approach used in the design of this vaccine. The outer membrane protein A and flagella used were derived from *S.* Enteritidis but previous studies show that they are found on other serovars including Dublin and Blegdam (Li et al., 1994). O antigens on *Salmonella* are shared with *E. coli* serovars, providing another direction the development of these vaccines can be explored (Wang and Reeves, 2000).

TABLES AND FIGURES

Table 4.1: Primer Sequences

Target	Primer	Sequence (5' - 3')	Reference
RPS13	F	CAAGAAGGCTGTTGCTGTTCG	(Hutsko et al., 2016)
	R	GGCAGAAGCTGTCGATGATT	
IL-1b	F	TCCTCCAGCCAGAAAGTGA	(Perez et al., 2017)
	R	CAGGCGGTAGAAGATGAAGC	
IFN-g	F	CTGATGGCGTGAAGAATGTG	(Shanmugasundaram, 2018)
	R	CTCCTCTGAGACTGGCTCCTTT	
IL4	F	AACATGCGTCAGCTCCTGAAT	(Röhe et al., 2017)
	R	TCTGCTAGGAACTTCTCCATTGAA	
IL10	F	CAGACCAGCACCAGTCATCA	(Shanmugasundaram, 2018)
	R	CGAACGTCTCCTGGATCTGC	
S. enterica	F	GCAGCGGTTACTATTGCAGC	(Markazi et al., 2018)
	R	CTGTGACAGGGACATTTAGCG	

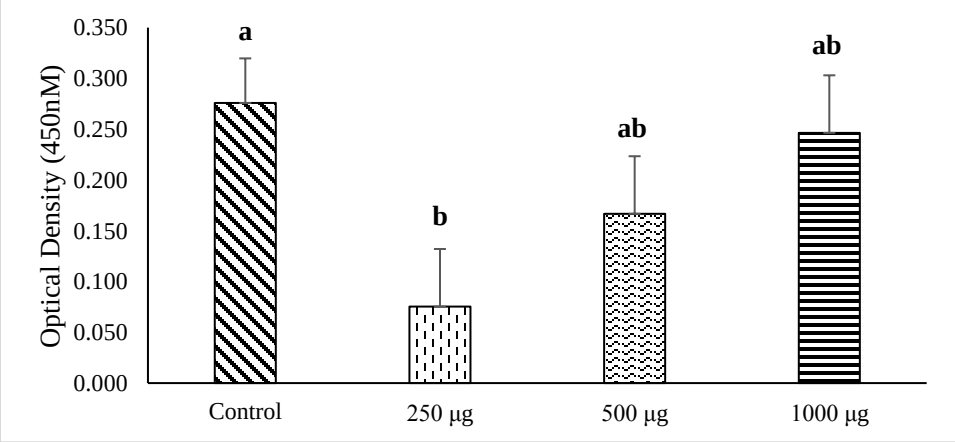

Figure 4.1: Effect of *Salmonella* nanoparticle vaccine on bile IgA antibodies in birds challenged with *Salmonella* Enteritidis

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Bile samples were collected on day 25 and analyzed for IgA content by ELISA. OD absorption values at 450 nM are reported. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

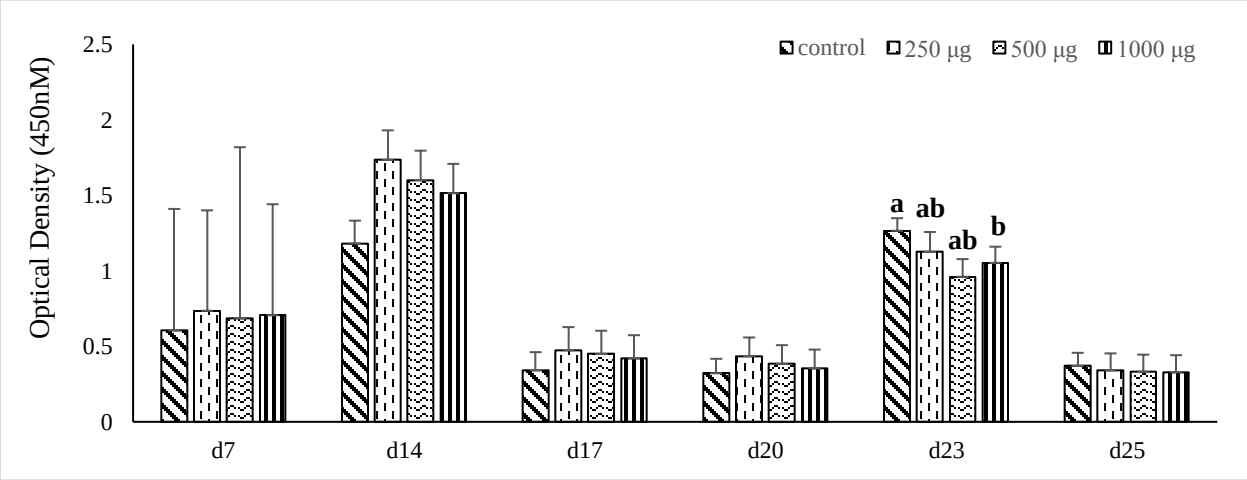

Figure 4.2: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on serum IgG antibodies

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Serum samples were collected on day 7, 14, 17, 20, 23, and 25 and analyzed for IgG content by ELISA. OD absorption values at 450 nM are reported. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

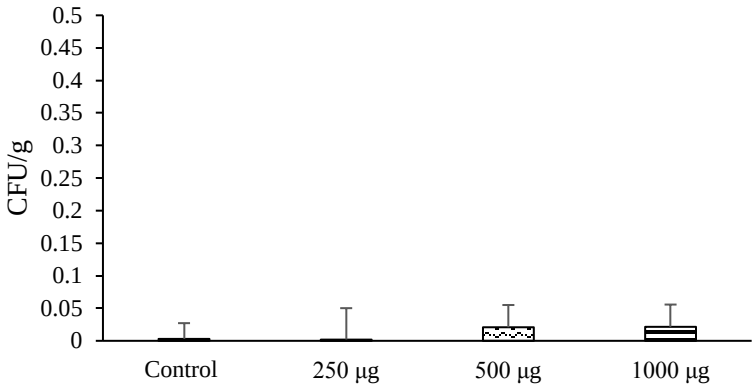

Figure 4.3: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on bacterial load of cecal contents

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal content samples were collected on day 25 and analyzed for *S. enterica* CFU concentration via qPCR. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

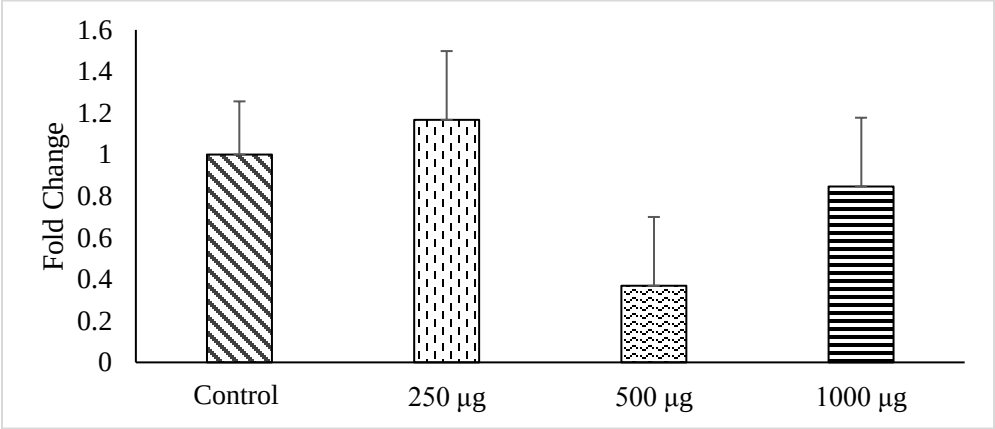

Figure 4.4: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on pro inflammatory cytokine IFN-γ

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal tonsil samples were collected on day 25 and analyzed for IFN- γ cytokine expression levels via qPCR. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

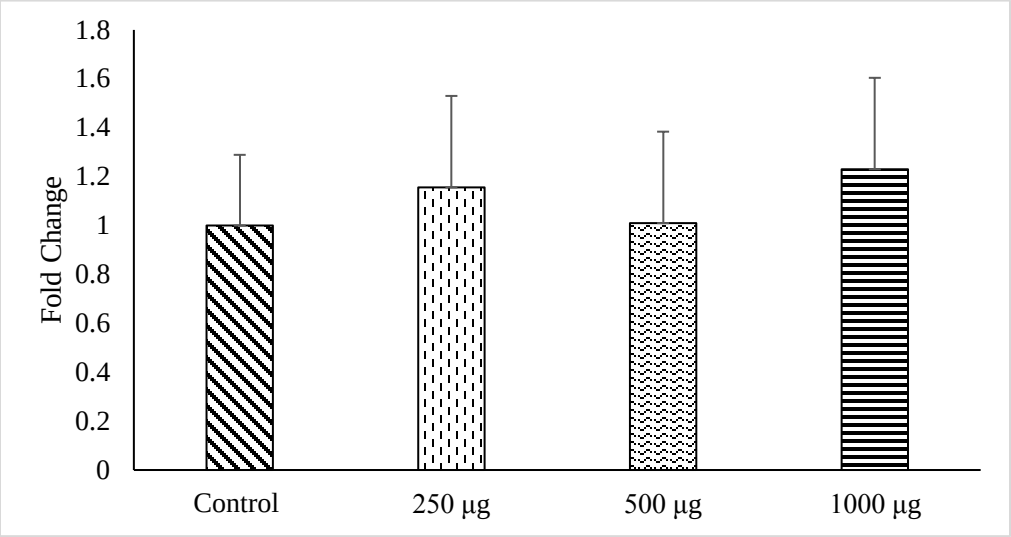

Figure 4.5: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on pro inflammatory cytokine IL-1β

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal tonsil samples were collected on day 25 and analyzed for IL-1β cytokine expression levels via qPCR. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

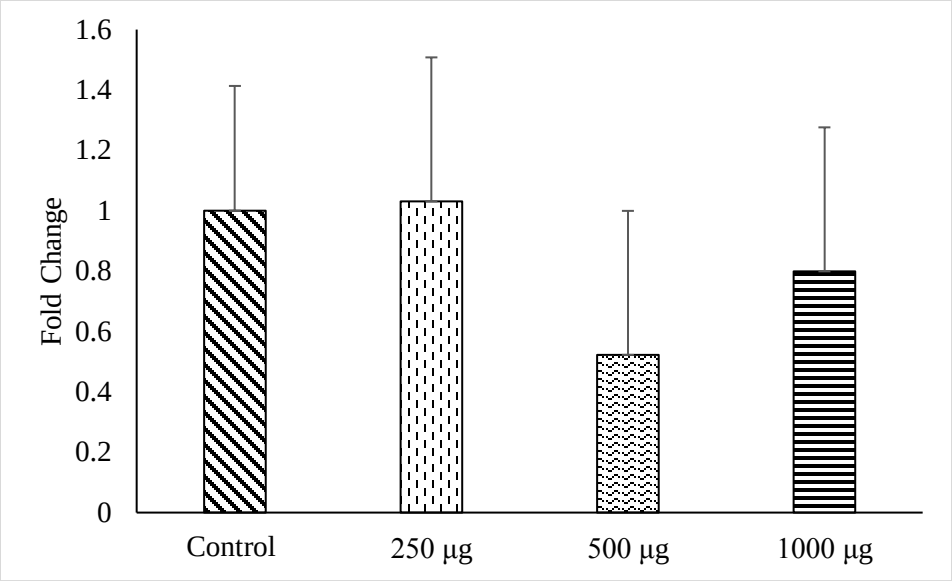

Figure 4.6: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on anti-inflammatory cytokine IL-4

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal tonsil samples were collected on day 25 and analyzed for IL-4 cytokine expression levels via qPCR. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

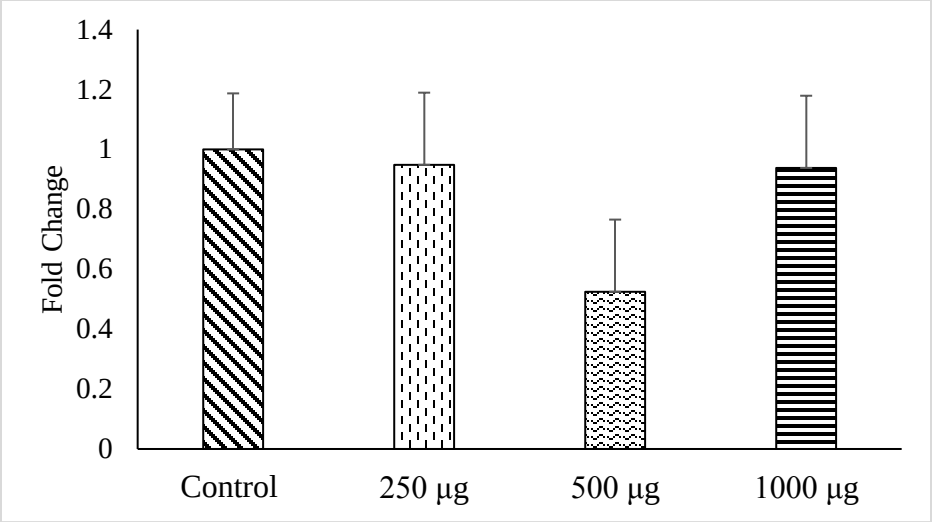

Figure 4.7: Effect of *Salmonella* antigen coated nanoparticle vaccine and *Salmonella enteria* Enteritidis challenge on anti-inflammatory cytokine IL-10

Broiler birds were inoculated orally with either 0 (control), 250, 500, 1000 µg per bird in 100 µL of PBS on d 0 and 7. All birds were challenged with 2.4 X 10^6 *Salmonella enteria* serovar Enteritidis wild-type on day 14. Cecal tonsil samples were collected on day 25 and analyzed for IL-10 cytokine expression levels via qPCR. Bars (+ SE) with no common superscripts differ significantly ($P < 0.05$), n= 3.

CHAPTER 5

CONCLUSIONS

Food-borne illnesses caused by *Salmonella enterica* infections are partially attributed to contamination of poultry carcasses in slaughter plants. With these studies, our objective was to determine the efficacy of a *S.* Enteritidis subunit nanoparticle vaccine that was orally administered to broilers. The bacterial components, flagellar protein and outer membrane protein A, were used to promote the uptake and transfer of the vaccine by M cells and processing of the vaccine by dendritic cells located in the intestinal lumen and Peyer's Patches. The ultimate goal was to induce the production of IgA antibodies and T cell differentiation to mitigate the *Salmonella* infection within the gut of the broiler chicks.

There are numerous *Salmonella* vaccines on the market for serovars including Enteritidis, Heidelberg, and Typhimurium but these vaccines are not marketable towards broilers due to mandated withdrawal periods or non-economically feasible administration methods. Preliminary studies showed that an orally administered *Salmonella*-loaded nanoparticle vaccine can induce the production of mucosal antibodies. This type of vaccine has a distinct advantage over commercially available oral *Salmonella* vaccines. It is a potent inducer of mucosal immunity while bypassing limitations for the other oral vaccines including regulatory withdrawal periods and consumer perception making it advantageous over current commercial vaccines. The polyanhydride nanoparticle subunit vaccine tested in the first study are proven to be an effective vaccine in inducing immunity against *S.* Enteritidis in as little as two doses when administered orally.

In the second study, the vaccine was tested against a challenge of *S.* Enteritidis wild-type. The effectiveness of the vaccine was shown initially with an increase in anti-*Salmonella* IgG antibodies and a decrease in IgA antibodies following the clearance of the bacteria. This may indicate that both IgG and IgA may play a role in clearing *Salmonella* infections. Further studies should be carried out to determine the importance of these antibodies and their role in preventing colonization or promoting clearance of bacterial infections in the gut, and the effectiveness of this vaccine against a persistent infection through the administration of a higher level of *S.* Enteritidis challenge.

A nanoparticle *S.* Enteritidis vaccine, would allow for protection in broiler chicks and help reduce the incidence of *S.* Enteritidis infections in humans. These studies show that polyanhydride is effective when used as a nanocarrier of *Salmonella* antigens and is effective at protecting broilers against *Salmonella* colonization and infection. Testing this vaccine with other problematic *Salmonella* serovars, either on its own or combined with commercially available vaccines would determine the cross-protection of the vaccine's antigens and their antigen's ability to induce immunity against said serovars. Finally, a viable alternative to oral gavage for the administration of the vaccine should be determined and reevaluation of optimal dosage in these alternative administration methods should be performed before this vaccine is marketed towards the broiler industry. Overall, this vaccine shows potential as a good alternative to conventional *Salmonella* vaccines that are currently being used in the commercial poultry industry.

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