EFFICACY OF CHITOSAN-BASED NANOPARTICLE VACCINE ADMINISTRATION IN BROILER BIRDS CHALLENGED WITH *SALMONELLA*

by

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(Under the Direction of Ramesh Selvaraj)

ABSTRACT

Salmonellosis, a zoonotic disease caused by *Salmonella*, is primarily attributed to the consumption of poultry eggs and meat. *Salmonella* enterica serovars enteritis (SE) and heidelberg (SH) are among the most frequent serotypes recovered from humans. Within few hours after chickens are orally infected, *Salmonella* can invade the intestinal tract and reach internal organs. Reducing SE or SH colonization in poultry may lead to a decrease of its transfer to humans, resulting in fewer outbreaks. Vaccines have proven to be a useful tool to control *Salmonella*. Their early protective efficacy is primarily conferred by the induction of antigen-specific antibodies. Killed *Salmonella* vaccines eliminate the probability of the live strain to regain its virulence, while oral administration of antigens efficiently stimulate mucosal and systemic immune responses. However, no oral killed vaccines are currently commercially available for broiler. This project characterizes the protective effects of an oral Chitosan Nanoparticle-*Salmonella* vaccine using broiler birds.

INDEX WORDS: *Salmonella*, Chitosan, Nanoparticles, Vaccination, Oral delivery, Broiler

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B.S., The University of Puerto Rico at Aguadilla, 2017

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2019

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May 2019

DEDICATION

I dedicate my thesis to my parents Yolanda and Pablo, and my siblings Erick, Omayra and Kristhine. Thank you for your unconditional and endless love and support. You are the spark that lights all my tomorrows.

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my advisor, Ramesh Selvaraj. Thank you for your guidance, patience and support. I am very thankful to have been your intern and continue my graduate education as your MS student.

I would also like to thank Woo Kim and Manpreet Singh for serving on my committee. I appreciate your guidance and feedback throughout my research. Thank you for the time you have taken to serve on my committee.

To Elsa Colón, Moises Cordero, Jose Cardé and Juliana Laspiur, thank you for your time and guidance during my undergraduate education. Your teachings have been the stepping stones that have allowed me to keep moving forward and reach my goals. I am forever appreciative of your time and support.

To Ashley Markazi, thank you for being an amazing mentor and friend during my internship at the Selvaraj lab. Your teachings and guidance have been a key component during my undergraduate and graduate education. I am forever grateful of the time you have taken to guide me and help me grow.

To members of the Selvaraj lab: Theros Ng, Muhammad Murtada, Gabriel Akerele, Jarred Oxford, Nour Ramadan, Ragini Reddyvari, Bailey Lester, and Revathi Shanmugasundaram. I am very grateful for all your help over the years. We have truly become a big family.

To my family and friends, I love you, and I am very thankful for your full and unconditional support through this journey. May the force be with you, always.

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CHAPTER 1

INTRODUCTION

Approximately 1.3 billion cases of human gastroenteritis due to *Salmonella* are reported annually worldwide, resulting in 3 million deaths (Gong *et al.* 2014). In the United States of America (USA), *Salmonella* is the leading foodborne pathogen, causing the largest number of deaths and has the highest cost burden (Batz *et al.* 2012). From the past decade, the highest numbers of *Salmonella* outbreaks are related to land animals, with more than 70% of human salmonellosis cases in the USA attributed to the consumption of contaminated chicken or eggs (Braden 2006, Pires *et al.* 2014, Andino and Hanning 2015). *Salmonella* enteritidis, *Salmonella* typhimurium and *Salmonella* heidelberg are the three most frequent serotypes recovered from humans each year (Gong *et al.* 2014).

Salmonella possesses effective acid tolerance mechanisms and upon ingestion it will survive passage through the low-pH conditions of the stomach (Hallstrom and McCormick 2011, Higginson *et al.* 2016), stimulate macrophages, evade killing by the host immune system (Pilonieta *et al.* 2014), and potentially transition to a systemic infection (Acheson and Hohmann 2001). Previous studies have shown that *S.* enteritidis can suppress nitric oxide (NO) production in infected chicken macrophage HD11 cells, while dead *S.* enteritidis stimulates a high level of NO production (He *et al.* 2013); compromising the cellular and downstream humoral immunity of the host. Hence, clearance of *Salmonella* in poultry requires strong humoral and cell-mediated immune responses (Van Immerseel *et al.* 2002, Raybourne *et al.* 2003, Neto *et al.* 2008, Markazi 2018).

Reducing *S.* enteritidis colonization in poultry may lead to a decrease human transmission, resulting in fewer cases of salmonellosis (Greig and Ravel 2009, Markazi 2018).

Vaccines have proven to be a useful tool to control *Salmonella* infections. Live attenuated vaccines produce both a strong humoral and cell-mediated responses (Lalsiamthara *et al.* 2016), however killed *Salmonella* vaccines are preferred as opposed to live vaccines due to the ability of the live strain to regain its virulence (Lauring *et al.* 2010, Kollaritsch and Rendi-Wagner 2012, Renu *et al.* 2018b) . In contrast, the route of administration for commercially available *Salmonella* killed vaccines in poultry poses a high disadvantage. These vaccines are injected manually in the breast muscle, which is time consuming, impractical for big poultry flocks, and decreases breast meat quality. Oral administration is typically referred to as the "ideal route" (Revolledo and Ferreira 2012, Gong *et al.* 2014) because it mimics natural infection, stimulates the mucosal and systemic immune responses, and decreases the cost of vaccination administration (Revolledo and Ferreira 2012). However, there are no oral killed vaccines currently commercially available for broilers due to the challenging acidic nature of the gastrointestinal tract (GIT) (Shaji and Patole 2008, Vela Ramirez *et al.* 2017, Renu *et al.* 2018b). Over the past few decades oral protein and peptide delivery carrier systems, such as nanoparticles, have been explored to overcome this problem (Shaji and Patole 2008, Salman *et al.* 2009a, Renu *et al.* 2018b).

Nanoparticle vaccines consist of a polymer coating that surrounds the vaccine antigen (Zhao *et al.* 2014) and protects the vaccine against chemical, enzymatic or immunological degradation (Tiwari *et al.* 2012, Sahdev *et al.* 2014, Zhao *et al.* 2014). The prolonged survivability of the vaccine within the GIT results in reducing the dosing frequency and the need for adjuvants, as they can act as adjuvants themselves (Tiwari *et al.* 2012), and also facilitating the presentation of the vaccine antigens to specific immune sites of the mucosal immune system (Cheung *et al.*

2015). Immunodominant components of the bacteria (lipopolysaccharide, fimbriae, and outer membrane proteins) also play a crucial role in increasing the vaccines efficiency (Coffman *et al.* 2010). Ligands can be conjugated to the surface of the nanoparticle to increase the presentation of the nanoparticle vaccine to a specific site within the GIT (Salman *et al.* 2009a, Renu *et al.* 2018b). For example, conjugating flagellin proteins to a nanoparticle *Salmonella* vaccine in mice mimicked the natural colonization of *S.* Enteritidis in the GIT, resulting in uptake of the antigen by the ileal Peyer's patches (Salman *et al.* 2009b), where aggregates of immune cells are located. Previous research has shown targeted delivery of protein antigen to dendritic cells (Zeng *et al.* 2013) and also indicate that several nanoparticles modulated immune responses via TLR (Luo *et al.* 2015). As a result, nanoparticles are advantageous for use as an oral vaccine, with an easier administration and a more effective local and intestinal immune responses.

Chitosan is a natural biodegradable copolymer derived from the partial deacetylation of chitin (Cheung *et al.* 2015). Previous research indicates that biodegradable chitosan nanoparticle (CNP) vaccines have ideal traits for delivering vaccine antigen loads orally (Wang *et al.* 2011b, Binnebose *et al.* 2015, Cheung *et al.* 2015). The amino and carboxyl groups in the chitosan molecule can be combined with glycoprotein in mucus to form a hydrogen bond, leading to an adhesive effect (Wang *et al.* 2011b, Cheung *et al.* 2015, Prajakta K.Khobragade 2015, Mohammed *et al.* 2017). This enables it to be internalized by M cells (Hallstrom and McCormick 2011) in the intestinal Peyer's patch and presented to underlying APCs for efficient uptake, processing and presentation of vaccine antigens. It has been demonstrated that copper-loaded chitosan nanoparticles (CNP-Cu) could improve growth performance and enhance immunity in rats (Du 2008, Wang *et al.* 2011a). CNP-Cu has also shown to improve growth performance in broiler birds

and to increase the concentrations of immunoglobulins complements, and lysozyme in serum, enhancing their immunological capacity (Wang *et al.* 2011a).

Despite studies reporting immunological effects of chitosan as a feed additive or chitosan nanoparticle vaccines in mice, there are very few studies that have assessed the effects of CNP vaccines in chickens. The overall goal of this research is to analyze the protective effects of an oral chitosan-based *Salmonella* nanoparticle vaccine, loaded with *S.* enteritidis outer membrane proteins (OMPs) and flagellin proteins, on broiler birds challenged with *Salmonella* enterica serovar enteritidis or heidelberg. Specifically, this research aims to

(1) Identify the nanoparticle vaccine dose that can provide optimal protection from *S.* enteritidis infection

(2) Quantify the efficiency of CNP-*Salmonella* vaccine to induce anti-*Salmonella* OMP and flagellar IgG and IgA specific antibodies in serum, cloacal swabs and bile

(3) Identify the CNP effect on broiler bird's production performance parameters, pro- and anti-inflammatory cytokines, and nitric oxide response

(4) Quantify the efficiency of the vaccine in decreasing shedding and colonization load of *Salmonella* enterica serovars enteritidis or heidelberg challenges in broiler birds.

CHAPTER 2

LITERATURE REVIEW

2.1 *SALMONELLA* INFECTION IN COMMERCIAL POULTRY

Introduction

Salmonellosis constitutes a worldwide major public health concern (Antunes *et al.* 2016). The main source of infection for humans is the contaminated poultry meat (Andino and Hanning 2015). A shift in *Salmonella* serotypes related to poultry and its production has been reported in different geographical areas over time (Antunes *et al.* 2016). The increasing globalization of poultry meat may contribute to the development of new challenges that lead to the demand of innovative approaches to improve salmonellosis control.

Salmonella as A Major Foodborne Pathogen

Salmonellosis is one of the most frequent food-borne zoonoses. It has been estimated that there are approximately 1.3 billion cases of human gastroenteritis due to *Salmonella* each year worldwide, and these result in 3 million deaths (Gong *et al.* 2014). In the US, *Salmonella* is the leading foodborne pathogen, causing the largest number of deaths and has the highest cost burden (BATZ *et al.* 2012). The annual costs associated with salmonellosis for 2010 were estimated at $2.71 billion for 1.4 million cases (Andino and Hanning 2015). From the past decade, the highest numbers of *Salmonella* outbreaks are related to land animals: with poultry being an important reservoir (Andino and Hanning 2015). More than 70% of human salmonellosis cases in the USA has been attributed to the consumption of contaminated chicken or eggs (Braden 2006, Pires *et al.* 2014, Andino and Hanning 2015). From 1998-2008, around 145 *Salmonella* outbreaks have been

associated with poultry while 117 outbreaks were related to eggs, causing illnesses in 2,580 and 2,938 people, respectively (Andino and Hanning 2015).

Poultry is the second most consumed meat globally (Food and Agriculture Organization of the United Nations 2014) and the per capita consumption of eggs in the USA keeps increasing (Conrad *et al.* 2017). In 2000, the U.S. consumption was estimated at 251 eggs per person versus 268.4 eggs per person in 2016 (US Department of Agriculture 2017). This trend has made *Salmonella* a prominent public health concern. *Salmonella* have many different serotypes. Typically, *Salmonella* enteritidis, *Salmonella* typhimurium and *Salmonella* heidelberg are the three most frequent serotypes recovered from humans each year (Gong *et al.* 2014). Understanding the differences between different prominent serotypes helps to better understand Salmonellosis and develop new strategies to decrease pathogen outbreaks.

Overview of Salmonella Characteristics

Salmonella is a facultative anaerobe, Gram-negative, flagellated, rod-shaped bacterial pathogen that can infect a large variety of hosts (Lahiri *et al.* 2010); with poultry being considered a major reservoir (Andino and Hanning 2015) for many of its serovars. *Salmonellae* are non-fastidious organisms (Public Health Laboratory Newtork 2000) as they do not have complex nutritional requirements and multiply under many environmental conditions outside the living hosts. They do not require NaCl for growth but can grow in the presence of 0.4 to 4% (Pui *et al.* 2011). Most *Salmonella* serotypes grow at temperature range of 2-54°C with optimum temperature of 35-37°C (Pui *et al.* 2011). They are often killed at temperature of 70°C or above and grow in a pH range of 4-9 with the optimum between 6.5 and 7.5. Like most microorganisms, their optimum water activity is between 0.99 and 0.94 yet can survive at a water activity <0.2, such as in dried foods. Complete inhibition of growth occurs at temperatures <7°C, pH <3.8 or water activity

<0.94 (Pui *et al.* 2011). These characteristics help *Salmonella* to thrive in many different environments and become a food safety concern.

Complex epidemiology of Salmonella

If not properly intervened with at an early stage, the complex epidemiology of this pathogen facilitates its contamination of poultry products and gives rise to numerous salmonellosis outbreaks. Humans infected by *Salmonella* display some common symptoms, e.g., infection with *S.* typhimurium and *S.* enteritidis results in gastroenteritis manifested by diarrhea, fever and abdominal cramps (Center of Disease Control and Prevention 2017a). In contrast, poultry infected with *Salmonella* are commonly asymptomatic carriers. This intracellular pathogen can colonize the gastrointestinal tract of birds without causing disease, which makes its epidemiology complex (Biologies 2013). These asymptomatic birds infected with *Salmonella* may shed the bacteria in the feces and as a result infect the environment and other closely housed birds (Biologies 2013). Recent research shows much of the contamination occurs inside the infected hens. The bacterium *Salmonella* enteritidis can infect the ovaries of egg-laying hens (Gantois *et al.* 2009) which then pass the contamination to the eggs prior to the formation of the shell. Young birds are more susceptible to *Salmonella* colonization of the gastrointestinal tract by vertical transmission from infected parents (by eggs) or by horizontal transmission (direct contact from infected to susceptible birds) at the hatcheries during feeding, handling, and transportation (Foley *et al.* 2011). Although significant advances have been made in reducing the prevalence of *Salmonella* contamination in processed poultry, its elimination in production facilities has not been achieved. Hence prevention is a key tool for producers e.g. probiotics (Ghadban 2002), antimicrobials and acidifiers, vaccines (Cervantes and Mam n.d.), etc.

2.2. MECHANISM OF INFECTION

Introduction

Salmonella infection (salmonellosis) is a common bacterial disease that affects the intestinal tract (Hallstrom and McCormick 2011). Because of this, the anatomy of the intestinal mucosal epithelium plays a key role in its pathogenesis. When designing a vaccine, understanding the pathogen is important to determine the point of intrusion and key cell types that will serve as targets to intervene with its invasion.

Anatomy of the intestinal mucosal epithelium

Understanding the anatomy of the intestinal mucosal epithelium is a key component to fight *Salmonella* invasion of the host. The mucus layer protects against *Salmonella* invasion of epithelial cells (Hallstrom and McCormick 2011). *Salmonella* must adhere to mucus components to remain in the intestines. The epithelial monolayer underlying the mucus layer contains different cell types with diverse roles (Hallstrom and McCormick 2011). One of the most important cell types are Microfold cells (M cells). Microfold cells sample mucosal contents (including intestinal antigens) and are the preferred route of entry by *Salmonella* (Hallstrom and McCormick 2011). This is because the surface of M cells is not covered by the mucus layer (Abbas Abul K. *et al.* 2015) and M cells do not process the antigen. Thus M cells inadvertently provide opportunities for bacterial pathogens to dock and invade (Hallstrom and McCormick 2011). By acting act as sentinels of the intestinal epithelium, M cells are crucial targets for potential *Salmonella* vaccines for poultry. Underlying the M cells is the sub-epithelial dome (SED) which houses Peyer's patches that contain germinal centers and dendritic cells (DCs) (Janeway 2012, Abbas Abul K. *et al.* 2015). Dendritic cells carry bacteria to the mesenteric lymph node, from which *Salmonella* can escape

due to their ability to interfere with the phagosome-lysosome fusion mechanism of macrophages (Buchmeier and Heffron 1991, Leoni Swart and Hensel 2012). This results in systemic disease.

Salmonella brief mechanism of infection

Salmonella possesses effective acid tolerance mechanisms (Higginson *et al.* 2016) and upon ingestion Salmonella will survive passage through the low-pH conditions of the stomach. In the small intestine, the bacterium adheres to and invades the intestinal epithelial cells (Hallstrom and McCormick 2011, Higginson *et al.* 2016). *Salmonella* can be transported through the mucosa, mostly via microfold (M) cells (Hallstrom and McCormick 2011), to gain access to the submucosa and underlying lymphoid tissue (Coburn *et al.* 2007). Subsequently, macrophages within the lymphoid tissue engulf *Salmonella* but are unable to kill them due to the ability of the bacteria to interfere with phagosome-lysosome fusion (Buchmeier and Heffron 1991, Leoni Swart and Hensel 2012). Thus, *Salmonella* resides and proliferates in these immune cells (House *et al.* 2001). The infection is then disseminated from the intestinal mucosa, resulting in bacteremia (Hallstrom and McCormick 2011) and eventually, invasion of distant organs (liver, spleen, gallbladder, and ovaries (McClatchy n.d.)). Cases of non-typhoidal *Salmonella* (NTS) infections, with serovars such as typhimurium and enteritidis, remain localized to the gastrointestinal tract, causing inflammation of the mucosa and secretory diarrhea (Mandal and Brennand 1988, Acheson and Hohmann 2001).

2.3 VACCIANTION OF COMMERCIAL POULTRY

Introduction

The practice of vaccination in the poultry industry has been a significant contributor for disease control (Andre *et al.* 2008). Vaccination can reduce disease incidence and reduce outbreak and mortality rates (Andre *et al.* 2008), thereby preventing economic loss for the production company as well as increasing food safety and tackling public health concerns. The fundamental concept behind vaccination is establishing herd immunity (Kim *et al.* 2011). This will reduce the probability of a bird or even a flock in a heavily populated region from becoming infected with a field challenge (Marangon and Busani 2006). For vaccines to be marketed for commercial use they must be efficient, safe, affordable, and suitable for mass application (McDougald 2008).

Salmonella vaccines for poultry

Pathogen reduction strategies at the farm level are the first step in a continuum that will assist in reducing the presence of food-borne pathogens in eggs and meat (OIE Terrestrial Animal Health Standards Commission 2015). Some intervention strategies that are implemented in poultry flocks and hatcheries to control *Salmonella* are feed and drinking water acidification with organic acids or supplementation of feed additives, such as pre-biotics, pro-biotics, and synbiotics, and early vaccination of chicks (Trampel *et al.* 2014). Vaccination has proven to be a useful tool to control *Salmonella* infections. Poultry vaccines are biological products that induce an immune response to the specific disease-causing agents (cobb 2013). There are 2 groups of vaccines: inactivated (killed) and attenuated live vaccines. They are both serotype-specific: they have a limited protective effect to the same *Salmonella* serotypes within their own group. Current live and killed vaccines aim to maintain the immune response while diminishing the ability of the microorganism to cause disease or immunosuppression (Brewer and Schijns 2009). The U. S. Food

and Drug Administration does not require mandatory vaccination because of the paucity of data on the efficacy of current vaccines and their apparent inadequacy in eradicating *Salmonella* (F.D.A. 2009). Instead they encourage producers to vaccinate if they think it will help reduce *Salmonella* load. Furthermore, available live *Salmonella* vaccines for poultry have two major concerns: the ability of live vaccine to shed and to persist in the environment and possible interference with the salmonellosis monitoring program (Guo *et al.* 2017). Also, the route of administration for commercially available *Salmonella* killed vaccines in poultry poses a high disadvantage. Killed *Salmonella* vaccines are injected manually in the breast muscle, which is time consuming (impractical for big poultry flocks) and decreases breast meat quality. Oral administration (via water and food) is typically referred to as the "ideal route" (Revolledo and Ferreira 2012, Gong *et al.* 2014). This is because it mimics natural infection, stimulates the mucosal and systemic immune responses, contributes to the quality assurance programs related to animal welfare and decreases the vaccination cost factor (Revolledo and Ferreira 2012). However, vaccination in broilers is limited because there are no killed oral vaccines currently available for broilers commercially: available *Salmonella* vaccines are often restricted to attenuated-live or killed (Meeusen *et al.* 2007). Until recently, injections remained the most common means for administering therapeutic proteins and peptides because of their poor oral bioavailability (Shaji and Patole 2008, Vela Ramirez *et al.* 2017). The main challenge has been to improve the oral bioavailability to at least 30-50% (Shaji and Patole 2008). Consequently, over the past few decades oral protein and peptide delivery carrier systems, such as nanoparticles, have been heavily explored.

Type of commercially available vaccines for Salmonella in poultry

There are four main global companies that manufacture and distribute *Salmonella* vaccines for use in poultry: 1) Zoetis, 2) IDT Biologika, 3) CEVA, and 4) ELANCO. Combined, these companies offer five *Salmonella* live vaccines for chickens, which are all administered by spray application, orally (via water) or injected. Likewise, there are also five killed *Salmonella* vaccines for chickens, however these must all be injected. Specific details of commercially available vaccines are listed below and summarized in Table 1:

1. **ZOETIS** (former subsidiary of Pfizer): Is a global animal health company that delivers quality medicines and vaccines, complemented by diagnostic products and genetic tests and supported by a range of services(Zoetis 2017a).

    a. **POULVAC® ST**: *Salmonella* typhimurium Vaccine, Live Culture: Helps stimulate cell-mediated immunity, is effective for helping prime the immune system. Administration by spray to healthy chickens 1 day-of-age as an aid in the reduction of *Salmonella* enteritidis, *Salmonella* heidelberg and *Salmonella* typhimurium colonization of the internal organs, including the intestines and ceca. A second dose should be given at 2 weeks-of-age in the drinking water (Zoetis 2017b).

    b. **POULVAC® SE**: *Salmonella* enteritidis Bacterin (contains three inactivated SE phage types: 4, 8 & 13a): POULVAC® SE is an inactivated vaccine that helps reduce colonization by *Salmonella* enteritidis (SE). Zoetis Global Poultry's Poulvac SE and SE-ND-IB (listed below) are the only vaccines that contain all 3 phage types, helping reduce *Salmonella* enteritidis colonization. Subcutaneous injection administration. Administer 2 separate doses 3-4 weeks apart (Zoetis 2017c).

c. **POULVAC® SE-ND-IB**: *Salmonella* enteritidis Bacterin–Newcastle-Bronchitis Vaccine (Mass. Type, Killed Virus): POULVAC® SE-ND-IB aids in the reduction of *Salmonella* enteritidis colonization of the internal organs and in the prevention of the signs and lesions associated with Newcastle disease and infectious bronchitis. Contains three inactivated SE phage (types 4, 8 a13a). Subcutaneous injection administration administer 2 separate doses 3 to 4 weeks apart (Zoetis 2017d).

2. **IDT Biologika**: IDT Biologika is a medium-sized company founded in Germany, where it is still headquartered. IDT Biologika operates subsidiaries worldwide. IDT Biologika uses biotechnology to manufacture vaccines and pharmaceuticals for national and international markets. In the 1990s, the company introduced the world's first *Salmonella* live vaccine for chickens, fostering the creation of a sophisticated immunization program (IDT Biologika 2017a).

    a. **SALMOVAC SE**, freeze-dried live *Salmonella* enteritidis vaccine for chickens: Oral vaccination of chickens' results in epidemiologically relevant reduction in the level of shedding and persistence of *Salmonella* enteritidis and *Salmonella* typhimurium organisms. Horizontal and vertical chains of infection are prevented or very substantially reduced in scope in flocks. Onset of immunity: 6 days after vaccination. Duration of immunity after single oral vaccination has been demonstrated up to 7 weeks after the vaccination (IDT Biologika 2017b).

    b. **ZOOSALORAL H**: *Salmonella* typhimurium live vaccine for chickens: Freeze-dried, for oral administration after reconstitution in drinking water. For

active immunization of hens (breeding and laying hens) against *Salmonella* typhimurium infection to reduce pathogen excretion and persistence of *S.* typhimurium field strains to an epidemiologically relevant degree, as well as heterologous immunization against *Salmonella* enteritidis infections (IDT Biologika 2017c).

3. **Ceva Santé Animale (CEVA)**: Is a global veterinary health company, headquartered in Libourne, France. Focused on research, development, production, and marketing of pharmaceutical products and vaccines for pets, livestock, swine and poultry. CEVA received the first USDA license for a poultry vaccine against *S.* enteritidis(CEVA 2017a).

    a. **LAYERMUNE® SE**: Standard in *Salmonella* protection: Is an inactivated (killed) bacterial vaccine (bacterin) that contains multiple selected strains of *Salmonella* enteritidis to aid in the prevention of infection of internal organs and colonization of the intestinal tract thereby reducing the risk of *S.* enteritidis shed in the environment and egg shell contamination. Used in breeding and laying chickens at least 12 weeks of age to aid in the control of disease caused by *S.* enteritidis strains that infect the ovary and consequently contaminate the egg contents. Vaccination of breeder chickens will reduce the *Salmonella* exposure of chicks in the hatchery. Two injections 4 -6 weeks apart and prior to the onset of lay will provide maximum protection. Revaccination just prior to molt is recommended (CEVA n.d.).

    b. **CEVAC CORYMUNE ® RANGE**: Broad Spectrum Infectious Coryza & *Salmonella* enteritidis Vaccine: Reduces the number of vaccinations by

combining the protection of Infectious Coryza, main viral diseases and *Salmonella* enteritidis in one vaccine(CEVA 2017b).

- **CEVAC® Corymune 4K** is the first inactivated vaccine combining Avibacterium paragallinarum serotypes A, B and C, and *Salmonella* enteritidis strain, homogenized with Aluminium Hydroxide adjuvant and thiomersal as a preservative. Recommended for the active immunization of breeder and laying-type chicken flocks against Infectious Coryza caused by *A.* paragallinarum and *Salmonella* enteritidis infection. Vaccine must be injected subcutaneously or intramuscular, at the dose of 0,5 ml per bird. Should be administered to breeder and laying-type pullets between 8 and 12 weeks of age (CEVA 2017b, 2018a).

- **CEVAC® Corymune 7K** contains an inactivated combination of *A.* paragallinarum serotypes A, B and C, and *S.* enteritidis strain, La Sota strain of Newcastle Disease virus, Massachusetts strain of the Infectious Bronchitis virus and B8/78 strain of the EDS virus, homogenized with oil adjuvant and thiomersal as a preservative. Recommended for the active immunization of breeder and laying-type chicken flocks against Infectious Coryza caused by Avibacterium paragallinarum, *Salmonella* enteritidis infection, Newcastle Disease, Infectious Bronchitis and Egg Drop Syndrome'76. Birds should be previously immunized with live vaccines against Newcastle Disease and Infectious Bronchitis and

4-6 weeks before, with an inactivated vaccine against Infectious Coryza and *Salmonella* enteritidis infection. Vaccine must be injected: subcutaneously or intramuscular, at the dose of 0,5ml per bird. Should be administered 2-4 weeks before the onset of the lay, between 14 and 18 weeks of age (CEVA 2017b, 2018b).

4. **ELANCO**: Is a global animal health company that offers a wide range of live and inactivated, commercially available poultry and autogenous biological vaccines. The commercial portfolio focuses on three areas: *Salmonella*, immunosuppression, and respiratory disease control(ELANCO 2017a).

    a. **AviPro® Megan® Vac 1**, live vaccine: This vaccine is recommended as an aid in the reduction of *Salmonella* typhimurium, *Salmonella* enteritidis and *Salmonella* heidelberg colonization of the internal organs of young growing chickens and as an aid in the reduction of *Salmonella* enteritidis colonization of the crop and digestive tract, including the ceca. The ovaries and oviducts were not evaluated and therefore this vaccine is not indicated for use in older chickens. Recommended for use at 1 day of age by spray. A second dose should be given at 14 days of age in the drinking water (ELANCO 2017b, 2018a).

    b. **AviPro® Megan® Egg**, live vaccine: Recommended for the vaccination of chickens to aid in the reduction of *Salmonella* enteritidis colonization of the internal organs, including the ovaries and oviduct, and the intestinal tract and ceca; and for turkeys as an aid in the prevention of *Salmonella* typhimurium

colonization of the liver and spleen. This vaccine is recommended for use at 2, 4 and 16 weeks of age by coarse spray application (ELANCO 2017b, 2018b).

c. **AviPro® 329 ND-IB2-SE4** – An inactivated vaccine for chickens: Offering broad protection as an aid in the prevention of Newcastle disease and Infectious Bronchitis (Mass. And Ark. Types) and as an aid in the reduction of *Salmonella* enteritidis colonization of internal organs, including the reproductive tract. Inject subcutaneously in the lower neck region using aseptic technique. Vaccinate between 12 and 16 weeks of age (ELANCO 2017).

Table 1. Summary of the commercially available vaccines for *Salmonella* in poultry.

COMPANY/ VACCINE	LIVE	KILLED	BIRD	ADMINISTRATION ROUTE
ZOETIS/ POULVAC® ST	X		Broiler/ layers	Spray
ZOETIS/ POULVAC® SE		X	Broiler/ layers	Injection
ZOETIS/ POULVAC® SE-ND-IB		X	Broiler/ layers	Injection
IDT BIO/ SALMOVAC SE	X		Broiler/ layers	Oral
IDT BIO/ ZOOSALORAL H	X		Breeder/ layers	Oral
CEVA/ LAYERMUNE® SE		X	Breeder/ layers	Injection
CEVA/ CORYMUNE ® RANGE		X	Breeder/ layers	Injection
ELANCO/ AVIPRO® MEGAN® VAC 1	X		*Young chickens	Spray
ELANCO/ AVIPRO® MEGAN® EGG	X		Layers/ turkeys	Spray
ELANCO/ AVIPRO® 329 ND-IB2-SE4		X	Breeder/layers	Injection

2.4 REVIEW OF IMMUNOLOGY OF *SALMONELLA* VACCINATION

Introduction

Vaccines are used to activate the innate immune response via antigen presenting cells (APC) in order to induce a protective adaptive immune response to a specific pathogen antigen (Pasquale *et al.* 2015a). In contrast, adjuvants are substances that are added to vaccines with the purpose of enhancing the immunogenicity of antigens that have insufficient immunostimulatory capabilities on their own (Pasquale *et al.* 2015b). Early adjuvants were used empirically (Pasquale *et al.* 2015b), however with increasing knowledge on how the immune system interacts with pathogens, our understanding of their roles and how these can be combined with vaccines to achieve a desired clinical benefit, has increased. The right combination of antigens and adjuvants can potentiate downstream adaptive immune responses, enabling the development of new efficacious vaccines (Pasquale *et al.* 2015a).

Adjuvants

An adjuvant is a substance that is formulated as part of a vaccine to enhance its ability to induce protection against infection (National Institute of Health 2018). The word "adjuvant" comes from the Latin adjuvare and means "to help"; hence they function to help activate the immune system, allowing the antigens in vaccines to induce a long-term protective immunity (National Institute of Health 2018). Adjuvants are commonly used in the search for new vaccines against challenging pathogens and for susceptible populations that respond poorly to traditional vaccines (Pasquale *et al.* 2015b). However, they are not needed for all vaccines; e.g. live-attenuated vaccines. This is because the vaccine strain can induce mild infection in recipients and triggers an immune response that is very similar to that induced by infection with wild-type strains (Pasquale *et al.* 2015b). Basically, these vaccines can initiate an innate immune response which

drives the subsequent adaptive response and ultimately eliminate the pathogen. It is theorized that the primary mechanism of action of adjuvants is on the innate immune response (Coffman *et al.* 2010, Pasquale *et al.* 2015a). Highly purified vaccine components frequently lack PAMPs (Pasquale *et al.* 2015a) and do not activate the initial innate immune response, rather an adaptive immune response occurs (Pasquale *et al.* 2015b). Adjuvants can act like PAMPs and trigger the innate immune response through a variety of mechanisms that lead to the recognition of the vaccine components as a "threat" (Pasquale *et al.* 2015b). This ultimately leads to the activation and maturation of APC (such as DC) and the subsequent initiation of downstream adaptive immune response (Coffman *et al.* 2010). By impacting the initiating signal to the innate immune system, the choice of adjuvant can direct the type of adaptive immune response triggered.

Virulence factors as adjuvants

Virulence factors can be used as adjuvants. These factors help bacteria to invade the host, cause disease, and evade host defenses (Cross 2008) and are shared among bacterium (Peterson 1996); making them potent adjuvants that can elicit an efficient immune response upon their recognition by the immune system. Adherence factors, that numerous pathogenic bacteria use to colonize mucosal sites and adhere to cells (Peterson 1996), can be used as adjuvants. Other examples are: a) surface components that allow the bacterium to invade host cells can be encoded on plasmids, b) bacterium capsules that protect them from opsonization and phagocytosis, c) endotoxins, like the lipopolysaccharide (LPS) endotoxins on Gram-negative bacterium, that cause fever, blood pressure changes, inflammation, lethal shock, and other toxic events, d) exotoxins that are produced and/or secreted from pathogenic bacteria, and e) siderophores (iron-binding factors that allow some bacteria to compete with the host for iron)(Peterson 1996, Pasquale *et al.* 2015b).

Flagellar and Outer Membrane Antigens as virulence factors:

Bacteria surface are covered with LPS while the outermost portion of the LPS is the O antigen (Bruslind 2019). Antibodies directed against the O antigens of Gram-negative bacteria are highly protective and many of the licensed vaccines against bacterial pathogens are directed against the capsular polysaccharides (Peterson 1996). These antigens are highly conserved within gram negative bacteria: constitutively expressed, accessible, and rarely subject to change (Puente *et al.* 1995, Peterson 1996, Singh *et al.* 2003). Essentially, OMPs possess intrinsic adjuvant capacity as they naturally exist in gram-negative bacteria; thus, they are capable of triggering a strong innate and subsequent adaptive immune response. The same concept is applied with flagellar proteins, as flagellum is the most common mechanism used by bacteria to swim through liquid media and invade its host (NIH Center for Macromolecular Modeling & Bioinformatics 2014). Flagella is the whole structure, while the slender threadlike portion of the flagella is called the H antigen (Center of Disease Control and Prevention 2017b). For ages, flagella have been generally regarded as important virulence factors, mostly because of its motility property (Duan *et al.* 2013).

Both Flagella (Mizel and Bates 2010) and OMPs (Tan *et al.* 2018) are *Salmonella* antigens that can be used as adjuvants, to induce a potent humoral immune response (HIR). Both proteins can be important virulence factors when designing a *Salmonella* vaccine. Flagella possess highly conserved regions in the flagellin protein among all bacteria, facilitating its recognition (Smith *et al.* 2003, Duan *et al.* 2013) by the innate immunity via Toll Like Receptor 5 (TLR5). Outer membrane proteins expressed in Gram-negative bacteria are known to be essential to bacterial survival within macrophages (Lindgren *et al.* 1996) and early studies have identified that the OMP C is highly conserved between several different serovars of *Salmonella* (Singh *et al.* 2003, Jha *et*

al. 2012). Ultimately, by using the intrinsic components from gram negative bacteria as vaccine adjuvants a more potent innate immune response can be triggered.

Conserved sequences within Outer Membrane & Flagellar proteins

Conserved proteins can be taken as antigens of interest and used to induce cross protection against a bacterial pathogen and multiple of its serovars. Outer membrane protein C is highly conserved within 11 different *Salmonella* serotypes, except for S. arizonae (Puente *et al.* 1995). Flagella possess highly conserved regions in the flagellin protein among all bacteria, and several patterns of *Salmonella* flagellin sequence are known to remain well conserved while others possess highly variable domains (Puente *et al.* 1995, Vonderviszt F. 2008, Duan *et al.* 2013). Both, Flagella and OMPs are *Salmonella* antigens that can be used as adjuvants, being key factors to induce a potent humoral immune response. By doing so, the infection would also be blocked at an early staring point, preventing bacteria to attach to a host cell and targeting it for destruction.

Immunology of Salmonella vaccination for poultry

In poultry, humoral and cell-mediated response (CMR) are much like mammalian counterparts (Davison *et al.* 2008a), meaning it consists of a multilayered network of cells and molecules that are actively working together at different point in times with distinct roles (Abbas Abul K. *et al.* 2015). This implies their immune response can be divided into an early-responding innate and slow-reacting adaptive defense system; both necessary for vaccination-induced immunity (Brewer and Schijns 2009). Subsequently, depending on the antigen in the vaccine, the birds' immune system will react and create a "memory" response of antibodies and immune cells (Davison *et al.* 2008b); the antibody response will become greater with frequent exposure to the same antigen – resulting in protection (maximizes immune response)(Abbas Abul K. *et al.* 2015). Because of this, flock vaccinations are usually carried out multiple times for the same disease.

However, secondary immunization can be avoided, and it is often preferred in the poultry industry, when adequate stimuli are provided during primary immunization (Brewer and Schijns 2009).

When designing a *Salmonella* vaccine both responses are important. The humoral immune response (HIR) response is of importance because the desired effect of the vaccine is to protect against *Salmonella* by generating antibodies that can immediately recognize and neutralize the bacterial pathogen and creating immunological memory (Davison *et al.* 2008b, Janeway 2012, Abbas Abul K. *et al.* 2015). While the CMR is just as important because *Salmonella* is an intracellular pathogen, thus antigen-specific CD4+ Th1 cells will recognize their antigen (Ag) and subsequently release cytokines that aid in the recruitment of macrophages to combat the infection (Janeway 2012, Abbas Abul K. *et al.* 2015).

2.5 REVIEW OF *SALMONELLA* AND CELLULAR IMMUNITY

Introduction

The upregulation of dendritic cells MHC II, CD40, B7 molecules are crucial for T-cell activation and triggering an efficient immune response (Janeway 2012, Abbas Abul K. *et al.* 2015). Upon vaccine-induced stimulation, these molecules must be able to properly increase the expression of such and trigger an effective receptor-ligand interaction. If efficient upregulation is not achieved, T cell unresponsiveness will lead to an anergic state (Abbas Abul K. *et al.* 2015) that can ultimately lead to an unbalance in the immune system and result in a chain of events where sever infections and diseases can take place. In addition, previous studies have shown that *S.* Enteritidis can suppress nitric oxide (NO) production, while dead *S.* Enteritidis stimulates a high level of NO production (He *et al.* 2013); compromising the cellular and downstream humoral immunity of the host.

Dendritic cells

Dendritic cells (DCs) are known as professional antigen presenting cells (APC) (Janeway 2012, Abbas Abul K. *et al.* 2015) and immune sentinels that play key roles in the regulation of immune responses to antigens (Abbas Abul K. *et al.* 2015). Efficient uptake, processing, and presentation of vaccine antigens by DC, is a prerequisite in shaping the nature of the adaptive immune response against a pathogen (Melief 2003). Immature DC capture the antigens and undergo a complex maturation process, marked by the release of cytokines and the increased expression of costimulatory molecules(Janeway 2012, Abbas Abul K. *et al.* 2015). Upon activation by mature DCs, CD4+ T cells differentiate into effector T cells and develop into either T helper type 1 cells (Th1) or Th2 cells, depending on the type and nature of invading pathogen (Abbas Abul K. *et al.* 2015). In most of the reported studies, the interaction of *Salmonella* with DCs has

been examined in mouse models, while a recent 2016 study explored *Salmonella* interaction in chicken bone marrow derived dendritic cells (chBM-DCs) (Kamble *et al.* 2016).

Dendritic cells and cross presentation

Salmonella's mechanism of invasion involves its uptake by M cells (Hallstrom and McCormick 2011) and subsequent processing by APC, such as DCs. Dendritic cells are not the only APC in the immune system (B-cells, macrophages), but they are particularly referred to as professional antigen presenting cells (Abbas Abul K. *et al.* 2015). They are the most efficient cross presenting cells. This is because their key role on the immune system (phagocytose, process antigens and migrate to secondary lymphoid organs to present these specifically to naïve CD4+ T cells(Janeway 2012, Abbas Abul K. *et al.* 2015) make it crucial to develop a full on downstream adaptative immune response. Cross presentation is the uptake, processing and presentation of extracellular antigens with MHC class I molecules to CD8+ T cells(Abbas Abul K. *et al.* 2015). The result of this process is called cross-priming and it is important because it permits the presentation of exogenous antigens, that are normally presented by MHC II on the surface of dendritic cells, to be presented through the MHC I pathway (Janeway 2012, Abbas Abul K. *et al.* 2015). The MHC I pathway is normally used to present cytosolic antigens (such as virus) that have infected a cell. However, cross presenting cells can utilize the MHC I pathway to remain uninfected, while still triggering an adaptive immune response. After vaccine induced activation, DC can migrate to lymph nodes and activate CD4+ T helper cells as well as cross prime CD8+ T cells (Abbas Abul K. *et al.* 2015).

Dendritic cells and T-cell anergy

T cell anergy is induced when the T cell receptor (TCR) stimulation "freezes" T cell responses until they receive an adequate subsequent antigenic signal (Janeway 2012, Abbas Abul

K. *et al.* 2015), known as co-stimulation, from an antigen-presenting cell (Melief 2003). Costimulatory signals on APC can rescue T- cells from anergy and stimulate them to produce the cytokines necessary for the proliferation and survival of T cells. CD4+ T cells respond to effective signals by producing interleukin 2 (IL-2) and by proliferating. An effective signal requires both ligation of TCR with the processed protein antigen presented by MHC II molecules on the surface and activation of costimulatory receptors, such as CD40 and CD40 ligand interaction and CD28 and B7 ligand interaction(Abbas Abul K. *et al.* 2015). When T cells receive only TCR signals in the absence of engagement of costimulatory receptors, they enter a state of unresponsiveness characterized by an inability to produce IL-2 or to proliferate upon re-stimulation (Abbas Abul K. *et al.* 2015).

Salmonella and macrophages

Salmonella spp. possesses effective acid tolerance mechanisms that contribute to its complex epidemiology and facilitates its survival through the gastrointestinal tract (GIT) upon ingestion (Higginson *et al.* 2016),(Hallstrom and McCormick 2011). It can gain entry into phagocytes, such as macrophages, via a combination of cell-initiated phagocytosis and bacteria-mediated invasion(He *et al.* 2013). These virulence effectors facilitate the internalization of *Salmonella*, which survives and replicate within *Salmonella*-containing vacuoles by interfering with the phagolysosome formation (Buchmeier and Heffron 1991, Leoni Swart and Hensel 2012). Previous studies have shown that *S.* Enteritidis can suppress nitric oxide (NO) production in infected chicken macrophage HD11 cells, while heat-killed *S.* Enteritidis stimulates a high level of NO production(He *et al.* 2013); compromising the cellular and downstream humoral immunity of the host. Hence clearance of *Salmonella* in poultry requires strong cell-mediated, and

subsequent humoral immune responses(Van Immerseel *et al.* 2002, Raybourne *et al.* 2003, Neto *et al.* 2008, Markazi 2018).

2.6 REVIEW OF *SALMONELLA* AND HUMORAL IMMUNITY

Introduction

Humoral immunity is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides (Abbas Abul K. *et al.* 2015). It contrasts with cell-mediated immunity. Its aspects involving antibodies are often called antibody-mediated immunity(Abbas Abul K. *et al.* 2015). The Enzyme-linked immunosorbent assay (ELISA) is an immunological assay that is commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples (Janeway 2012, Abbas Abul K. *et al.* 2015). When using the ELISA technique for *Salmonella* detection, the common Ig levels measured are IgG or IgY (avian equivalent(Davison *et al.* 2008b)) and IgA levels.

Enzyme Linked Immunosorbent Assay (ELISA)

Depending on the form of the disease, *Salmonella* may be detected in feces; placenta, fetal tissues and vaginal discharge; blood; or various internal organs at necropsy(Biologies 2013). Serology can be useful for diagnosis in a herd or flock(Biologies 2013). Limitations of this diagnostic test is that antibodies do not appear until two weeks after infection, and antibodies may also be present in uninfected animals(Biologies 2013). In addition, if there are low levels of pathogenic bacteria, below the sensitivity level of the assay, these might not be detected(Abbas Abul K. *et al.* 2015). A common serologic test is the Sandwich-Enzyme Linked Immunosorbent Assay (ELISA). Another approach used in research to test the effectiveness of a treatment is reducing *Salmonella* is the collection of cecal content and/or cecal tonsils, or other organs, to quantify *Salmonella* levels via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

Sandwich ELISA requires a compatible antibody/antigen pair that permits the recognition of your target epitopes on the same antigen. The first antibody is called the capturing

antibody(Abbas Abul K. *et al.* 2015) and it is coated on the plate and used to immobilize the antigen upon binding during incubation with the sample. Free antigen is removed by a washing step and then a detecting antibody is added to bind the captured antigen and enable subsequent detection. Sandwich ELISA is divided into 2 main systems: 1) Direct Sandwich: Uses a conjugated detecting antibody to an enzyme (fluorescence tag). Following incubation with the antibody-antigen complex immobilized on the wells, signal detection is performed upon successive addition of substrate, or 2) Indirect Sandwich: Using either an unconjugated or biotinylated detecting antibody that once is bound to the antibody-antigen complex on the well is subsequently detected by either an anti-species antibody or streptavidin conjugated to an enzyme or fluorescent tag. The increasing color response observed is directly proportional to the antigen of interest and vice versa. Using a stopping solution, the optical density values can be read (Farzan *et al.* 2007, Abbas Abul K. *et al.* 2015).

When using the ELISA technique for *Salmonella* detection, the common Ig levels measured are IgG or IgY (avian equivalent) and IgA levels. This is because different types of antibodies have specific functions. *Salmonella* can cause bacteremia, so looking at IgG in serum samples is useful because this antibody is secreted by the plasma cells in the blood; it is the most abundant antibody in the blood (Abbas Abul K. *et al.* 2015). Monitoring IgA levels are important when sampling directly from the intestinal epithelial wall, with cloacal swabs, or when looking into bile samples. This is because *Salmonella* is an intestinal pathogen and IgA is the major class of antibody present in the mucosal secretions of most mammals (Woof and Kerr 2004). This antibody represents a key first line of defense against invasion by inhaled and ingested pathogens at vulnerable mucosal surfaces (Woof and Kerr 2004). IgA can also be found at significant

concentrations in the serum of many species, where it functions as a second line of defense to eliminate pathogens that have breached the mucosal surface (Woof and Kerr 2004).

Other antibodies and their specific functions include: 1) IgD functions mainly as an antigen receptor on B cells that have not been exposed to antigens, 2) IgE binds to allergens and triggers histamine release from mast cells and basophils and is involved in allergy and parasitic worm protection, 3) IgM is either expressed on the surface of B cells (monomer) or in a secreted form (pentamer) with very high avidity. It is known to eliminate pathogens in the early stages humoral immunity before there is enough IgG (Abbas Abul K. *et al.* 2015).

2.7 REVIEW OF BIODEGRADABLE NANOPARTICLES

Introduction

In brief, nanoparticles (NP) have multiple characteristics that make them highly suitable for vaccine delivery. Because of their size similarity to cellular components, they can enter living cells using the cellular endocytosis mechanism (pinocytosis) (Lindgren *et al.* 1996). This nature enables the enhancement of antigen processing by professional antigen present cells (APCs), particularly DCs. Previous research has shown targeted delivery of protein antigen to dendritic cells (Zeng *et al.* 2013) and indicate that several nanoparticles modulated immune responses via TLR (Luo *et al.* 2015).

Biodegradable Nanoparticles as Potential Carriers for Salmonella Vaccine

Polymeric NP have been used to entrap antigen for delivery to certain cells due to their slow biodegradation rate (Zhao *et al.* 2014) they can trigger a long-lasting immune response. They can also act as adjuvants themselves(Tiwari *et al.* 2012), reducing the need for these. Furthermore, biodegradable polyanhydride and chitosan nanoparticle vaccines have been shown to have ideal traits for delivering vaccine antigen loads orally (Binnebose *et al.* 2015, Cheung *et al.* 2015). Polyanhydride nanoparticles degrade into dicarboxylic acids upon scission of the anhydride bond, rendering them highly biocompatible (Binnebose *et al.* 2015). While chitosan is a natural biodegradable copolymer derived from the partial deacetylation of chitin (Khobragade *et al.* 2015). Its composition (randomly distributed N-acetyl glucosamine and D- glucosamine) make it a cationic polymer with mucoadhesive properties (Cheung *et al.* 2015, Khobragade *et al.* 2015) which enables it to be internalized by M cells in the intestinal Peyer's patch and presented to underlying APCs for efficient uptake, processing and presentation of vaccine antigens. It is also approved by the U.S. FDA for tissue engineering and drug delivery (Mohammed *et al.* 2017).

Basic Characteristics of Chitosan as a Mucoadhesive Polymer

Delivery in the gastrointestinal tract (GIT) requires a mucoadhesive delivery system (Hombach and Bernkop-Schnürch 2010, Khobragade *et al.* 2015), such as chitosan nanoparticles, that allows adhesion to the mucus membrane and release of the load over time, with the potential to reduce dosing frequency. Chitin is the second most abundant natural polymer in nature (after cellulose) (Khobragade *et al.* 2015) and it is found in the structure of a widespread number of invertebrates (crustaceans, exoskeleton, and insect cuticles) and cell wall of fungi(Cheung *et al.* 2015). Chitin from natural sources is found bound to proteins and minerals, while chitosan is produced commercially by the chemical deacetylation of chitin under alkaline condition(Cheung *et al.* 2015, Prajakta K.Khobragade 2015). This natural polymer acts a penetration enhancer by opening the tight junctions of the epithelium and facilitating paracellular and transcellular transport of drugs (Mohammed *et al.* 2017). As a polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine, the amino and carboxyl groups in the chitosan molecule can be combined with glycoprotein in mucus to form a hydrogen bond, leading to an adhesive effect (Wang *et al.* 2011a). Chitosan mainly combines with anionic group of mucus i.e. sialic acid and sulfonic group substituent. This ionic interaction between the cationic primary amine of chitosan (pKa ~6.5) and anionic sialic acid group of mucus constitutes its mucoadhesion (Khobragade *et al.* 2015, Mohammed *et al.* 2017). The greater the molecular weight and higher the degree of deacetylation of chitosan, the stronger will be its adhesivity (Wang *et al.* 2011a, 2011b).

2.8 REVIEW OF PRELIMINARY WORK: *SALMONELLA*-NP VACCINE

Introduction

Much work has been done on bioengineering a *Salmonella* subunit vaccine, using a polyanhydride nanoparticle (PNP) containing immunogenic *Salmonella* outer membrane proteins (OMPs), flagellar (F) protein-entrapped and surface F-protein-coated PNPs (OMPs-F-PNPs) using a solvent displacement method (Renu *et al.* 2018b). The candidate vaccine is acid tolerant and had ideal physicochemical properties for oral delivery in layer birds. Layer chickens inoculated orally with OMPs-F-PNPs had substantially higher OMPs-specific IgG response and OMPs-F-PNPs vaccine cleared *Salmonella* cecal colonization in 33% of vaccinated birds (Renu *et al.* 2018b). This pilot study demonstrated that targeted delivery of this NP vaccine to ileum mucosal immune sites of chickens and induced specific immune response to lessen Sa*lmonella* colonization in intestines (Renu *et al.* 2018b).

Salmonella-Chitosan Nanoparticle Vaccine

A targeted mucoadhesive chitosan-based *Salmonella* nano-vaccine for oral delivery in poultry was engineered (Sankar Renu, Ashley D. Markazi, Santosh Dhakal, Yashavanth Shaan Lakshmanappa, Revathi Shanmugasundaram 2018). The vaccine contained immunogenic *Salmonella* outer membrane proteins (OMPs) and flagellar (F) protein-entrapped and a surface F-protein-coat. The candidate vaccine was analyzed for its resistance in acidic microenvironment and physicochemical properties for oral delivery. In terms of morphology, the vaccine showed an average size of 168 nm. The optimal size for uptake of particulate vaccine by antigen-presenting cells and Peyer's patches M cells is 100-500 nm (Howe *et al.* 2014). The entrapment efficiency of chitosan nanoparticles was 70%, meaning that only 30% of the proteins were not successfully entrapped/adsorbed into NP; improving one of the main challenges for proteins and peptide oral

delivery to more than 50% (Shaji and Patole 2008). Chitosan nanoparticles have been shown to exhibit a pH-dependent drug release because of the solubility of chitosan (Mohammed *et al.* 2017). The synthetized chitosan nanoparticles were found to be stable in both acidic and alkaline pH conditions with less than 10% and 0% protein release respectively. The biocompatibility of chitosan nanoparticles using a hemolysis assay resulted in 0% hemolysis, demonstrating that the nanoparticles were biocompatible in chickens. In addition, layer chickens vaccinated orally with this nano-vaccine had significantly higher OMPs-specific intestinal mucosal antibody response (Renu *et al.* 2018). This study demonstrated the capability of this nano-vaccine to target ileal Peyer's patches and induce specific local intestinal immunity by *ex vivo* and *in vivo* studies. All results indicated that the candidate oral *Salmonella* NP vaccine has the potential to lessen salmonellosis in poultry (Renu *et al.* 2018). Further studies are currently in process with the purpose of fully exploiting the products efficacy on broiler birds.

CHAPTER 3

EFFICACY OF CHITOSAN-BASED NANOPARTICLE VACCINE ADMINISTRATION IN BROILER BIRDS CHALLENGED WITH *SALMONELLA* ENTERICA SEROVAR ENTERITIDIS[1]

[1] K. Acevedo, S. Renu, T. Ng, M. Mortada, G. Akerele, J. Oxford, R. Shanmugasundaram, B. Lester, R. Gourapura, R. Selvaraj. To be submitted to *Poultry Science*.

ABSTRACT

This study analyzed the protective effects of an oral chitosan-based *Salmonella* nanoparticle vaccine (CNP) loaded with *Salmonella* outer membrane proteins (OMPs) and flagellin proteins. Day-old Cobb-500 broilers (n=18) were orally gavaged with PBS or 500, 1000 or 2000 µg of OMPs + flagellar proteins. A booster was given at 1wk-of-age. At 2wk-of-age, birds were challenged with 5.4 x 10^5 CFU of live *S.* enteritidis orally. All birds were euthanized at 11d-post challenge. Vaccination did not affect (P>0.05) body weight gain or feed conversion ratio when compared to challenged control group. Macrophage nitric oxide production 11d-post-*Salmonella* challenge was higher (P<0.05) in the 500µg vaccinated group. Broiler birds vaccinated orally with 1000µg CNPs showed higher (P<0.05) anti-*Salmonella* OMPs-specific serum IgG and IgA titers in serum. Cloacal anti-*Salmonella*-OMP IgA titers were substantially higher in the 1000µg treatment group (P<0.05), whereas cloacal anti- *Salmonella*-flagellin IgA titers were higher (P<0.05) in the 500µg vaccinated group. At 25d-of-age, birds that were vaccinated with 1000µg CNPs had higher (P<0.05) bile anti-*Salmonella* OMPs and flagellin-specific IgA titers (P<0.05). *Salmonella* enteritidis population on cecal content at 11d-post challenge showed a numerical decrease in birds with 1000µg CNP vaccine when compared to the unvaccinated control. At 11d-post challenge, the 1000µg CNP vaccine dose: increased (P<0.05) pro-inflammatory cytokine IL-1β and anti-inflammatory cytokine IL-10 in cecal tonsils, while decreasing (P<0.05) pro-inflammatory cytokines IL-1β and IL-4 on the liver and increasing IL-1β (P<0.05) in the spleen. Results demonstrate that the CNP vaccine had no adverse effects on bird's production performance or immunological health. CNP 1000µg dose showed potential to mitigate salmonellosis in poultry. It can be concluded that vaccinating birds with 1000 µg of CNP can provide optimal protection from *S.* enteritidis infection.

INTRODUCTION

Approximately 1.3 billion cases of human gastroenteritis due to *Salmonella* are reported annually worldwide, resulting in 3 million deaths (Gong *et al.* 2014). *Salmonella* enterica serovars: enteritidis, typhimurium and heidelberg are the three most frequent serotypes recovered from humans each year (Gong *et al.* 2014). More than 70% of human salmonellosis cases in the USA has been attributed to the consumption of contaminated chicken or eggs (Andino and Hanning 2015), most likely via contamination from chicken intestinal contents (Braden 2006, Pires *et al.* 2014).

Salmonella spp. possesses effective acid tolerance mechanisms that contribute to its complex epidemiology and facilitates its survival through the gastrointestinal tract (GIT) upon ingestion (Higginson *et al.* 2016),(Hallstrom and McCormick 2011). *Salmonella* stimulates macrophages, evade killing by the host immune system (Pilonieta *et al.* 2014), and potentially transitions to a systemic infection (Acheson and Hohmann 2001). Previous studies have shown that *S.* enteritidis can suppress nitric oxide (NO) production in infected chicken macrophage HD11 cells, while dead *S.* enteritidis stimulates a high level of NO production (He *et al.* 2013); compromising the cellular and downstream humoral immunity of the host. Hence clearance of *Salmonella* in poultry requires strong humoral and cell-mediated immune responses(Van Immerseel *et al.* 2002, Raybourne *et al.* 2003, Neto *et al.* 2008, Markazi 2018). Reducing *S.* enteritidis colonization in poultry may lead to a decrease of its transfer to humans, resulting in fewer cases of salmonellosis(Greig and Ravel 2009, Markazi 2018).

Vaccines have proven to be a useful tool to reduce the burden of infectious diseases (Andre *et al.* 2008). Live attenuated vaccines produce a strong humoral and cell-mediated responses (Lalsiamthara *et al.* 2016), however killed *Salmonella* vaccines are preferred as opposed to live ones due to the ability of the live strain to regain its virulence(Lauring *et al.* 2010, Kollaritsch and Rendi-Wagner 2012, Renu *et al.* 2018b) . In contrast, the route of administration for commercially available *Salmonella* killed vaccines in poultry poses a high disadvantage. These vaccines are injected intramuscularly, which is labor intensive, impractical for big poultry flocks, and decreases breast meat quality. Therefore, the "ideal route" of *Salmonella* vaccine administration is oral administration since it mimics natural infection, stimulates the mucosal and systemic immune responses, and can be mass administered (Revolledo and Ferreira 2012, Gong *et al.* 2014). However, there are currently no oral killed vaccines commercially available for broilers due to the challenging acidic nature of the gastrointestinal tract (GIT) (Shaji and Patole 2008, Vela Ramirez *et al.* 2017, Renu *et al.* 2018b). Oral protein and peptide delivery carrier systems, such as nanoparticles, have been heavily explored to overcome this problem(Shaji and Patole 2008, Salman *et al.* 2009a, Renu *et al.* 2018b).

Nanoparticle (NP) vaccines comprise a polymer coating, consisting of immunodominant components of the bacteria, that surrounds the vaccine antigen(Zhao *et al.* 2014). Thereby, protecting the vaccine against chemical, enzymatic or immunological degradation (Tiwari *et al.* 2012, Sahdev *et al.* 2014, Zhao *et al.* 2014). This results in prolonged survivability of the vaccine within the GIT, which reduces the dosing frequency and need for adjuvants, as they can act as adjuvants themselves(Tiwari *et al.* 2012); while facilitating a targeted delivery of the vaccine antigens to specific immune sites of the mucosal immune system (Cheung *et al.* 2015). Even so, ligands can be conjugated to the surface to increase the presentation of the NP vaccine to a specific

site within the GIT(Salman *et al.* 2009a, Renu *et al.* 2018b). For example, conjugating flagellin proteins to a NP *Salmonella* vaccine in mice mimicked the natural colonization of *S.* Enteritidis in the GIT, resulting in uptake of the antigen by the ileal Peyer's patches (Salman *et al.* 2009b).

Chitosan is a natural biodegradable copolymer derived from the partial deacetylation of chitin(Cheung *et al.* 2015). Studies have shown that biodegradable chitosan nanoparticle (CNP) vaccines have ideal traits for delivering vaccine antigen loads orally (Wang *et al.* 2011b, Binnebose *et al.* 2015, Cheung *et al.* 2015). The amino and carboxyl groups in the chitosan molecule can be combined with glycoprotein in mucus to form a hydrogen bond, leading to an adhesive effect (Wang *et al.* 2011b, Cheung *et al.* 2015, Prajakta K.Khobragade 2015, Mohammed *et al.* 2017). This enables it to be internalized by M cells (Hallstrom and McCormick 2011) in the intestinal Peyer's patch and presented to underlying APCs for efficient uptake, processing and presentation of vaccine antigens.

It has been demonstrated that Copper-loaded chitosan nanoparticles (CNP-Cu) could improve growth performance and enhance immunity in rats (Du 2008, Wang *et al.* 2011a). CNP-Cu has also shown to improve growth performance in broiler birds and significantly the concentrations of immunoglobulins on serum; enhancing their immunological capacity (Wang *et al.* 2011a). More importantly, previous research regarding our vaccine candidate for this study, demonstrated the vaccines potential against *S.* enteritidis challenge in layer birds and lead us to further explore its protective effects on broiler birds. The chitosan nanoparticle vaccine contains the immunogenic outer membrane proteins (OMPs), flagellar (F) protein of *S.* enteritidis and it is surface-decorated with F-protein (OMPs-F-CS NPs). Results showed the vaccine successfully targeted the intestinal Peyer's patches immune cells of birds, its ability to substantially induce antigen specific mucosal antibody and T cell responses, and its potential to mitigate salmonellosis

in layer chickens challenged with *S.* enteritidis (Sankar Renu, Ashley D. Markazi, Santosh Dhakal, Yashavanth Shaan Lakshmanappa, Revathi Shanmugasundaram 2018).

Despite studies reporting immunological effects of chitosan as a feeding additive or CNP vaccines in mice, there are very few studies that have assessed the effects of CNP vaccines in chickens. This pilot trial aimed to 1) Identify the vaccine dose that can provide optimal protection from *S.* enteritidis infection, and 2) Characterize its immunological effects of the vaccine on broiler birds challenged with *S.* Enteritidis. We hypothesized that the oral delivery of CNP, loaded with *S.* enteritidis immunogenic antigens (flagellin and outer membrane proteins) and surface-coated with flagellin proteins, will induce anti- *Salmonella* IgG and IgA in serum, fecal swabs and bile, and decrease *Salmonella* shedding/load in broiler birds.

MATERIAL AND METHODS

Preparation of loaded Chitosan Nanoparticle vaccine. Engineering of the CNP vaccine was done at the Food Animal Health Research Program, OARDC and Department of Veterinary Preventive Medicine, The Ohio State University, USA; as described in its patent by corresponding authors (10336-342WO1 2017).

Birds. One-day-old Cobb-500 chicks (n=18) were used in the present study. All Birds were confirmed to be *Salmonella* negative by streaking cloacal swabs on Xylose Lactose Tergitol™ 4 (XLT4) agar plates, before bacterial challenge. Birds were provided ad libitum intake of water and feed and were housed in individual battery cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Georgia.

Treatments and challenge. At 0d of age, chickens were orally vaccinated with 0.1 mL of PBS (Mock) or 500µg, 1000µg, or 2000µg OMP + flagellar proteins/dose/bird loaded into CNP matrix with surface tagged flagella. The same dose and route of delivery was repeated at 1 wk of

age. *Salmonella* enterica serovar enteritidis-wildtype pure culture was grown in 10 mL tryptic soy broth (TSB) at 37^{o}C without shaking. After 8h of incubation different dilutions were plated on XLT4 agar plate and bacterial colonies were isolated. On day of challenge, TSB was inoculated with one bacterial colony for a six hours incubation period. One hundred microliters of the bacterial suspension were transferred into 40 mL of fresh TSB and incubated at 37^{o}C (5% CO2) for 1 hour until concentration of the bacteria reached approximately 10^6 CFU/mL, as estimated spectrophotometrically at 600 nm wavelength and confirmed by a previously done standard curve. The bacteria were serially diluted and plated on XLT4 agar plate to confirm concentration by enumeration of *S.* enteritidis colonies (CFUs) using ImageJ Software (version 1.52k). At 2 wk of age, birds were challenged using an oral gavage with 0.1 mL of 5.4 x 10^5 CFU of live *S.* enteritidis. Body Weight Gain (BWG) and Feed conversion ratio (FCR) was also monitored throughout the entire study. Chickens were euthanized by cervical dislocation at 11 d post-*Salmonella* challenge for sample collection.

Effect of CNP–Salmonella vaccine on macrophage nitric oxide production post-Salmonella enterica serovar enteritidis challenge. Spleen samples from 25d old birds on control and treatment groups were harvested in 2ml of RPMI-1640 medium (GE Healthcare Life Sciences, UT) and kept on ice. Spleens were crushed using 45µ cell strainers (Fisher scientific), adding 3mL of media and centrifuged at 500 xg for 15 minutes at 4 ºC. The buffy coat was transferred to 3 mL RPMI-1640 medium containing 1% Penicillin-Streptomycin (Sigma-Aldrich). An equal volume of the PBMC solution was carefully layered on Ficoll-Paque PLUS (density 1.077 g/mL) (GE Healthcare, PA). The solution was centrifuged at 500 xg for 30 minutes, and the PBMC layer was transferred and washed with 5 mL of media. The obtained pellet was resuspended in RPMI-1640 complete media (1% Penicillin-Streptomycin, 5% Fetal Bovine Serum (Sigma-Aldrich), 5%

Chicken Serum (Sigma-Aldrich), and plated in TC-treated culture dish plates (Corning). Cells were incubated for 24 hours at 37 ºC, 5% CO_2, cell density was adjusted to 10^6 cells/mL with complete medium and 200 µl at 1 x 10^5 cells/well were plated to flat bottom 96 well plate (Greiner bio-one, NC) in triplicates. Cells were stimulated for nitrite production with 1 µg/mL *Salmonella* enterica serovar enteritidis LPS in complete medium and incubated for 72h at 37 ºC, 5% CO_2.

Samples were centrifuged at 2000rpm for 5 minutes at 4ºC. One hundred microliters of the supernatants were transferred to a new plate, and 100 µL of Griess reagent was subsequently added/well. Samples were incubated for 5 minutes and the absorbance was read at 540nm using an ELISA plate reader. The nitrite content of the supernatant was determined using a sulfanilamide/N-(1-naphthyl) ethylenediamine dihydrochloride solution (R2233500, Ricca Chemical Company, Arlington, TX) following the manufacturer's instructions. Nitrite concentrations were determined from a standard curve drawn with different concentrations of sodium nitrite solution (Fig 1, A).

Effect of CNP-Salmonella vaccine on anti-Salmonella IgA and IgG antibody titers. Serum samples were analyzed for anti-*Salmonella* OMPs-specific IgG antibody response using ELISA. Blood samples were collected at d0, d7 pre- *Salmonella* challenge; d14, d17 post- *Salmonella* challenge. Blood samples were centrifuged at 5000xg for 10 min, and the serum-containing supernatants were collected and stored at -80°C until use. Cloacal swabs were collected in 2 ml PBS at d17, d20, d23, and d25 post- *Salmonella* challenge, vortex, centrifuged at 3000xg for 10 min, and supernatants were collected and stored at -80°C until use. Bile samples were collected at d25 from the gallbladder using an insulin syringe and aliquots were directly stored at -80°C until use.

High-binding-flat bottom 96-well plates (Greiner Bio-one, NC) were coated with pre-titrated amount of OMPs or Flagellin (2 µg/mL or 7.5 µg/mL for IgG or IgA ELISA, respectively) diluted in 0.05 M sodium carbonate-bicarbonate coating buffer, pH 9.6 and incubated overnight at 4˚C. Plates were washed three times with PBS containing Tween-20 (PBST) (0.05%) and blocked with 5% skim milk powder in 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 triplicates to the wells. For fecal supernatants, 50 µl of each undiluted sample were added in triplicates to the wells. Samples were then incubated for 2h at room temperature. Plates were washed three times in PBST, 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 50 µl/well of TMB peroxidase substrate were added to each well. The reaction was stopped after 6 min by adding 2M sulfuric acid. The OD was measured at 450 nm using spectrophotometry. The corrected OD was obtained by subtracting the treatment group OD from the blank control OD.

Effect of CNP–Salmonella vaccine on Salmonella population in the ceca post-Salmonella enterica serovar enteritidis challenge. At 11 d post-*Salmonella* challenge, whole ceca were collected following euthanasia and samples were stored at −80°C until DNA extraction. Cecum samples were homogenized, and DNA was extracted. Briefly, cecal contents (0.1 g) were diluted in 1 mL of 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 2 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, 5M NaCl and 100% 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. The isolated DNA was dissolved in Tris-EDTA (pH 8.0) buffer, and the concentration was determined by using NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The *Salmonella* load of the ceca was analyzed by real-time PCR, using the 16S ribosomal DNA primers and *Salmonella* specific DNA primers. Primers are shown in Table 2. For analysis, samples were diluted to a 100ng/µl final concentration. 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. To evaluate the relative proportion of bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100% (Shanmugasundaram *et al.* 2013).

Effect of CNP-Salmonella vaccine on IL-1β, IFNγ, IL-10, IL-4 mRNA transcription in the cecal tonsils, liver and spleen. Cecal tonsil, liver and spleen samples were collected at 11d post-*Salmonella* challenge. Total RNA was extracted by using TRIzol reagent (Invitrogen). The isolated RNA was dissolved in Tris-EDTA (pH 7.5) buffer, and the concentration was determined by using NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The cDNA synthesis was achieved with 1 µg of total RNA. The mRNA transcripts of pro-inflammatory cytokines IL-1β, IFNγ, and anti-inflammatory cytokines IL-10, IL-4, and the house keeping gene RPS13 were analyzed by real-time PCR using the iQ™ SYBR® Green Supermix (Bio-Rad, CA). The threshold cycle (Ct) values were determined by iQ5 software (Bio-Rad, Hercules, CA) as described earlier.

Target gene transcripts were normalized to the RPS13 housekeeping gene. 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). Primers are described in Table 2.

Statistical Analysis. Data were analyzed by a One-way ANOVA using JMP software (JMP, SAS Institute Inc., Cary, NC). When results were significant ($p< 0.05$) differences between means were analyzed using Tukey's Honest Significant Difference test.

RESULTS

Pre- and post-challenge body weight and feed conversion ratio. There was no significant difference in BWG recorded between any of the treatment groups during the 25d period of the experiment. Likewise, there were no significant differences on FCR between any of the treatment groups pre or post- *S.* enteritidis challenge. Figure1, A and Figure1, B illustrate the mean BWG and FCR, respectively, reported as final performance at d25.

Effects of CNP on macrophage-nitric oxide production post- Salmonella enterica serovar enteritidis challenge. In birds vaccinated with 500µg CNP vaccine dose, splenic macrophages stimulated with 1 µg/mL *S.* enteritidis LPS produced significantly greater levels (P=0.0094) of nitric oxide compared to that of the mock (PBS), 1000µg, and 2000µg CNP vaccine doses (Fig. 2). Results showed a dose-dependent manner (Fig 2, A).

Effect of CNP- Salmonella vaccine on serum anti-Salmonella IgG antibody titers. There were no significant differences in anti-*Salmonella* OMPs-specific serum IgG titers among treatment groups pre-*Salmonella* challenge (P=0.2901) (Fig. 3). However, there were significant differences among treatments in birds at 2wk (8hr post-*Salmonella* challenge) (P=0.0029). Birds vaccinated with the 1000µg CNP vaccine dose had significantly higher anti-*Salmonella*

OMPs-specific IgG titers on serum, compared to that from mock (PBS-treated) group (Fig. 3). On d17 bird's vaccine with 500µg and 2000µg CNP vaccine dose, had significantly higher (*P*= 0.0047) anti-*Salmonella* OMPs specific IgG on serum. Similarly, for d20 birds vaccinated with the 500µg CNP vaccine dose showed a significant increase for anti-*Salmonella* OMPs specific IgG on serum, while birds vaccinated with the 2000µg dose had a significant decrease (Fig. 4) (*P*= 0.0231), when compared to control. No significant differences between any treatment groups were observed on d23 *(P*=0.0372). Finally, on d25 birds the mock group had significantly higher anti-*Salmonella* OMPs specific IgG in serum, while the 2000µg CNP vaccine dose showed a significant decrease (Fig. 4) (*P*= 0.0011), when compared to that of the control group.

There were no significant differences among treatment groups pre-*Salmonella* challenge for anti-*Salmonella* OMPs-specific IgA titers on serum (Fig. 5) (*P*=0.9700). However, there were significant differences among treatments in birds at 2 wk of age (post- *Salmonella* challenge) (*P*= 0.0009). Birds vaccinated with 1000µg CNP had significantly higher anti- *Salmonella* OMPs-specific IgA titers on serum (*P*=0.0009), compared to that from mock, 2000µg, and 500µg groups by 185%, 117% and 375%, respectively (Fig. 5).

No significant results (*P*>0.05) were found for all time points, between any of the treatment groups for anti-*Salmonella* Flagellin-specific IgG and IgA titers on serum.

Effect of CNP- Salmonella vaccine on cloacal swabs anti-Salmonella IgA antibody titers. Anti-*Salmonella* OMP IgA titers from cloacal swabs was 208% higher (*P*=0.0551) in the 1000µg treatment group at 2wk (post-challenge) compared to that from the 2000µg group (Fig. 6). Anti- *Salmonella* flagellin-specific IgA titers from cloacal swabs, was higher (*P*=0.0223) in the 500µg vaccinated group at 2wk (post-challenge) compared to that of the mock and 1000µg vaccine dose by 142% and 218%, respectively (Fig. 7).

Effect of CNP-Salmonella vaccine on bile anti-Salmonella IgA antibody titers. There were significant differences in bile IgA titers among treatments at d25 (post-challenge). Birds that were vaccinated with 1000µg CNP had higher (*P*=0.0026) bile anti-*Salmonella* OMPs-specific IgA titers compared to that from the mock, 2000µg, and 500µg treatment groups by 73%, 73% and 63%, respectively (Fig. 8). Anti-*Salmonella* flagellin-specific IgA on bile was higher (0.0058) in the 1000µg vaccinated group than the control and 500µg group by 12%, and 13%, respectively (Fig. 9).

Effect of CNP–Salmonella vaccine on Salmonella population in the ceca post-Salmonella enterica serovar enteritidis challenge. No significant differences were observed for bacterial colonization of cecal content at 11d post-challenge (*P*=0.224) (Fig. 10).

Effect of CNP- Salmonella vaccine on IL-1β, IL-10, IL-4, IFNγ mRNA transcription in the cecal tonsils. Significant results were found for cecal tonsil IL1β mRNA amount. IL 1β was found to be 10 times lower in the 500µg vaccinated group while the 1000µg and 2000µg vaccine doses were 2 times higher, when compared to control (*P*=0.0004) (Fig. 11, A). Cecal tonsil IL10 was 2-fold lower in the 500µg treatment while the 1000µg treatment was times 3.3 times higher, when compared to that of the control (*P*= 0.019) (Fig. 11, B). There were no significant (*P*>0.05) differences on IL-4 and IFNg mRNA transcripts (Fig. 11, C-D; respectively) among treatment groups at 11 d post-*Salmonella* challenge (*P*=0.1146; *P*= 0.6905, respectively).

Effect of CNP- Salmonella vaccine on IL-1β, IL-10, IL-4, IFNγ mRNA transcription in the spleen. Spleen IL1β mRNA amount was 2-fold higher in the 1000µg vaccinated group compared to that of the mock treatment (*P*=0.0057) (Fig. 11, A). Birds on the mock group (PBS) had significantly less IL-4 mRNA amount while no significant differences were found between vaccine doses, when compared to control (*P*=0.0011) (Fig. 12, C). There were no significant (*P*>0.05)

differences on IL-10 and IFNg mRNA transcripts (Fig. 12, B; Fig. 12, D, respectively) among treatment groups at 11d post-*Salmonella* challenge (*P*=0.2497; *P*= 0.4261, respectively).

Effect of CNP-Salmonella vaccine on IL-1β, IL-10, IL-4, IFNγ mRNA transcription in the liver. Significant results were found for liver IL-1β mRNA amount. IL-1β mRNA content with the 500µg CNP vaccine dose was 2.6 times higher while the 1000µg treatment was 0.7 times lower, when compared to the control (*P*= 0.0432) (Fig 13, A). Significant results were found for liver IL-4 mRNA amount. IL-4 mRNA amount was 3-fold lower in the 1000µg vaccinated group compared to that of the mock group (*P*= 0.009) (Fig 13, C). There were no significant (*P*>0.05) differences on IL-10 and IFNγ mRNA transcripts (Fig 13, B; Fig 12 D, respectively) among treatment groups at 11d post- *Salmonella* challenge (*P*= 0.0749; *P*=0.1215, respectively).

DISCUSSION AND CONCLUSION

It has been demonstrated that Copper-loaded chitosan nanoparticles (CNP-Cu) could improve growth performance in rats (Du 2008) and broiler birds (Wang *et al.* 2011a). However, the oral administration of CNP vaccine resulted in no significant difference in the mean BWG and FCR recorded between any of the treatment groups pre or post- *S.* enteritidis challenge from d0 - d25 (Fig 1, A, B, respectively). Different optimal levels could contribute to the differences in experimental animals(Wang *et al.* 2011a). Therefore, it can be concluded that 2000µg, 1000µg, and 500µg CNP vaccine doses have no adverse effects on bird's production performance parameters.

Salmonellae persist and multiply within macrophages (MacLennan 2014). Activated macrophages inhibit pathogen replication by releasing a variety of effector molecules, including nitric oxide (NO) (Tripathi *et al.* 2007). Previous *in vitro* studies have shown that *S.* enteritidis can suppress NO production in infected chicken macrophage-like HD11 cells, while dead *S.* enteritidis

stimulates a high level of NO production (He *et al.* 2013). In birds vaccinated with 500µg CNP vaccine dose, splenic macrophages stimulated with 1 µg/mL *S.* Enteritidis LPS produced significantly greater levels (P=0.0094) of nitric oxide compared to that of the mock, 1000µg, and 2000µg CNP vaccine doses (Fig. 2, B). Results showed a dose-dependent manner (Fig 2, B). Results were consistent with previous findings were Nitric oxide synthase (iNOS) mRNA expression was upregulated in CNP-*Salmonella* vaccine in treated layer chickens (10336-342WO1 2017). NO-dependent tissue injury has been implicated in a variety of autoimmune diseases (Abramson *et al.* 2001), suggesting that increasing NO levels could result in adverse health effects in other species. Hence 1000µg CNP *Salmonella* vaccine dose showed the release of substantial NO levels enough to fight infection while preventing any potential NO-dependent tissue injury in broiler birds.

Antibodies have a vital role in eliminating extracellular bacteria (Abbas Abul K. *et al.* 2015). Previous studies indicate that antibodies can neutralize *Salmonella* that are not shielded by residing inside host cells(MacLennan 2014). Theoretically, *Salmonella*e are susceptible to antibodies at different points of the invasion: a) following primary invasion, b) when initially entering the circulation, and c) when transiting between phagocytes via the blood or extracellular fluids(MacLennan 2014). ELISA was used to analyze the vaccine effect on broiler bird's humoral immunity. Anti-*Salmonella* OMPs-specific IgG and IgA antibodies were detectable in serum, pre-challenge and post-challenge-after an 8hr delay blood draw. There were no significant differences in anti- *Salmonella* OMPs-specific serum IgG (Fig. 3) and IgA (Fig. 5) titers among treatment groups pre-*Salmonella* challenge. However, all vaccine doses numerically increased anti-*Salmonella* OMPs-specific serum IgG antibody titers at d7, displaying potential to induce a humoral immune response. Birds vaccinated with 500µg CNP vaccine dose had significantly

higher anti-*Salmonella* OMPs specific IgG titers on d17 and d20 (Fig. 4), and substantially higher anti-*Salmonella* flagellin-specific IgA titers from cloacal swabs at d14 post-challenge (Fig 7). The 500µg vaccine dose displayed potential to induce an effective humoral immune response, however due to the exceedingly high amounts of NO levels it can induce, this vaccine dose was not considered an optimal dose for administration.

On broiler birds vaccinated with 1000µg CNP dose *S.* enteritidis challenge substantially boosted serum anti-*Salmonella* OMP specific IgG (Fig. 3) and IgA (Fig. 5) antibody responses at d14 post- challenge, cloacal anti-*Salmonella* OMP specific IgG titers at d25-post challenge (Fig. 6), and bile anti-*Salmonella* OMP (Fig. 8) and flagellin (Fig. 9) specific IgA titers. This dose conferred an optimal vaccine mediated protection as it substantially increased Anti-*Salmonella* IgG and IgA titers in serum, cloacal swabs and bile samples. Along with the moderate NO levels it can induce, this vaccine displayed great potential to confer an efficient innate and humoral immune response against *S.* enteritidis.

Birds dosed with 2000µg CNP vaccine had significantly higher anti-*Salmonella* OMPs specific IgG titers on d17 while having a significant decrease on d20 (Fig. 4), displaying a typical humoral immune response where antibody titers decline with time after immunization (Abbas Abul K. *et al.* 2014). Upon termination on d25, birds in the mock group had significantly higher anti- *Salmonella* OMPs specific IgG on serum, while the 2000µg CNP vaccine dose showed a significant decrease (Fig. 4). A possible explanation for higher OMP-specific IgG titers in the mock group is the assumption that a persistently high antibody response is indicative of a persistent infection (Hansen *et al.* 2006). An infected animal that has been immunized and has successfully cleared an infection follows a typical adaptive antibody response: it will develop a high concentration of antibodies against the antigen which will decline with time after each

immunization (Abbas Abul K. *et al.* 2015), while a carrier animal will usually maintain persistently high antibody levels in blood, as previously documented with *S.* dublin in cattle (Hansen *et al.* 2006). Despite the small amounts of NO levels this vaccine dose induced, it showed potential to induce an effective humoral immune response against *S.* enteritidis in broiler birds.

Overall, results were consistent with other research regarding to chitosan nanoparticle vaccines as an effective vaccine delivery system. Copper-loaded chitosan nanoparticles (CNP-Cu) have shown to enhance immunity in rats (Du 2008), and to increase the concentrations of immunoglobulins, complements and lysozyme in serum (Wang *et al.* 2011a). Moreover, the vaccine under study has previously shown potential to increase the humoral immune response of layer birds (Renu *et al.* 2018). Results show CNP- *Salmonella* vaccine successfully managed to substantially increase antigen specific anti-*Salmonella* IgG and IgA antibody titers in broiler birds infected with *S.* enteritidis; with the 1000µg CNP vaccine dose displaying potential to provide protection from *S.* enteritidis infection.

Salmonella enteritidis frequently colonizes the gastrointestinal tract of poultry (Andino and Hanning 2015); hence *Salmonella* population on cecal contents was quantified by Real-Time PCR. Although there were no significant differences for bacterial colonization of cecal content 11d post-challenge, results showed a numerical decrease of *Salmonella* enteritidis population on cecal contents from birds orally vaccinated with the 1000µg CNP vaccine dose, when compared to control (Fig. 10). The 1000µg CNP vaccine dose demonstrated potential to mitigate *S.* enteritidis population on ceca, but further studies need to be done to further exploit this.

Our current experiment utilized Real-Time PCR analysis to monitor pro- and anti-inflammatory cytokine response on cecal tonsils. In addition, because the colonization of *Salmonella* enteritidis in the liver and spleen has been reported to occur 1d after inoculation (Van

Immerseel *et al.* 2002, Coble *et al.* 2011), our study looked into these organs as well. Previous research has been demonstrated that the chitosan composition of the nanoparticle itself has a strong potential to increase both cellular and humoral immune responses, and elicit a balanced Th1/Th2 response (Wen *et al.* 2011). Following the application of CNP-*Salmonella* vaccine, broiler birds displayed a wide variety of Th1- and Th2-type responses. Results showed that different doses of the vaccine induced substantially high IL1β mRNA levels on cecal tonsils, spleen and liver samples (Fig 11, A; Fig 12, A; Fig 13, A). A possible explanation for increased mRNA content of pro-inflammatory cytokine IL1β, is that the adjuvant composition can be one that induces an immune response predominantly of the Th1 type (10336-342WO1 2017). On the other hand, the only vaccine dose that substantially increased anti-inflammatory cytokine IL-10 levels was the CNP 1000µg vaccination dose (Fig 11, B). This cytokine is of particular interest because during infections caused by extracellular or highly inflammatory bacteria, IL-10 production reduces host tissue damage and facilitates host survival (Peñaloza *et al.* 2018). When measuring the expression of IFNg, an important activator of macrophages(Abbas Abul K. *et al.* 2015), no significant differences on mRNA transcripts were seen within treatments (Fig 11, D; Fig 12, D; Fig13 D). IL-4, a Th2 cytokine which decreases the production of Th1 cells, macrophages, and IFN-gamma (Abbas Abul K. *et al.* 2015), was significantly decreased on spleen samples from the mock group (Fig. 12, C) while birds treated with different vaccine doses displayed higher amounts ($P>0.05$). Results show that all CNP vaccine doses induced protective Th1 and Th2 cytokine mRNA expression levels. However, given that 1000µg CNP dose was the only one to substantially increase IL-10 levels, this vaccine dose showed the most potential to induce a more potent anti-inflammatory response against extracellular bacteria *S.* enteritidis.

In conclusion, the efficacy of orally delivered chitosan-based nanoparticle vaccines was demonstrated against a *Salmonella* enteritidis infection in broiler birds. CNP vaccine does not affect production performance on birds. In addition, CNP can trigger substantial NO levels in response to *S.* enteritidis infection. In vaccinated birds, CNPs vaccine predominantly induced antigen specific IgG, and mucosal IgA response. Moreover, CNP vaccine induced balanced Th1 and Th2 cytokine mRNA expression levels. Thus, these study results show that CNP vaccine is a potential vaccine for oral delivery of antigens against *Salmonella* in poultry, and that 1000µg dose of the vaccine can provide optimal protection from *S.* enteritidis infection. Further studies are required to improve the vaccine efficacy to mitigate *Salmonella* in poultry.

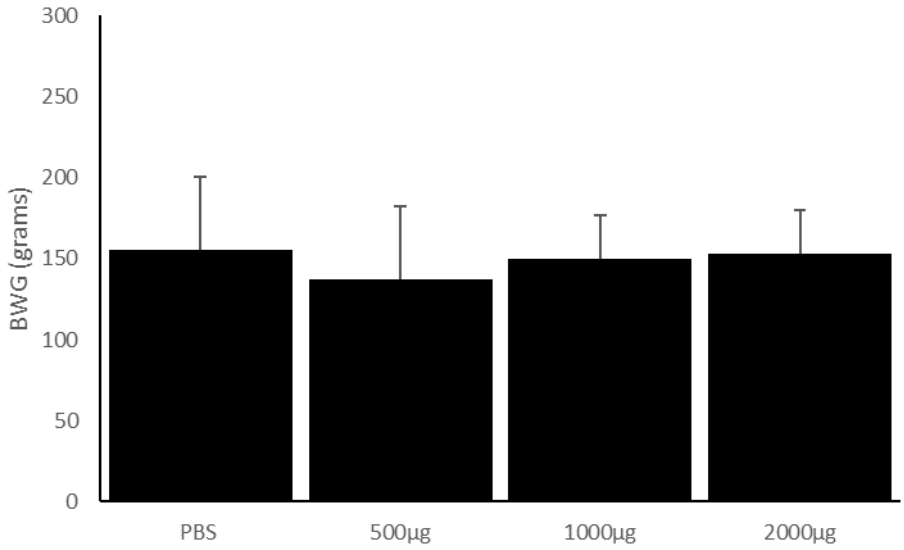
TABLES

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

Target	**Primer**	**Sequence (5' – 3')**	**Reference**
IL-1β	F	TCCTCCAGCCAGAAAGTGA	(Shanmugasundaram *et al.* 2012)
	R	CAGGCGGTAGAAGATGAAGC	
IL-10	F	GAGGAGCAAAGCCATCAAGC	(Luoma 2016)
	R	CTCCTCATCAGCAGGTACTCC	
IL-4	F	AACATGCGTCAGCTCCTGAAT	(Renu *et al.* 2018a)
	R	TCTGCTAGGAACTTCTCCATTGAA	
IFNg	F	GTGAAGAAGGTGAAAGATATCATGGA	(Markazi 2018)
	R	GCTTTGCGCTGGATTCTCA	
16S	F	AGAGTTTGATCCTGGCTCAG	(Luoma 2016)
	R	GACTACCAGGGTATCTAATC	
S. enterica	F	GCAGCGGTTACTATTGCAGC	(Luoma 2016)
	R	CTGTGACAGGGACATTTAGCG	

FIGURES

A.



B.

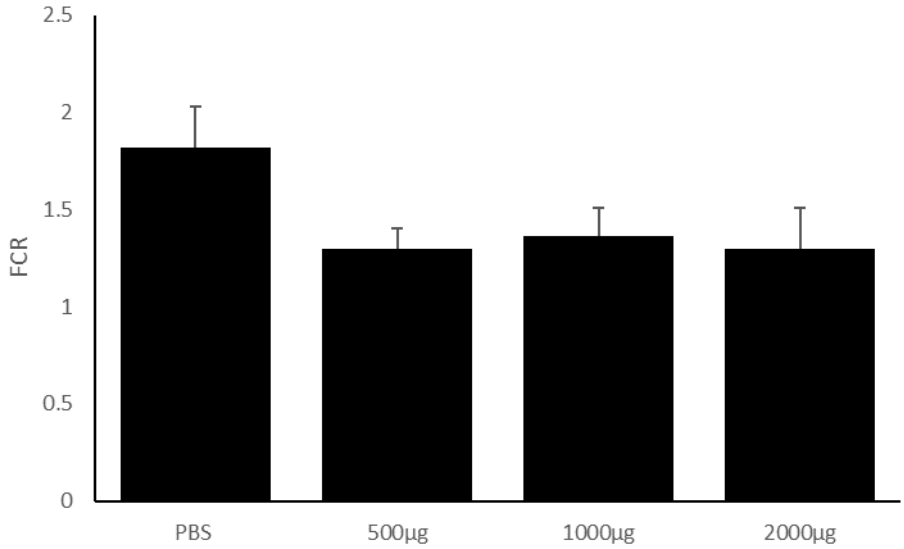


Figure 1. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on performance characteristics of broilers birds.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were

challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Production performance parameters were monitored throughout the study and chickens were euthanized by cervical dislocation at d25 for sample collection. A - Body weight gain. B- Feed conversion ratio. Results are reported as final performance at d25. Bars (+SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P=0.7759$; Panel B, $P=0.7254$.

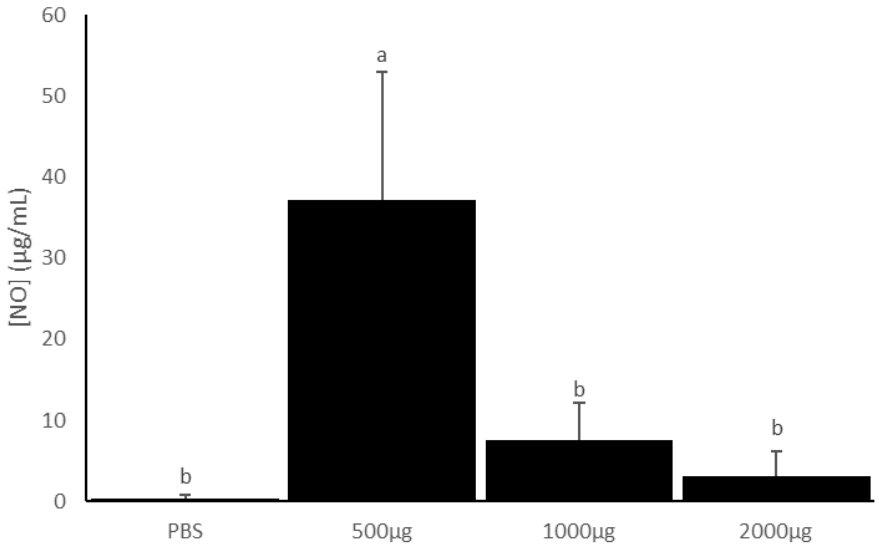


Figure 2. *Effect of CNP–Salmonella vaccine on macrophage nitric oxide production post-Salmonella enterica serovar enteritidis challenge.* Splenic macrophages from 25d old birds were isolated and cells were stimulated for nitrite production with 1 µg/mL *S.* enteritidis LPS. Nitrite concentrations of splenic macrophages for each treatment group. Results are reported as average optical density (OD) values. Bars (+SE) with no common superscript differ ($P \leq 0.05$). P value: $P=0.0094$.

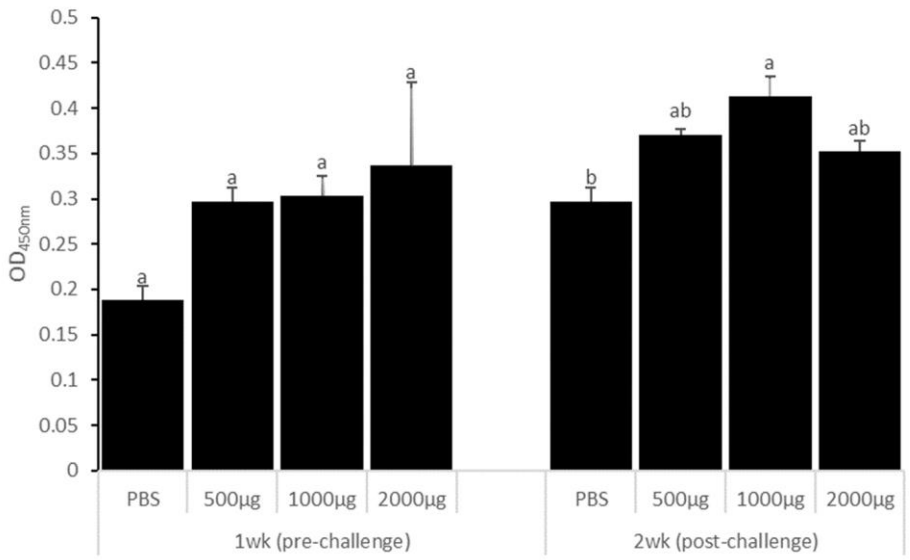


Figure 3. *Effect of Chitosan Nanoparticle (CNP)- Salmonella vaccine on anti-Salmonella IgG antibody titers pre- and post-Salmonella challenge.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Blood samples were collected at 1 and 2 wk of age and analyzed for anti-*Salmonella* OMP-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: 1 wk, $P=0.2901$; 2 wk, $P=0.0029$.

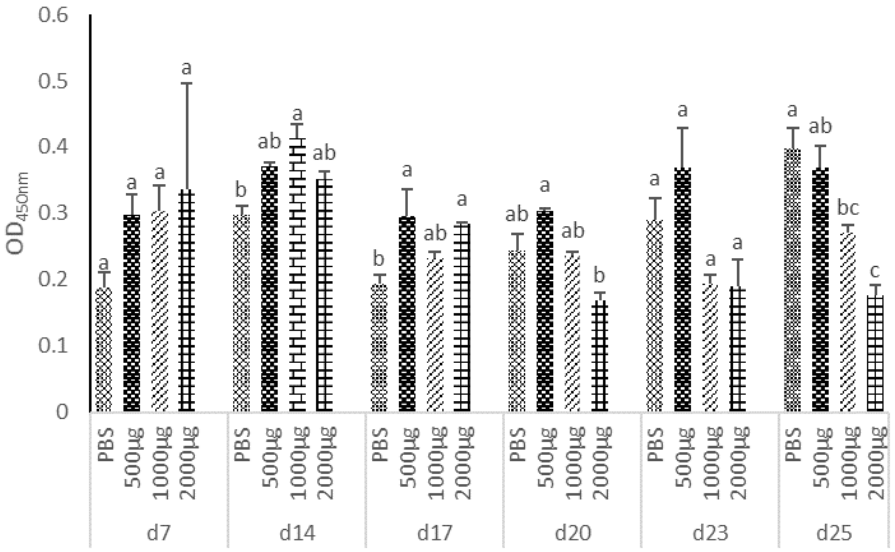


Figure 4. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgG antibody titers in serum.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Blood samples were collected at 1 and 2 wk of age and analyzed for anti-*Salmonella* OMP-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: d7, P=0.2901; d14, P=0.0029; d17, P=0.0047; d20, P=0.0231; d23, P=0.0372; d25, P= 0.0011.

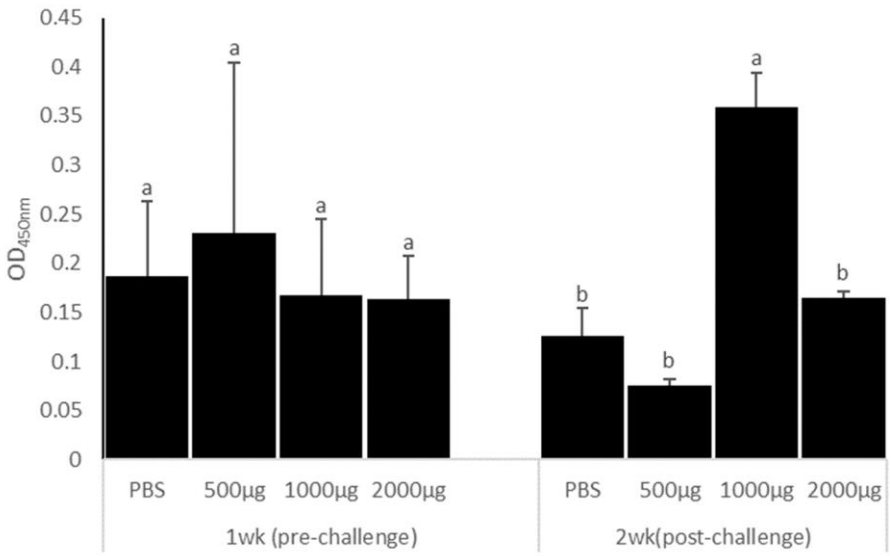


Figure 5. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre- and post-Salmonella challenge.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Blood samples were collected at 1 and 2 wk of age and analyzed for anti-*Salmonella* OMP-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: 1 wk, $P = 0.9700$; 2 wk, $P = 0.0009$.

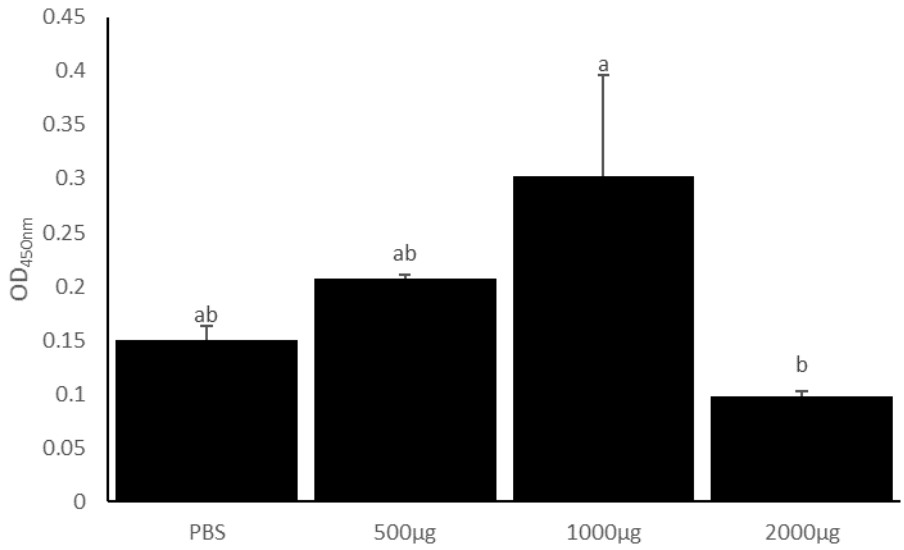


Figure 6. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers in cloacal swabs: OMP.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Cloacal swab samples were collected at d17, d20, d23, and d25 and analyzed for anti-*Salmonella* OMP-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: $P=0.0551$.

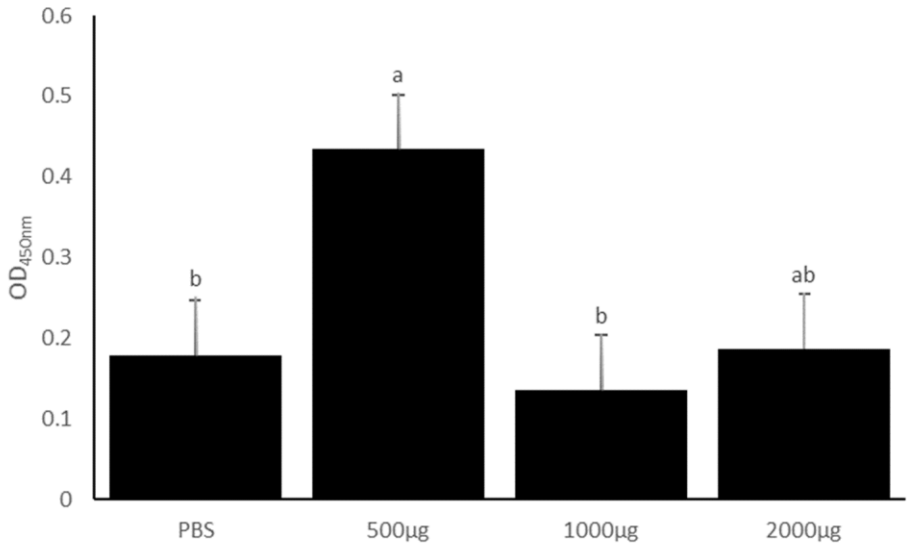


Figure 7. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers in cloacal swabs: Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Cloacal swab samples were collected at d17, d20, d23, and d25 and analyzed for anti-*Salmonella* Flagellin-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: $P=0.0223$.

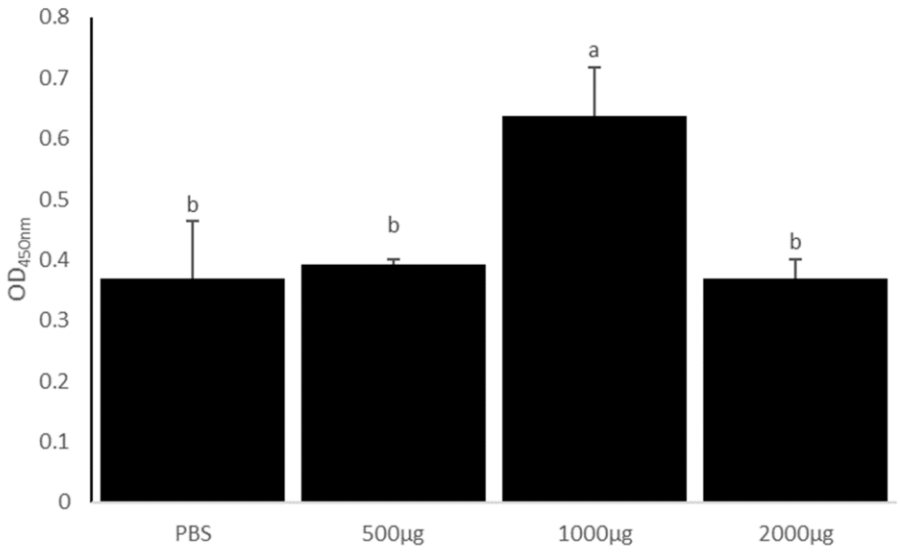


Figure 8. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on bile anti-Salmonella IgA antibody titers: OMP.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Bile samples were collected at 11d post-*Salmonella* challenge and analyzed for anti-*Salmonella* OMP-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: $P=0.0026$.

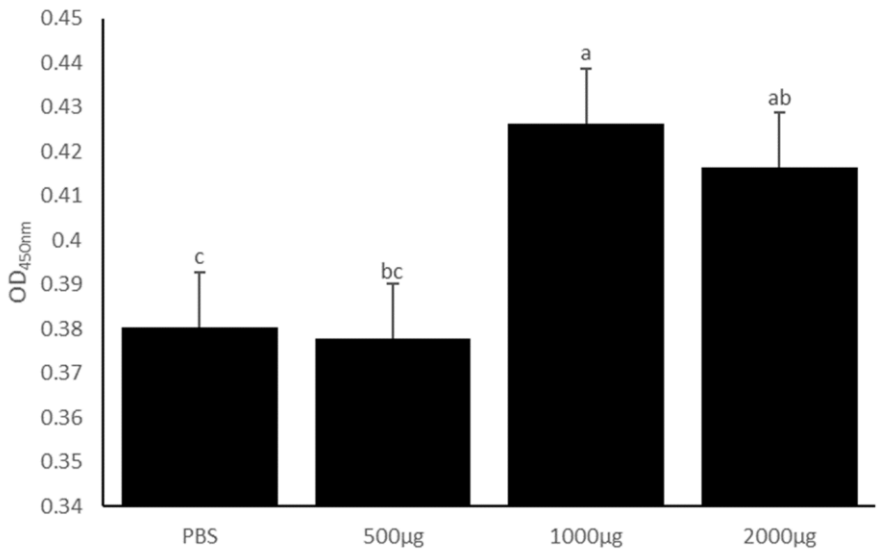


Figure 9. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on bile anti-Salmonella IgA antibody titers: Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4×10^5 CFU/mL). Bile samples were collected at 11d post-*Salmonella* challenge and analyzed for anti-*Salmonella* Flagellin-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: $P=0.0058$.

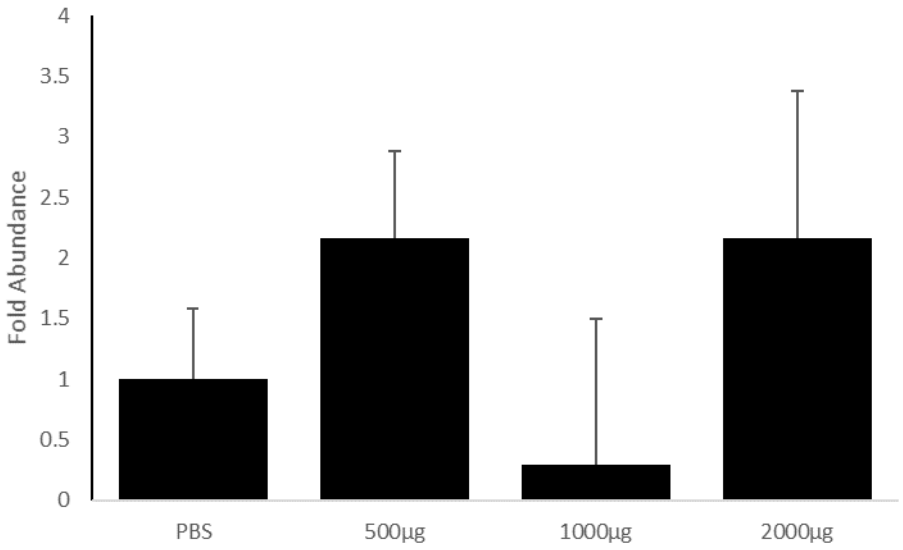
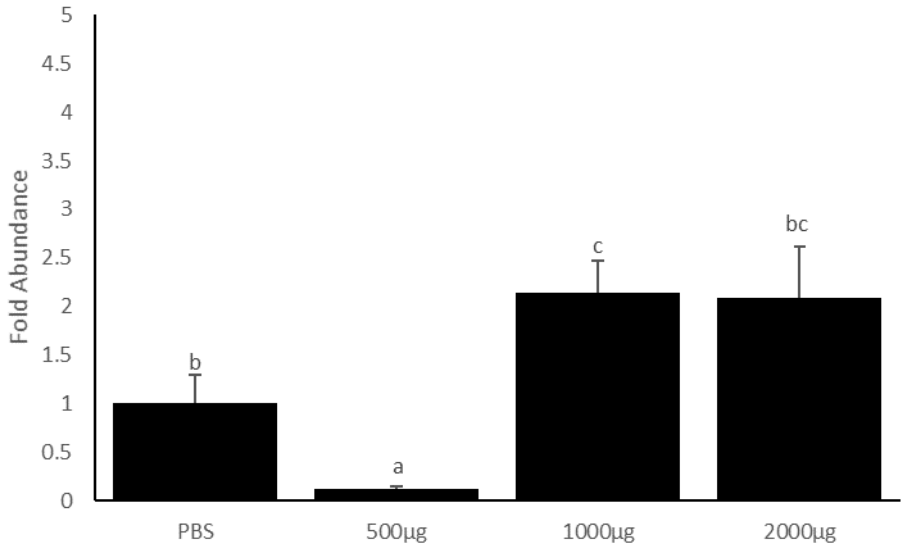


Figure 10. *Real-time PCR quantification of S. enteritidis on cecal content colonization 11d post challenge.* To evaluate the relative proportion of bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: $P=0.224$.

A.


Fold Abundance
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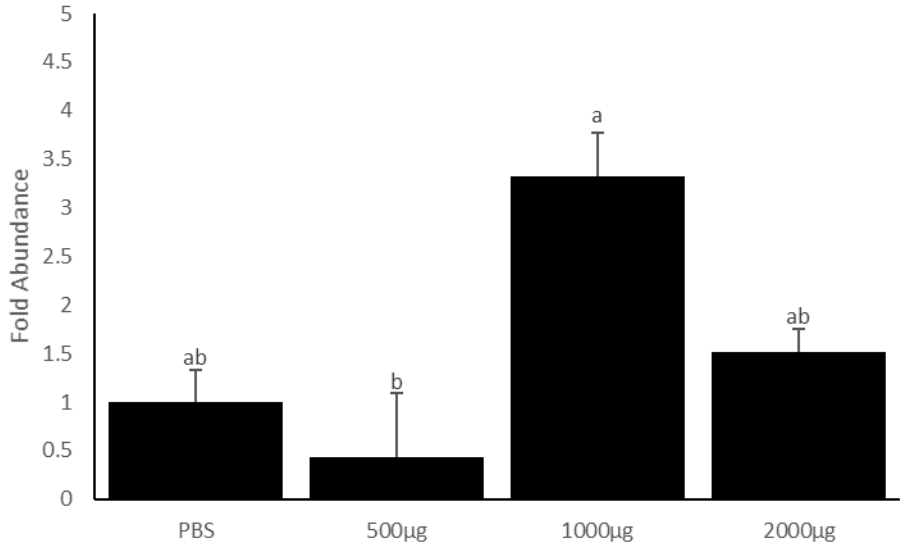
B.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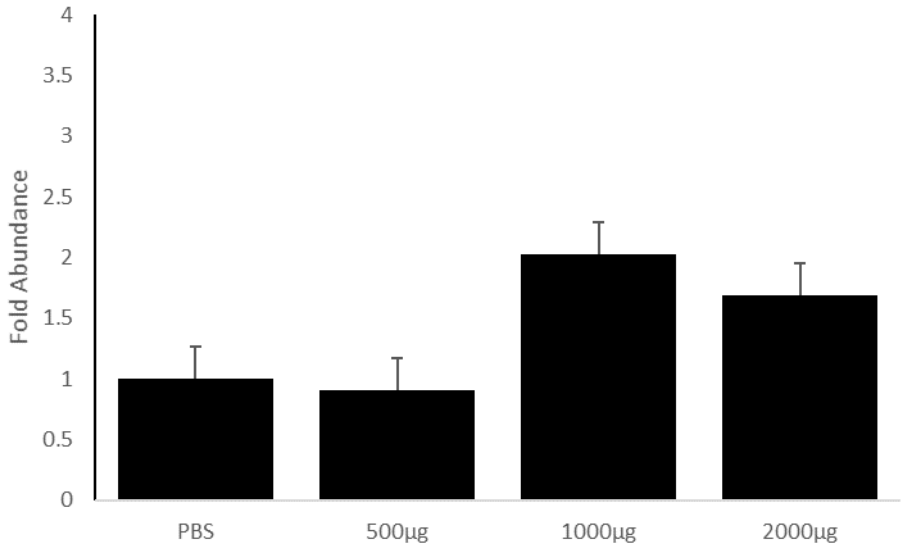

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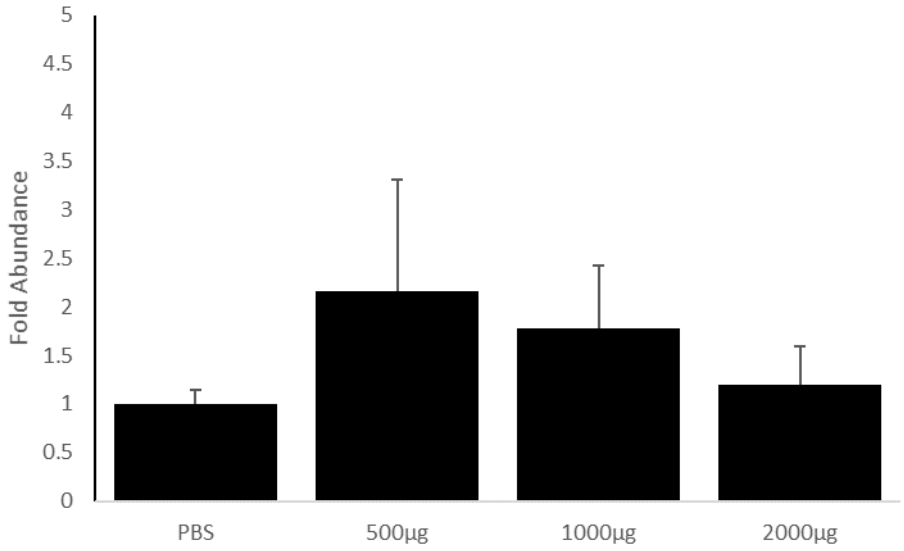
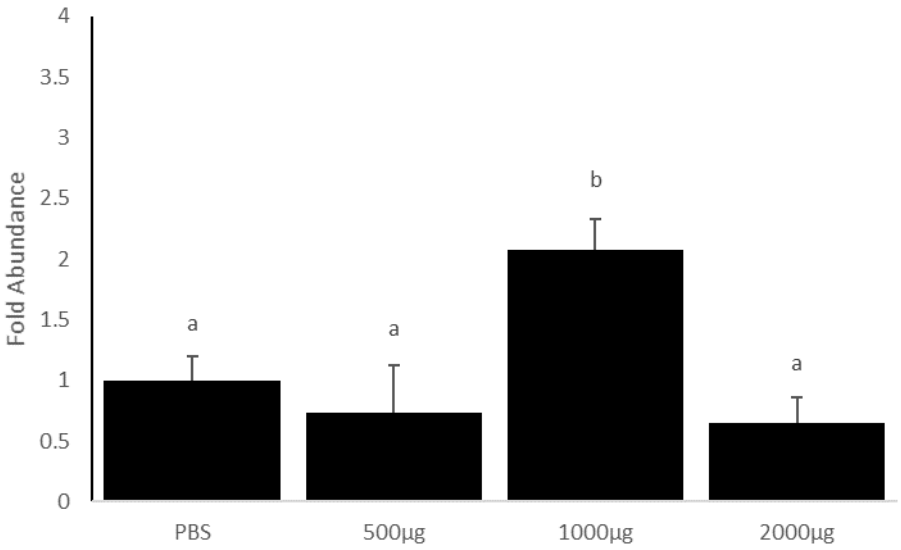


Figure 11. *Effect of CNP- Salmonella vaccine on IL-1, IFNγ, IL-10, IL-4 mRNA transcription in the cecal tonsils.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Cecal tonsil samples were collected

at 11 d post-*Salmonella* challenge and analyzed for mRNA content after correcting for 13RPs13 mRNA content and normalizing to the mRNA content of the mock group. A - IL1 mRNA content. B - IL10 mRNA content. C - IL 4 mRNA content. D – IFNg mRNA content. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P=0.0004$; Panel B, $P=0.019$; Panel C, $P=0.1146$; Panel D, $P=0.6905$.

A.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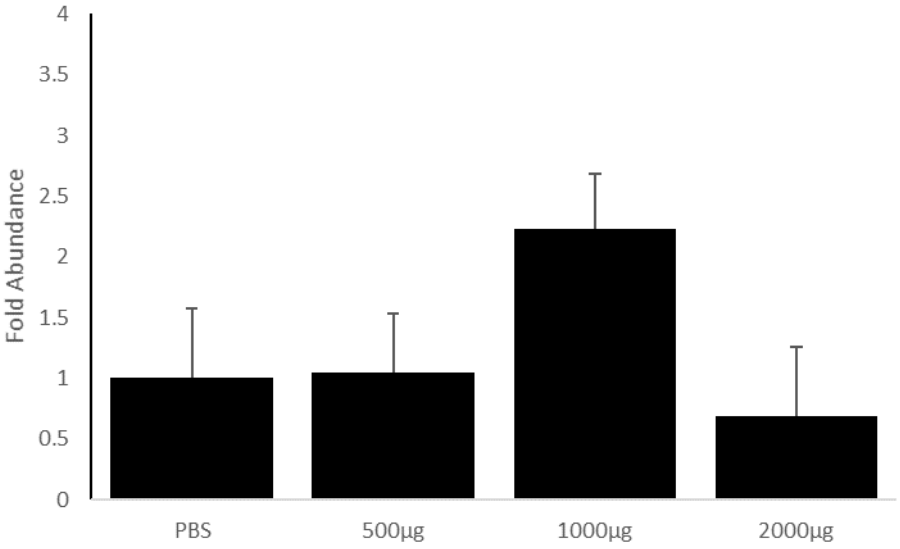
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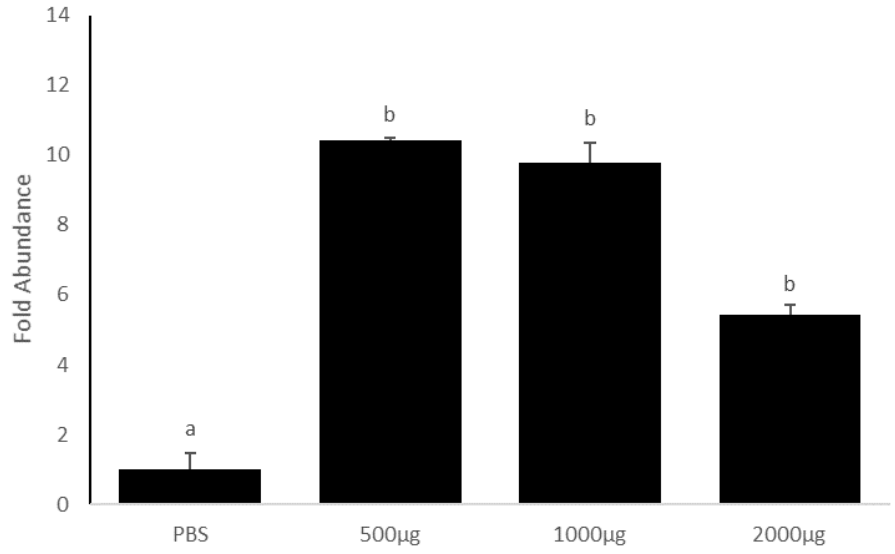


D.

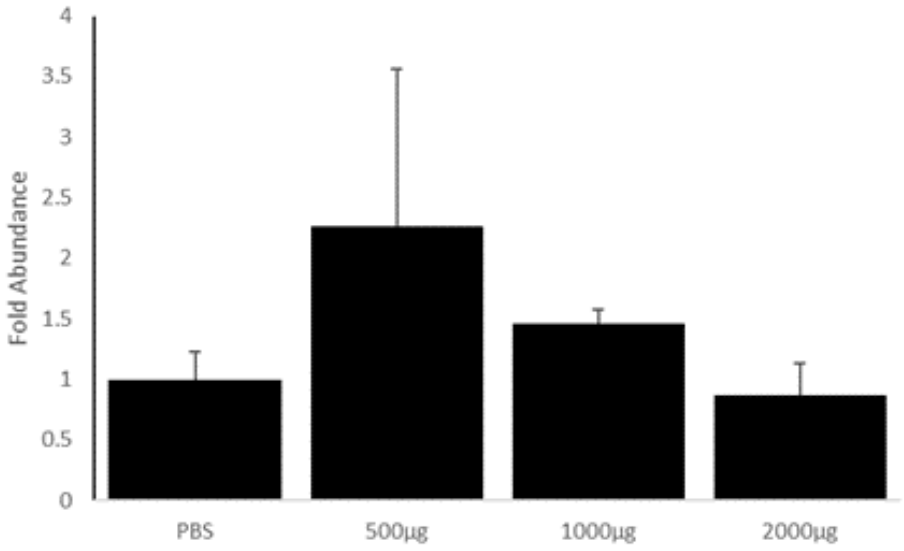
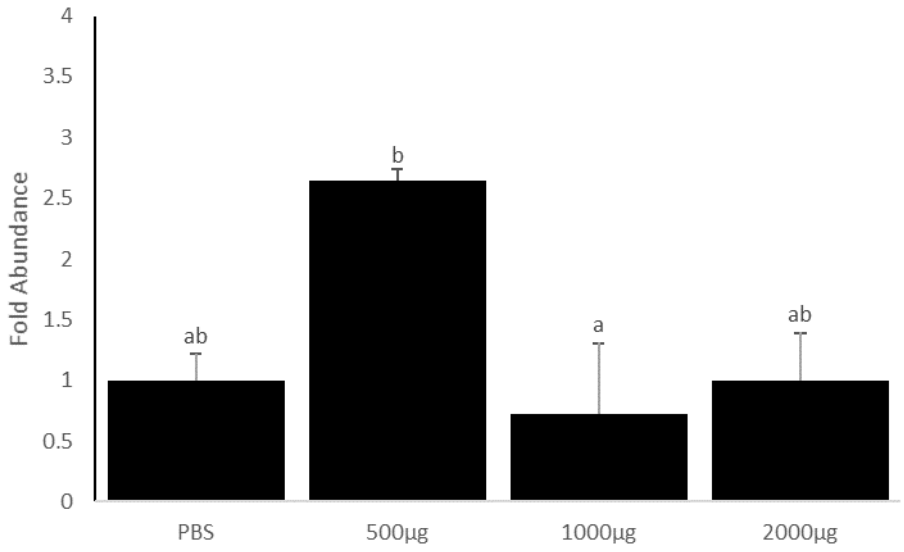


Figure 12. *Effect of CNP- Salmonella vaccine on IL-1, IFNγ, IL-10, IL-4 mRNA amounts in the spleen.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Spleen samples were collected at

11d post-*Salmonella* challenge and analyzed for mRNA content after correcting for 13RPs13 mRNA content and normalizing to the mRNA content of the mock group. A - IL1 mRNA content. B – IL10 mRNA content. C - IL4 mRNA content. D – IFNg mRNA content. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P=0.0057$; Panel B, $P=0.2497$; Panel C, $P=0.0011$; Panel D, $P=0.4261$.

A.


Fold Abundance
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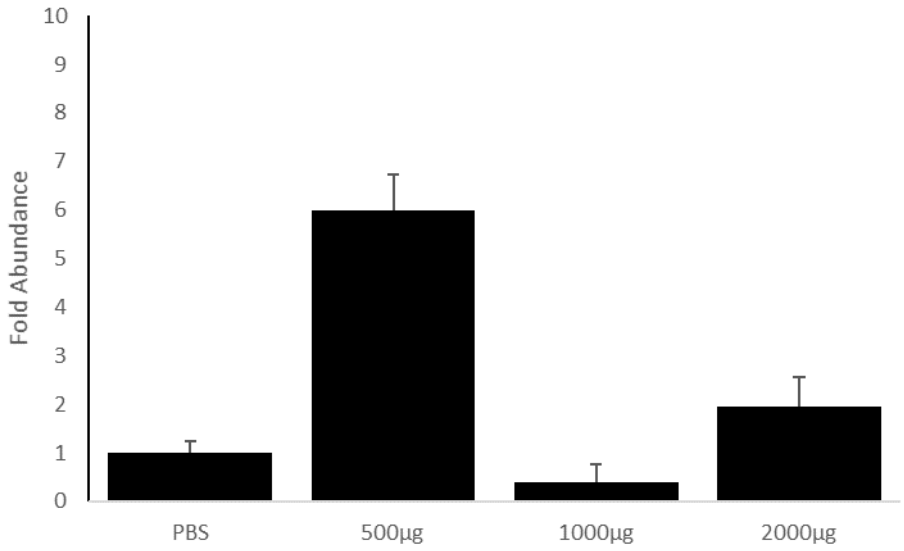
B.


Fold Abundance
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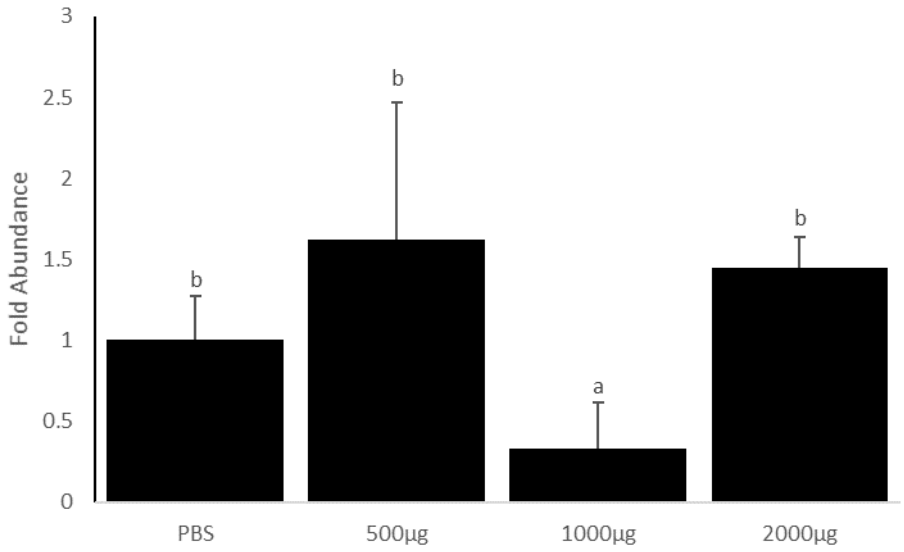
C.



D.

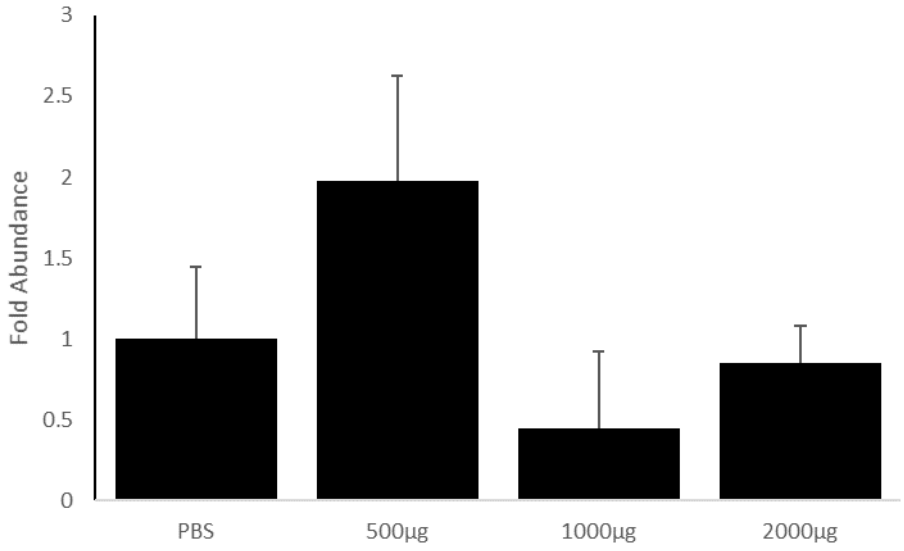
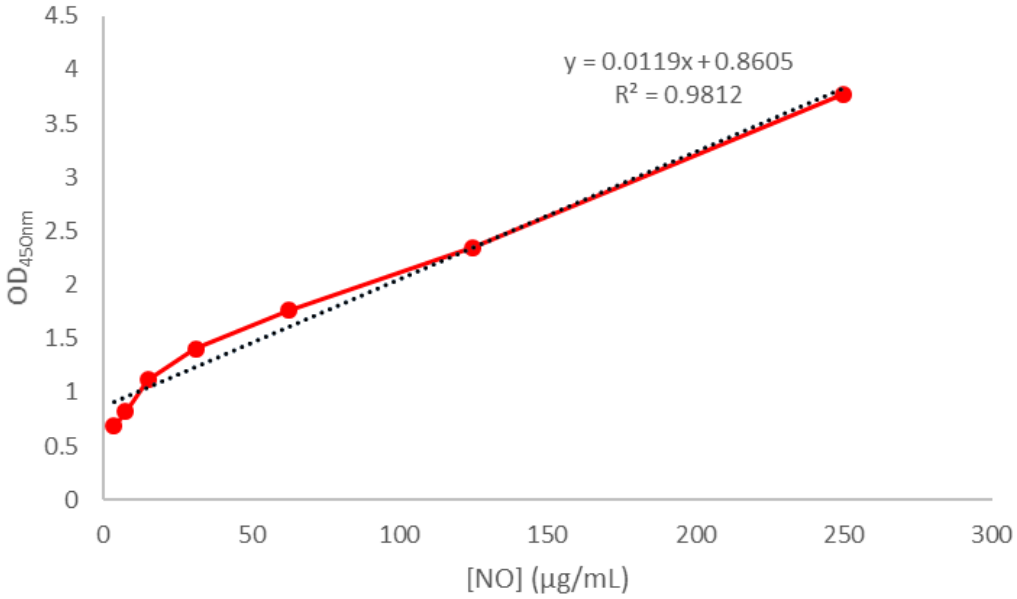


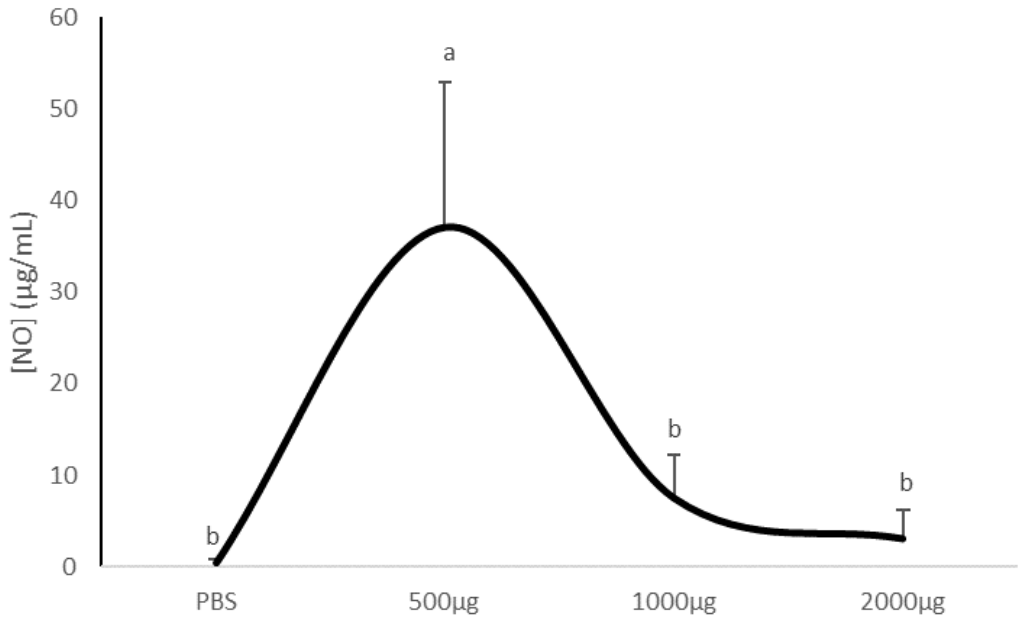
Figure 13. *Effect of CNP- Salmonella vaccine on IL-1, IFNγ, IL-10, IL-4 mRNA transcription in the liver.* At 0d of age, chickens were orally vaccinated with either PBS (Mock) or different doses of OMPs + flagellin proteins: 500µg, 1000 µg, or 2000 µg, loaded into CNP. Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar enteritidis (5.4 x 10^5 CFU/mL). Liver samples were collected at 11d

post-*Salmonella* challenge and analyzed for mRNA content after correcting for 13RPs13 mRNA content and normalizing to the mRNA content of the mock group. A - IL1 mRNA content. B – IL 10 mRNA content. C - IL4 mRNA content. D – IFNg mRNA content. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P=0.0432$; Panel B; $P=0.0749$; Panel C, $P= 0.009$; Panel D, $P= 0.1215$.

SUPPLEMENTAL MATERIALS



Supplemental figure 1, A. *Effects of CNP on macrophage-nitric oxide production post-Salmonella enterica serovar enteritidis challenge: Standard Curve.* Nitrite concentrations were determined from a standard curve drawn with different concentrations of sodium nitrite solution: 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, 7.8125 µM, 0 µM.



Supplemental figure 2, A. *Effects of CNP on macrophage-nitric oxide production post-Salmonella enterica serovar enteritidis challenge: Dose-dependent response.* In birds vaccinated with 500µg CNP vaccine dose, splenic macrophages stimulated with 1 µg/mL *S.* enteritidis LPS produced significantly greater levels (p= 0.0094) of nitric oxide compared to that of the mock (PBS), 1000µg, and 2000µg CNP vaccine doses. Results showed a dose-dependent manner, with the lowest vaccine dose generating the highest nitric oxide levels and vice versa.

CHAPTER 4

EFFICIENCY OF CHITOSAN-BASED NANOPARTICLE VACCINE TO DECREASE COLONIZATION AND SHEDDING AND PROVIDE CROSS PROTECTION IN BROILER BIRDS CHALLENGED WITH SALMONELLA ENTERICA SEROVARS: ENTERITIDIS AND HEIDELBERG[2]

[2] K. Acevedo, S. Renu, T. Ng, M. Mortada, G. Akerele, J. Oxford, R. Shanmugasundaram, B. Lester, R. Gourapura, R. Selvaraj. To be submitted to *Poultry Science*.

ABSTRACT

This study analyzed the protective effects of an oral chitosan-based *Salmonella* nanoparticle vaccine (CNP) engineered with *Salmonella* outer membrane and flagellin proteins. A total of 264, one-day-old Cobb-500 broilers (n=6/pen) were orally gavaged with PBS or 1000µg CNP or Poulvac ST-live commercial vaccine (CV). A booster was given at 7d of age. At 14d of age, birds were orally challenged with 10^5 CFU of live *S.* enteritidis (SE) or *S.* heidelberg (SH). All birds were euthanized at 4d post-challenge. There were no differences ($P>0.05$) for body weight gain and feed conversion ratio between any treatment groups. Birds vaccinated with CNP or CV showed higher ($P<0.05$) anti-*Salmonella* OMPs-specific serum IgG titers at 8h post-challenge in response to SH and SE challenge. CNP and CV numerically increased ($P>0.05$) antibody titers in response to the booster, showing potential to induce a protective immune response against SE and SH. At 4d post-SH challenge, birds that were vaccinated with CNP and CV had higher ($P<0.05$) bile anti-*Salmonella* flagellin-specific IgA titers ($P<0.05$). Both, CNP vaccine and CV decreased ($P<0.05$) Anti-*Salmonella* OMP-specific IgG titers on serum at 2d post-SE challenge or 4d post-SH and SE challenge. Estimates for SH population in liver and spleen showed CNP and CV numerically decreased bacterial population when compared to control. *Salmonella* enteritidis and heidelberg population on cecal content at 2d post challenge showed a numerical decrease in birds vaccinated with CNP and CV vaccine when compared to the unvaccinated control. CNP vaccination had no effect ($P>0.05$) on cecal tonsils IL-1β and IL-10 cytokine mRNA expression. Results demonstrate that CNP can induce an immune response similar to that of Poulvac ST, in birds challenged with SH and SE, and that this vaccine has the potential to reduce SH population in liver and spleen, and SE and SH cecal colonization.

INTRODUCTION

In the United States of America (USA), *Salmonella* is the leading foodborne pathogen, causing the largest number of deaths in humans and has the highest cost burden of any foodborne pathogen (Batz *et al.* 2012). *Salmonella* enterica has been estimated to cause 93.8 million infections globally each year, with 155,000 deaths (Majowicz *et al.* 2010). Infection by this bacterium is most frequently caused by consumption of contaminated poultry meat and eggs (Andino and Hanning 2015). Within a few hours after chickens are orally infected, *Salmonella* can invade the intestinal tract and reach internal organs such as the liver and spleen (Jones *et al.* 2016). *Salmonella* enterica serovars enteritidis and heidelberg are among the most frequent serotypes recovered from humans each year (Gong *et al.* 2014). Over the years, *Salmonella* heidelberg (SH) has gained prominence in North America poultry production and other countries (Borsoi *et al.* 2011). *Salmonella* heidelberg has been isolated and reported from poultry its products in Brazil since 1962, while *Salmonella* enteritidis (SE) has been recognized as a serious problem in poultry and public health since 1993(Borsoi *et al.* 2011). The more that is learned about the more prominent serotypes helps us better understand illness and develop new strategies to decrease pathogen outbreaks.

Vaccination is an ideal strategy for the prevention of salmonellosis (Nandre *et al.* 2015). Cross-protection can enhance the clearance of pathogens through the acquired immune response (Nnalue 1990, Nandre *et al.* 2015). A vaccine constructed for a single serovar of *Salmonella* may induce immunity against other heterologous serovars of *Salmonella* (Beal *et al.* 2006), this is a common tool employed by many vaccine companies. Conserved proteins can be taken as antigens

of interest and used to induce cross protection against multiple serovars. For example, outer membrane protein (OMP) C is highly conserved within 11 different *Salmonella* serotypes, with exception of *S.* arizonae (Puente *et al.* 1995). Flagella also possess highly conserved regions among all bacteria, and several patterns of *Salmonella* flagellin sequences are known to be well conserved while others possess highly variable domains (Puente *et al.* 1995, Vonderviszt F. 2008, Duan *et al.* 2013). Both, Flagella (Mizel and Bates 2010) and OMP (Tan *et al.* 2018) are *Salmonella* antigens that possess intrinsic adjuvant capacity to induce an innate and subsequent humoral immune response in poultry, as they naturally exist in gram-negative bacteria.

Studies have shown that biodegradable chitosan nanoparticle (CNP) vaccines have ideal traits for delivering vaccine antigen loads orally (Wang *et al.* 2011b, Binnebose *et al.* 2015, Cheung *et al.* 2015). The amino and carboxyl groups in the chitosan molecule can be combined with glycoprotein in mucus to form a hydrogen bond, leading to an adhesive effect (Wang *et al.* 2011b, Cheung *et al.* 2015, Prajakta K.Khobragade 2015, Mohammed *et al.* 2017). This enables it to be internalized by M cells (Hallstrom and McCormick 2011) in the intestinal Peyer's patch and presented to underlying antigen presenting cells (APCs) for efficient uptake, processing and presentation of vaccine antigen. The vaccine candidate for this study was engineered using a chitosan nanoparticle matrix, loaded with a crude-enriched OMP and Flagellin extract from *S.* enteritidis and surface tagged with flagellin proteins, which could potentially to provide cross-protection for other *Salmonella* serovars. Other studies involving this nano vaccine have showed it provides a targeted delivery of its antigen to the intestinal Peyer's patches immune cells, as well its ability to substantially induce antigen specific mucosal antibody and T cell responses and its potential to mitigate salmonellosis in layer chickens challenged with *S.* enteritidis (Sankar Renu,

Ashley D. Markazi, Santosh Dhakal, Yashavanth Shaan Lakshmanappa, Revathi Shanmugasundaram 2018).

Our current study was done to analyze the efficacy of a novel Chitosan-nanoparticle (CNP) *Salmonell*a vaccine to provide cross protection against SE and SH challenge in broiler birds. We hypothesized that the oral delivery of CNP will induce anti- *Salmonella* IgG and IgA in serum, fecal swabs and bile, and decrease *Salmonella* shedding/load in broilers.

MATERIAL AND METHODS

Preparation of loaded Chitosan Nanoparticle vaccine. Engineering of the CNP vaccine was done at the Food Animal Health Research Program, OARDC and Department of Veterinary Preventive Medicine, The Ohio State University, USA; as described in its patent by corresponding authors (10336-342WO1 2017).

Commercial vaccine. A live commercial vaccine (Poulvac ST, Zoetis, NJ) that contains attenuated strains of *S.* enteritidis, *S.* heidelberg, and *S.* typhimurium was chosen as a control.

Animal husbandry. Day-old Cobb-500 chicks were used in the present study (n=264). Birds were provided water and feed *ad libitum* and were housed in floor pens prepared with fresh pine shavings (6 birds/pen). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Georgia. Body weight gain (BWG) and Feed conversion ratio (FCR) was recorded at all time points.

Treatments and challenge. At 0d of age, chickens were orally vaccinated with PBS (Mock), 1000µg preparation of loaded CNP vaccine, or a live commercial vaccine. The same dose and route of delivery was repeated at 7d of age. *Salmonella* enteritidis and *Salmonella* heidelberg isolates, resistant to novobiocin (Sigma–Aldrich, St. Louis, MO), were maintained in tryptic soy broth or tryptic soy agar at 40°C. XLT4 agar (Sigma–Aldrich, St. Louis, MO) containing 25

mg/mL novobiocin was used to culture the resistant isolates. Inoculum for infection was prepared from 18- to 24-h cultures. The bacteria was cultured until the final concentration reached approximately 10^6 CFU/mL, estimated spectrophotometrically at 600 nm and a pre-determined standard curve. A stock solution (1×10^6 CFU/mL) was prepared in sterile PBS (pH 7.2). The bacteria were serially diluted and plated on XLT4 agar plate to confirm concentration by enumeration using ImageJ Software (version 1.52k). At 14d of age, birds were challenged using an oral gavage with 0.1 mL of 10^5 CFU of live *S.* enteritidis or heidelberg. Body Weight Gain (BWG) and Feed conversion ratio (FCR) was also monitored during the study. Chickens were euthanized by cervical dislocation at 4d post- *Salmonella* challenge for sample collection.

Effect of CNP-Salmonella vaccine on anti-Salmonella IgA and IgG antibody titers. Serum samples were analyzed for anti-*Salmonella* OMPs-specific IgG antibody response using ELISA. Blood samples were collected at d0 and 6 (pre-challenge) and d13, 16, 18 (post-challenge). Blood samples were centrifuged at 5000xg for 10 min, and the serum-containing supernatants were collected and stored at -80°C until use. At the same time points, cloacal swabs were collected and then placed in 2 ml PBS and vortexed. The samples were then centrifuged at 3000xg for 10 min, and the supernatants were collected and stored at -80°C. Bile samples were collected at 4d post challenge from the gallbladder using a syringe and then were stored at -80°C.

High-binding- flat bottom 96-well plates (Greiner Bio-one, NC) were coated with pre-titrated amount of OMPs or Flagellin (2 µg/mL or 7.5 µg/mL for IgG or IgA ELISA, respectively) diluted in 0.05 M sodium carbonate-bicarbonate coating buffer, pH 9.6 and incubated overnight at 4˚C. Plates were washed three times with PBS containing Tween-20 (PBST) (0.05%) and blocked with 5% skim milk powder in 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 triplicates to the wells. For fecal supernatants, 50 µl of each undiluted sample were added in triplicates to the wells. Samples were then incubated for 2h at room temperature. Plates were washed three times in PBST, 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 50 µl/well of TMB peroxidase substrate were added to each well. The reaction was stopped after 6 min by adding 2M sulfuric acid. The OD was measured at 450 nm using spectrophotometry. The corrected OD was obtained by subtracting the treatment group OD from the blank control OD.

Effect of CNP–Salmonella vaccine on Salmonella population in the liver, spleen, and ceca post-Salmonella enterica serovar enteritidis or heidelberg challenge. The number of *Salmonella* in liver and spleen samples was determined by the Most Probable Number (MPN) method. Liver samples were homogenized in 4x volume of tryptic soy broth (TSB). Spleen samples were homogenized in 2x volume of TSB. Samples were prepared in 10-fold dilution series, and then 1mL of each dilution were inoculated into triplicate broth culture tubes for incubation. Following incubation, all tubes are examined for turbidity and the pattern of growth in the tubes was scored against an MPN table from the U.S. Food and Drug Administration's Bacterial Analytical Manual (Sutton 2010).

At 4d post-*Salmonella* challenge, whole ceca were collected following euthanasia and samples were stored at −80°C until DNA extraction. Cecum samples were homogenized, and DNA was extracted. Briefly, cecal contents (0.1 g) were diluted in 1 mL of 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 2 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, 5M NaCl and 100% isopropanol were added to the cell lysate and centrifuged at 18,000xg for 2 min. DNA pellets were then resuspended, washed in 70% ethanol, and then resuspended in 100 µl of TE buffer. The isolated DNA was dissolved in Tris-EDTA (pH 8.0) buffer, and the concentration was determined using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The *Salmonella* load of the ceca was analyzed by Real-Time PCR, using 16S ribosomal DNA primers and *Salmonella* specific DNA primers. Primers are shown in Table 3. For RT-PCR analysis, samples were diluted to a 100ng/µl final concentration. 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. To evaluate the relative proportion of bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100% (Shanmugasundaram *et al.* 2013).

Effect of CNP-Salmonella vaccine on IL-1β, IL-10, mRNA transcription in the cecal tonsils. Cecal tonsil samples were collected at 4d post-challenge. Total RNA was extracted by using TRIzol reagent (Invitrogen). The isolated RNA was 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. The mRNA transcripts of pro-inflammatory cytokine IL-1β, anti-inflammatory cytokine IL-10, and the house keeping gene GAPDH were analyzed by real-time PCR using the iQ™ SYBR® Green Supermix (Bio-Rad, CA). The threshold cycle (Ct) values were determined by iQ5 software (Bio-Rad, Hercules, CA) as

described earlier. Target gene transcripts were normalized to the RPS13 housekeeping gene. 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). Primers are described in Table 3.

Statistical Analysis. MPN-non-parametric data was transformed to Log10 for normal distribution. All data was examined by One-wayANOVA using JMP software (JMP, SAS Institute Inc., Cary, NC). When results were significant ($P < 0.05$) differences between means were analyzed using Tukey's Honest Significant Difference test.

RESULTS

Pre- and Post-Challenge Body Weight and Feed Conversion Ratio. There were no significant difference in BWG or FCR between any of the treatment groups pre or post-*S.* enteritidis or heidelberg challenge.

Salmonella heidelberg serovar-effect of CNP-Salmonella vaccine on Serum anti-Salmonella IgG antibody response. There were no significant results for anti-*Salmonella* specific-OMP IgG titers on serum samples from d6 for 1^{ry} immunization (P=0.3977) and d13 for booster (P=0.0711) (Fig. 14). However, when compared to the control group, the birds vaccinated with the commercial vaccine (CV) and chitosan nanoparticle (CNP) vaccine had higher levels (P=0.0365) of anti-*Salmonella* specific-OMP antibodies (by 253% and 173%, respectively). at 8h post-challenge (Fig. 14). No significant differences were observed for anti-*Salmonella* specific-OMP IgG antibody levels for 2d post-challenge (P=0.1268) or 4d post-challenge ($P = 0.0446$) (Fig. 15). There were no significant differences for anti-*Salmonella* specific-flagellin IgG antibodies at d6 for 1^{ry} immunization (P=0.0525) and d13 for booster (P=0.0525), where a numerical increase of antibody titers in response to the CNP booster was shown. There were no

significant results for serum IgG antibodies at any time point post SH challenge (8h post-challenge (*P*=0.4841); 2d post-challenge (*P*=0.1344); 4d post-challenge (*P*=0.1552)) (Fig. 16).

Salmonella heidelberg serovar-effect of CNP-Salmonella vaccine on cloacal swabs anti-Salmonella IgA antibody response. There were significant results for anti-*Salmonella* specific-OMP IgA antibody levels from cloacal swabs taken on d6 for the 1^{ry} immunization. A significant increase (*P*<.0001) of antibody titers for birds in the mock group was shown by 86.1% and 107% when compared to the CNP and CV treatment groups, respectively (Fig. 17). Conversely, no significant results (*P*=0.0786) were observed on d13 for the booster (Fig. 17). No significant results were observed for anti-*Salmonella* specific-OMP IgG antibody levels at 8h post-challenge (*P*=0.8201) (Fig. 18). There was a decrease in antibody levels at 2d post challenge (*P*=0.0005) for the CNP and CV treatment group by 65.1% and 71.2%, respectively, when compared to control (Fig. 18). Similar results were observed at 4d post-challenge (p<.0001) (Fig. 18) with CNP and CV vaccinated birds having reduce antibody levels by 71.8% and 66.63%, respectively, when compared to the unvaccinated birds. For anti-*Salmonella* flagellin-specific IgA antibody titers, there were no significant results (*P*>0.05) at d6 for the 1^{ry} immunization, d13 for the booster, 8h post-challenge, or 2d post-challenge. However, significant reduction in antibody levels was observed in the CV (64.36%) and CNP (51.2%) vaccinated birds 4d post-challenge (*P*=0.0255), when compared to the control (Fig. 19).

Salmonella heidelberg serovar-effect of CNP-Salmonella vaccine on bile anti- Salmonella IgA antibody response. A significant increase was observed for specific flagellin IgA antibody levels at 8h post-SH challenge (*P*= 0.0435) in birds vaccinated with the CV (6.36%),

similar to that of the CNP (4.59%), when compared to NV-control (Fig. 20). No significant results were observed for anti-*Salmonella* specific-OMP IgA antibody levels (*P*=0.1391) (Fig. 21).

Salmonella enteritidis serovar-effect of CNP-Salmonella vaccine on serum anti-Salmonella IgG antibody response. No significant differences (*P*>0.05) were observed for anti-*Salmonella* specific-OMP IgG levels at d6 for 1^{ry} immunization and d13 for booster (Fig. 22). However, significant antibody levels (*P*=0.0377) were obtained at 8h post-challenge in response to *S.* Enteritidis challenge. The Anti-*Salmonella* OMP-specific IgG antibody levels from the CV and the CNP treatment groups were higher by 253% and 173%, respectively, when compared to the NV-control (Fig. 22). Anti-*Salmonella* OMP IgG antibody levels were significantly decreased at 4d post-challenge (*P*=0.027) (Fig. 23). The CV (72.63%) and CNP (72.62%) vaccinated birds had significantly lower anti-*Salmonella* OMP IgG titers, when compared to control. No significant results (*P*>0.05) were obtained for anti-*Salmonella* flagellin-specific IgG titers (Fig. 24).

Salmonella enteritidis serovar-effect of CNP-Salmonella vaccine on cloacal swabs anti-Salmonella IgA antibody response. No significant differences were obtained for anti-*Salmonella* OMP-specific IgA antibody titers at d6 for 1^{ry} immunization (*P*=0.4676) and d13 for booster (*P*= 0.4584) (Fig. 25), and 8h post-challenge (*P*=0.8200) (Fig. 26). Statistically significant results were obtained at 2d post-challenge (P=0.0464), where CNP showed a significant decrease of 66.5% in antibody titers, similar to that of the CV treatment by 51.42%, when compared to control (Fig. 26). Similar results were observed at 4d post-challenge with the CV and CNP vaccinated birds having significantly decreased IgA levels (*P*=<.0001) by 65.13% and 59%, respectively, when compared to that of the control (Fig. 26). No significant results (*P*>0.05) were observed for anti-*Salmonella* flagellin-specific IgA antibody titers at any timepoint (Fig. 27).

Salmonella enteritidis serovar-effect of CNP-Salmonella vaccine on bile anti-Salmonella IgA antibody response. No significant results were obtained for anti- *Salmonella* flagellin-specific IgA titers (P=0.1524) (Fig. 28) or anti-*Salmonella* OMP-specific IgA titers (P=0.8935) (Fig. 29).

Effect of CNP–Salmonella vaccine on Salmonella population in the liver 4d post-Salmonella challenge. The MPN results are shown in Tables 4 and 5. No significant results were observed for *Salmonella* heidelberg in the liver at 4d post-challenge (P=0.5639) (Fig. 30). There was no bacterial colonization of the liver in response to *S.* enteritidis challenge.

Effect of CNP–Salmonella vaccine on Salmonella population in the spleen 4d post- Salmonella challenge. The MPN results are shown in Tables 6 and 7. No significant results were obtained for *Salmonella* heidelberg in the spleen at 4d post-challenge (P=0.4988) (Fig. 31). There was no bacterial colonization of the spleen in response to *S.* Enteritidis challenge.

Effect of CNP–Salmonella vaccine on Salmonella population in the ceca 2d Post-Salmonella enterica serovar enteritidis or heidelberg challenge. No significant results (P>0.05) were obtained for *Salmonella* heidelberg colonization of cecal content 2d post-challenge (Fig. 32). *Salmonella* enteritidis population on cecal content had a significant decrease (P=0.011) in birds vaccinated with CNP and CV, when compared to that of the unvaccinated control group (Fig. 33).

Effect of CNP-Salmonella vaccine on IL-1β, and IL-10 mRNA cecal tonsil transcription. No significant results (P>0.05) were obtained for cecal tonsil IL-1β mRNA transcription or IL-10 mRNA content at 4d post-*S.* heidelberg (Fig. 35, B; Fig. 34, B, respectively) and 4d post-*S.* enteritidis challenge (Fig. 35, A; Fig. 34, A, respectively).

DISCUSSION AND CONCLUSION

This study evaluated the protective effects of an oral chitosan-based *Salmonella* nanoparticle vaccine (CNP) on broilers birds challenged with 10^5 CFU of live *S.* enteritidis (SE) or *S.* heidelberg (SH). Oral administration of CNP vaccine resulted in no significant differences in the BWG and FCR between any of the treatment groups pre or post-SE or SH challenge. This result was consistent with previous studies using CNP to immunize layer hens (Renu *et al.* 2018), and a pilot trial using broilers (Acevedo *et al.* 2019). From this study it can be concluded that 1000µg CNP vaccine has no adverse effects on bird's performance parameters.

*Salmonella*e are susceptible to antibodies following primary invasion, when initially entering the circulation, and when transiting between phagocytes via the blood or extracellular fluids(MacLennan 2014). Thus, antibodies have a vital role in protection of host during an infection.

No significant results were obtained for any antibody titers after the primary immunization, instead a numerical increase was observed (Figs. 14-19; Figs. 22-27). Initial antigen exposure elicits an extrafollicular response that results in the rapid but low appearance of IgG antibody titers (Siegrist 2008). As B cells proliferate and differentiate into plasma cells, IgG antibody titers increase up to a peak value (Siegrist 2008). A secondary immune response given by a booster vaccine can reactivate immune memory and results in a rapid increase of IgG antibodies (Siegrist 2008, Abbas Abul K. *et al.* 2015). In this study, anti-*Salmonella* specific-OMP IgA antibody titers from cloacal swabs of birds treated with CNP and CV were significantly lower after 1^{ry} immunization, but once the booster was given the antibody levels were equivalent for all treatment groups (Fig. 17). A possible explanation for significantly higher antibody levels in the mock group at an early stage could be a) the transfer of maternal antibodies from hens to the chicks via the egg

(Hamal *et al.* 2006) or b) pre-existing *Salmonella* colonization from exposure to exogenous microbes either in the hatchery or farm environment (Russell 2013). It has been reported that within broiler hatcheries, 74% of pad samples placed under newly hatched chicks contained *Salmonella* spp. (Cox *et al.* 1990), and although hatcheries and farms take multiple preventive measures to decrease *Salmonella* emergence, there is currently no silver bullet for *Salmonella* mitigation. A numerical increase in antibody levels was observed on d13 in response to the vaccine booster (Figs. 14-19, and Figs. 22-27), indicating that CNP and CV boosters stimulated an additional immune response. Anti-*Salmonella* OMPs-specific IgG antibody levels were detectable in serum, pre- and post-SE or SH challenge. Broiler birds vaccinated orally with CNP or CV had higher ($P<0.05$) anti-*Salmonella* OMPs-specific serum IgG antibody titers at 8h post-challenge (Fig. 14; Fig. 22). The CNP vaccine increased antibody levels in a similar manner to that generated by the CV treatment group, both induced a substantial amount of antibodies in response to SH and SE challenge. Similar results were obtained for anti-*Salmonella* flagellin-specific IgA levels from bile in response to SH challenge at 8h post-challenge, where both CNP and CV vaccine provided significant ($P<0.05$) amounts of antibody levels at 8h post-challenge (Fig. 20). Results are in accordance to existing literature showing that re-exposure to the same antigen will reactive pre-existing memory cells leading to more memory cells, and therefore a quicker and greater response (Janeway 2012). Future research with CNP vaccine can explore the administration of a 2nd booster, depending on the number of antigens in the vaccine and its virulence, repeated doses might aid to ensure immunity is developed to provide adequate and long-lasting protection (Hamboursky 2015). As expected, both CNP vaccine and CV decreased ($P<0.05$) anti-*Salmonella* OMP-specific IgG titers in serum at either 2d post-SE challenge or 4d post-SH and SE challenge (Fig. 15; Fig. 23). Long-lived plasma cells that have reached survival

niches in the bone marrow continue to produce antigen-specific antibodies, which then decline over time (Siegrist 2008). This generic pattern may not apply to live vaccines triggering long-term IgG antibodies for extended periods(Siegrist 2008). However, killed *Salmonella* vaccines are preferred to live ones due to the ability of the live bacteria strain to regain its virulence(Lauring *et al.* 2010, Kollaritsch and Rendi-Wagner 2012, Renu *et al.* 2018b). In contrast, higher antibody titers in the non-vaccinated group at 2d and 4d post-SE or SH challenge can be explained by the assumption that persistently high antibody responses are indicative of a persistent infection (Hansen *et al.* 2006). This is because an immunized animal that has successfully cleared an infection follows a typical adaptive antibody response in which it will develop a high concentration of antibodies against the antigen and these will decline with time after each immunization (Abbas Abul K. *et al.* 2015). Carrier animals will usually maintain persistently high antibody levels in blood, as seen with S. dublin in cattle (Hansen *et al.* 2006) .

Overall, the results were consistent with other research regarding to chitosan nanoparticle vaccines as an effective vaccine delivery system. Copper-loaded chitosan nanoparticles (CNP-Cu) have been shown to enhance immunity in rats (Du 2008), and have also shown to increase the concentrations of immunoglobulins, complements, and lysozyme in serum; enhancing their immunological capacity (Wang *et al.* 2011a). In addition, a study with CNP- *Salmonella* vaccine indicated that the candidate oral *Salmone*lla NP vaccine had the potential to lessen salmonellosis in layer chickens (Renu *et al.* 2018). This was confirmed for broiler birds with our previous pilot trial (Acevedo *et al.* 2019). Results show CNP- *Salmonella* vaccine managed to substantially increase the quality of the humoral immune response from broiler birds infected with *S.* enteritidis or *S.* heidelberg; displaying potential to provide cross protection against these serovars.

Within a few hours after chickens are orally infected, *Salmonella* can invade the intestinal tract and reach internal organs such as the liver and spleen (Jones *et al.* 2016). Reducing SE and SH colonization in poultry may lead to a decrease in cases of human salmonellosis (Greig and Ravel 2009, Markazi 2018). Our study estimated the SH and SE population on liver and spleen using the most probable number methodology (MPN), and Real Time-PCR for cecal content. There was no SE colonization in liver and spleen (Tables 5 and 7, respectively). Real-Time PCR results showed that birds vaccinated with CNP and CV had a significant decrease on SE colonization in cecal contents at 2d post-challenge (Fig. 33). Results are consistent with previous findings with the same vaccine candidate against SE challenge in layer hens (Renu *et al.* 2018); hence, CNP also showed potential to mitigate SE population in cecal content of broiler birds. No significant differences were observed for SH colonization in the liver or spleen at 4d post-challenge (Fig. 30; Fig. 31, respectively). Similarly, Real Time-PCR results showed no significant differences for SH colonization in cecal content at 2d post-challenge (Fig. 32). However, estimates for SH population showed CNP and CV vaccination numerically decreased bacterial population in liver and spleen (Tables 4 and 6, respectively), as well as in cecal content (Fig. 32), when compared to control; thus, the vaccines' potential to reduce SH population in liver, spleen and ceca should be further explored.

Our current experiment utilized real-time PCR analysis to monitor pro- and anti-inflammatory cytokine response in cecal tonsils. According to literature the adjuvant composition can be one that induces an immune response predominantly of the Th1 type (10336-342WO1 2017). Other research studies have shown that the chitosan composition of the nanoparticle itself has a strong potential to elicit a balanced Th1/Th2 response (Wen *et al.* 2011). No significant

differences were obtained for either cytokines mRNA expression (Fig. 34, Fig. 35); hence, it can be concluded that the vaccine has no adverse effects to the bird's production parameters.

In conclusion, the efficacy of orally delivered chitosan-based nanoparticle vaccines was demonstrated against a *Salmonella* enteritidis and *Salmonella* heidelberg infection in broilers. CNP vaccine does not affect production performance or IL-1β and IL-10 cytokine levels in the cecal tonsils of vaccinated birds, showing its potential as a safe vaccine candidate. CNP vaccination predominantly induced antigen specific anti-*Salmonella* IgG and mucosal IgA response in broilers. In this study, chitosan nanoparticle vaccine has shown potential to mitigate cecal colonization of *Salmonella* enteritidis in broiler birds. Further research needs to be done to further explore the vaccines potential to mitigate SH population in liver and spleen. Results show that CNP vaccine is a potential vaccine candidate for oral delivery of antigens against *Salmonella* in poultry. This nano-vaccine can provide protection from *S.* enteritidis and *S.* heidelberg infection.

TABLES

Table 3. Real- time PCR primers for cytokine and bacterial analysis.

Target	**Primer**	**Sequence (5' – 3')**	**Reference**
GAPDH	F	TCCTGTGACTTCAATGGTGA	N/A
	R	CACAACACGGTTGCTGTATC	
IL-1β	F	TCCTCCAGCCAGAAAGTGA	(Shanmugasundaram *et al.* 2013)
	R	CAGGCGGTAGAAGATGAAGC	
IL-10	F	GAGGAGCAAAGCCATCAAGC	(Luoma 2016)
	R	CTCCTCATCAGCAGGTACTCC	
16S	F	AGAGTTTGATCCTGGCTCAG	(Luoma 2016)
	R	GACTACCAGGGTATCTAATC	
S. enterica	F	GCAGCGGTTACTATTGCAGC	(Luoma 2016)
	R	CTGTGACAGGGACATTTAGCG	

FIGURES

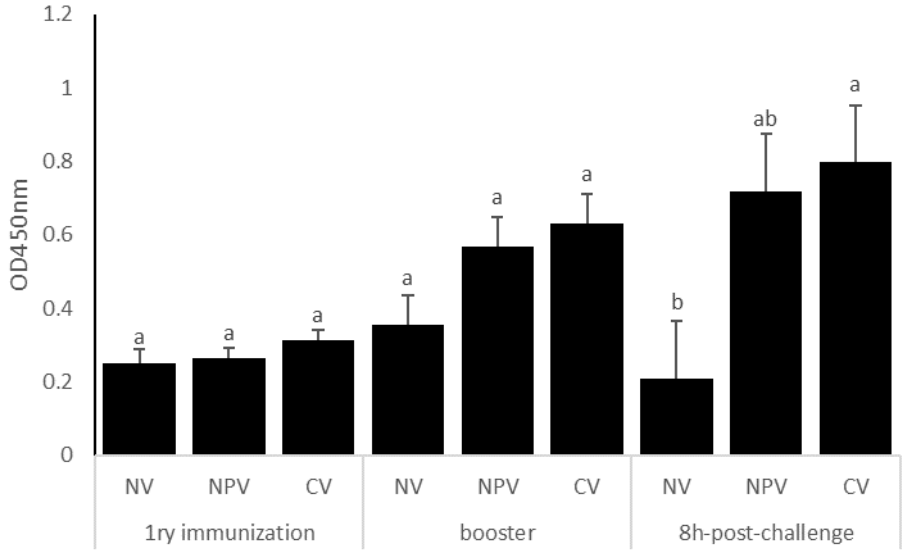


Figure 14. *Effect of Chitosan Nanoparticle (CNP)- Salmonella vaccine on anti- Salmonella IgG antibody titers pre- and post-Salmonella heidelberg challenge: Serum-OMP*. At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses were repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14 at 8h post *S.* challenge and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, $P=0.398$; booster, $P=0.071$; 8h post-challenge, $P=0.037$.

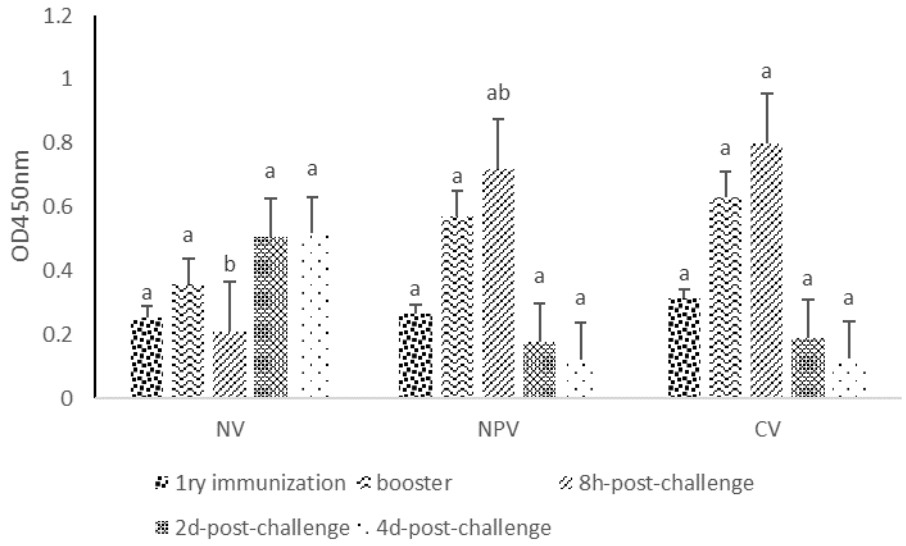


Figure 15. *Effect of Chitosan Nanoparticle (CNP)- Salmonella vaccine on anti- Salmonella IgG antibody titers pre- and post-Salmonella heidelberg challenge: Serum-OMP-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 1wk of age. At 2wk of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post *S.* challenge and were analyzed for anti- *Salmonella* OMP-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: 1^{ry} immunization, P=0.3977; booster, P= 0.0711, 8h post-challenge, P=0.0365; 2d post-challenge, P= 0.1268; 4d post-challenge, P= 0.0446.

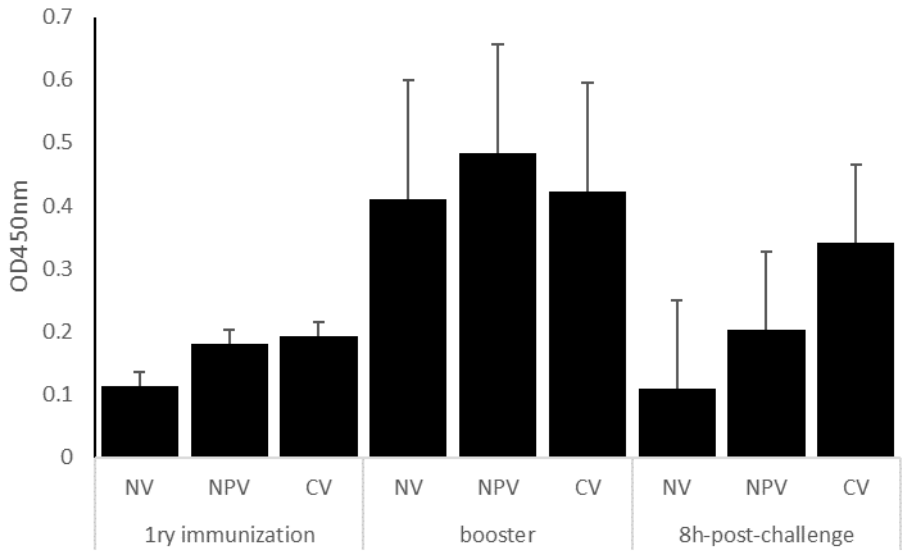


Figure 16. *Effect of Chitosan Nanoparticle (CNP)- Salmonella vaccine on anti- Salmonella IgG antibody titers pre- and post-Salmonella heidelberg challenge: Serum-Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post *S.* challenge and were analyzed for anti-*Salmonella* Flagellin-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: 1^{ry} immunization, $P=0.053$; booster, $P=0.054$; 8h post-challenge, $P=0.4841$.

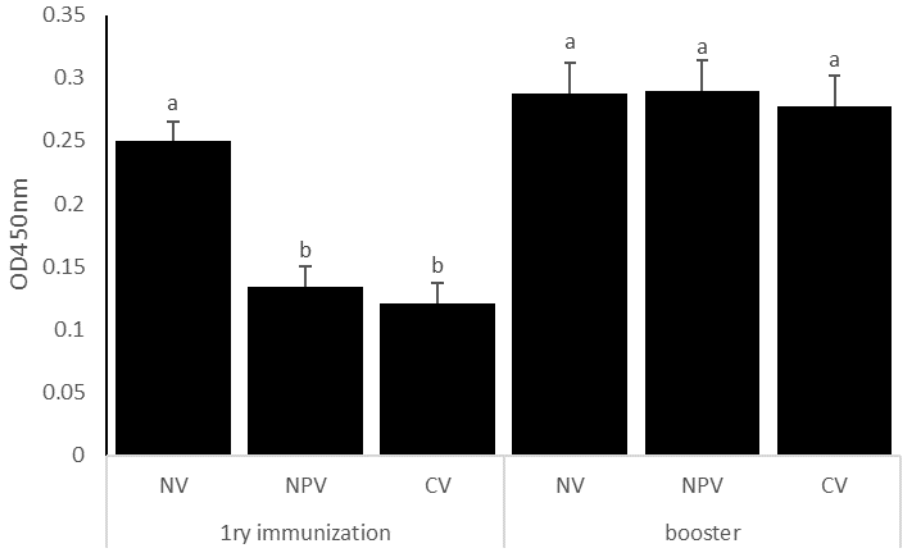


Figure 17. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti- Salmonella IgA antibody titers pre-Salmonella heidelberg challenge: Cloacal Swab-OMP.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Cloacal swabs were collected at d6, d13 pre-*Salmonella* challenge and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, P=<.0001; booster, P=0.0786.

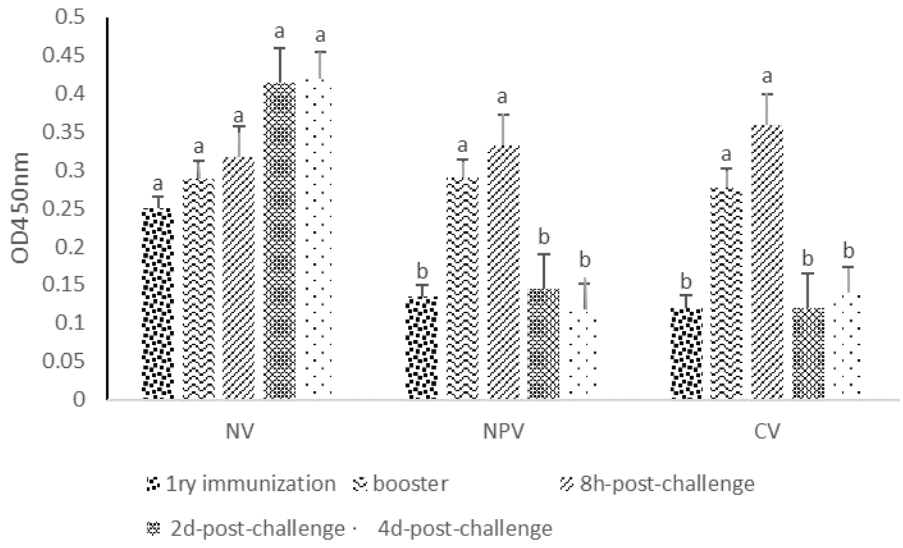


Figure 18. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre- and post-Salmonella heidelberg challenge: Cloacal Swab-OMP-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Cloacal swabs were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post-*S.* challenge, and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, P=<.0001; booster, P= 0.0786; 8h post-challenge, P= 0.8201; 2d post-challenge, P= 0.0005; 4d post-challenge, P=<.0001.

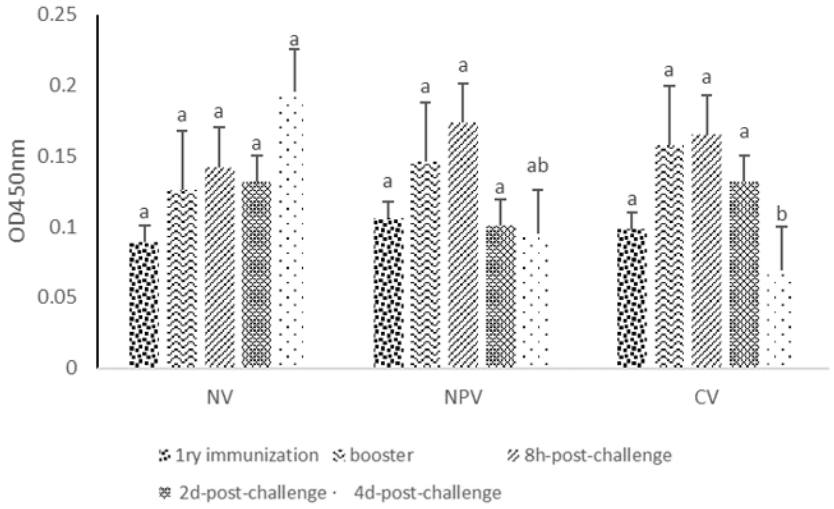


Figure 19. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre- and post-Salmonella heidelberg challenge: Cloacal Swab-Flagellin-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7dof age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Cloacal swabs were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post-*S.* challenge, and were analyzed for anti-*Salmonella* Flagellin-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: 1^{ry} immunization, P=0.6321; booster, P=0.8663; 8h post-challenge, P= 0.4025; 2d post-challenge, P= 0.1404; 4d post-challenge, P= 0.0255.

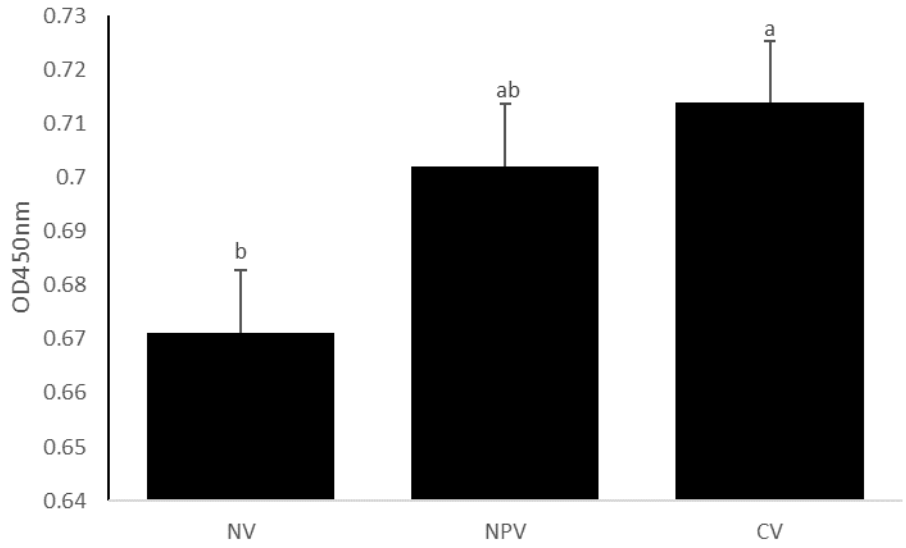


Figure 20. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers 4d post-Salmonella heidelberg challenge: Bile-Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Bile samples were collected at 8h post-*Salmonella* challenge and were analyzed for anti-*Salmonella* Flagellin-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: $P=0.0435$.

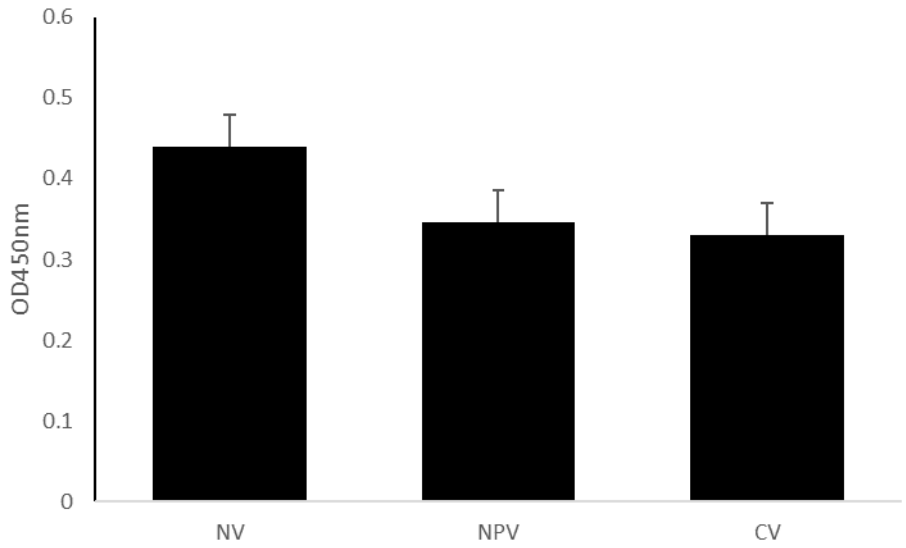


Figure 21. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers 4d post-Salmonella heidelberg challenge: Bile-OMP.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Bile samples were collected at 8h post-*Salmonella* challenge and were analyzed for anti-*Salmonella* OMP-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: $P = 0.1391$.

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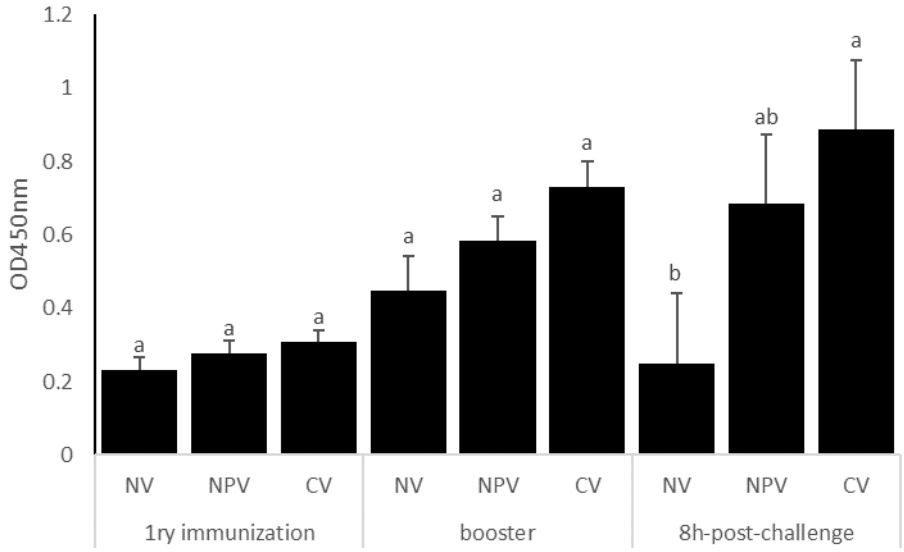


Figure 22. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgG antibody titers pre- and post-Salmonella enteritidis challenge: Serum-OMP*. At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14 at 8h post-*S.* challenge and were analyzed for anti-*Salmonella* OMP-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: 1st booster, $P = 0.3031$; 2nd booster, $P = 0.0817$; 8h post-challenge, $P = 0.0377$.

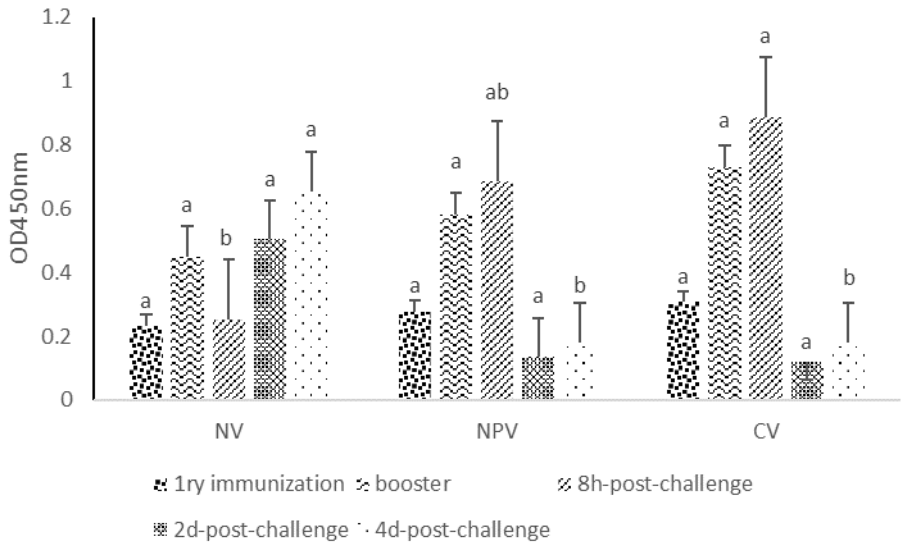


Figure 23. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgG antibody titers pre- and post-Salmonella enteritidis challenge: Serum-OMP-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post *S.* challenge and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, P=0.3031; booster, P=0.0817; 8h post-challenge, P=0.0377; 2d post-challenge, P= 0.1003; 4d post-challenge, P= 0.027.

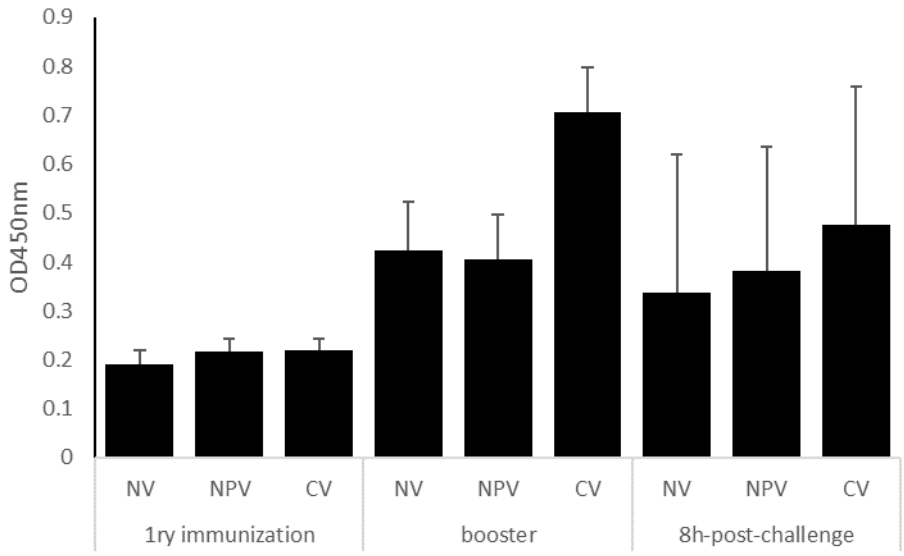


Figure 24. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgG antibody titers pre- and post-Salmonella enteritidis challenge: Serum-Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Blood samples were collected at d6, d13 pre-*Salmonella* challenge and d14 at 8h post-*S.* challenge and were analyzed for anti-*Salmonella* Flagellin-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: 1^{ry} immunization, $P = 0.7277$; booster, $P = 0.0685$; 8h post-challenge, $P = 0.9401$.

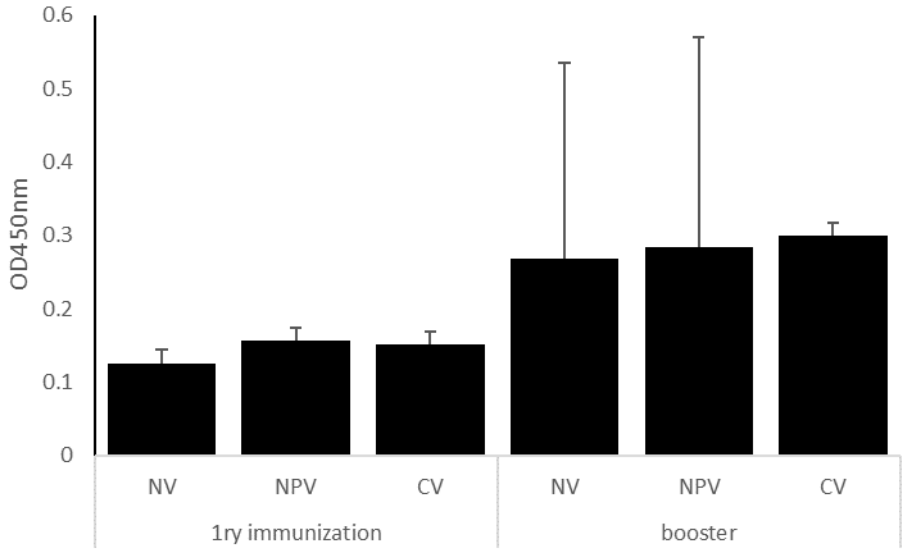


Figure 25. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre-Salmonella enteritidis challenge: Cloacal Swab-OMP.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar heidelberg (10^5 CFU/mL). Cloacal swabs were collected at d6, d13 pre-*Salmonella* challenge and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, $P = 0.4676$; booster, $P = 0.4584$.

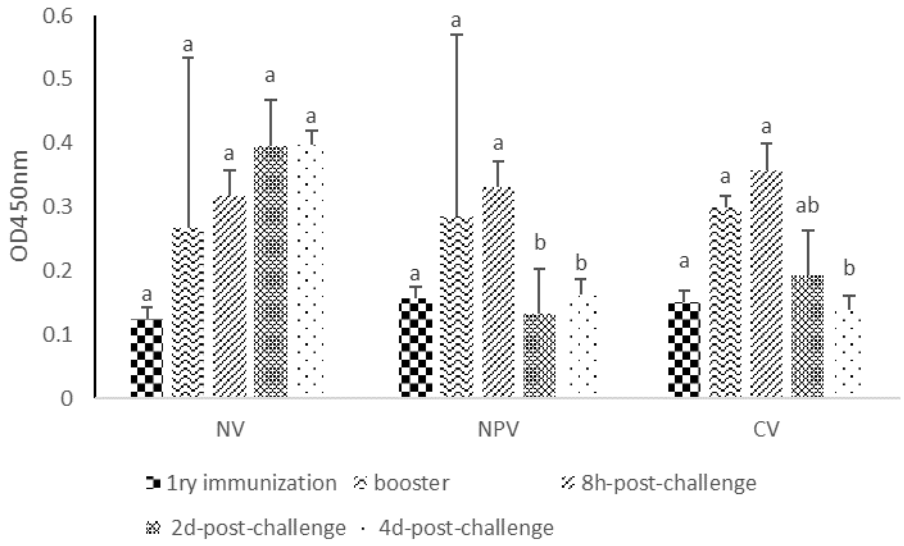


Figure 26. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre- and post-Salmonella enteritidis challenge: Cloacal Swab-OMP-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Cloacal swab samples were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post-*S.* challenge and were analyzed for anti-*Salmonella* OMP-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: 1^{ry} immunization, P=0.4676; booster, P=0.4584; 8h post-challenge, P=0.8200; 2d post-challenge, P=0.0464; 4d post-challenge, P=<.0001.

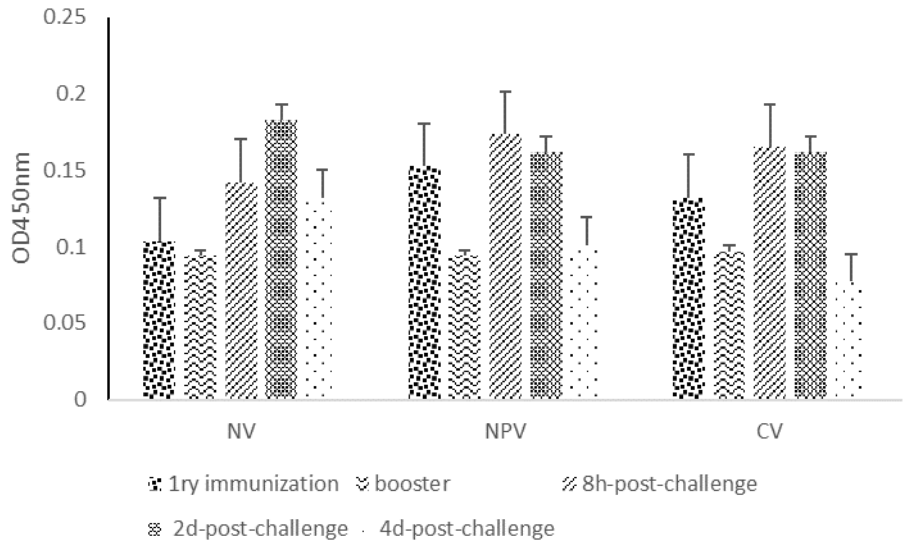


Figure 27. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers pre- and post-Salmonella enteritidis challenge: Cloacal Swab-Flagellin-All time points.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Cloacal swab samples were collected at d6, d13 pre-*Salmonella* challenge and d14, d16, d18 post-*S.* challenge and were analyzed for anti-*Salmonella* Flagellin-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: 1st booster, P=0.4899; 2nd booster, P=0.7976; 8h post-challenge, P=0.5389; 2d post-challenge, P=0.2654; 4d post-challenge, P= 0.1404.

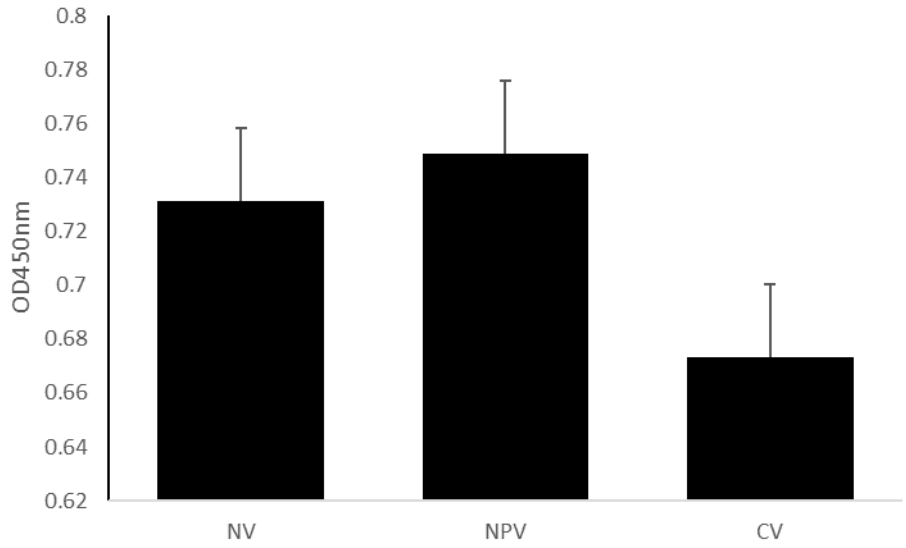


Figure 28. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody titers 4d post-Salmonella enteritidis challenge: Bile-Flagellin.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Bile samples were collected at 8h post-*Salmonella* challenge and were analyzed for anti-*Salmonella* Flagellin-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: $P=0.1524$.

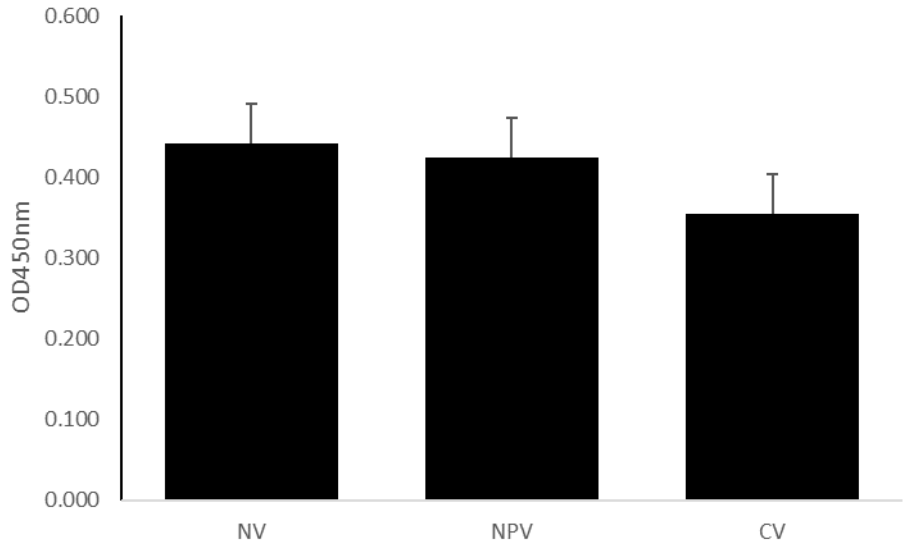


Figure 29. *Effect of Chitosan Nanoparticle (CNP)-Salmonella vaccine on anti-Salmonella IgA antibody Titers 4d post-Salmonella enteritidis challenge: Bile-OMP.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis (10^5 CFU/mL). Bile samples were collected at 8h post-*Salmonella* challenge and were analyzed for anti-*Salmonella* OMP-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: $P = 0.8935$.

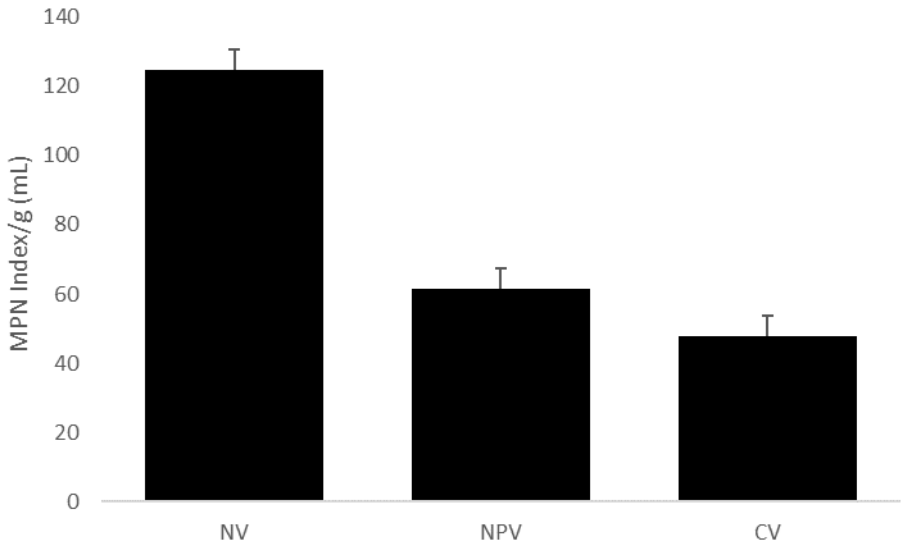


Figure 30. *Effect of CNP–Salmonella vaccine on Salmonella population in the liver 4d post-Salmonella enterica serovar heidelberg challenge.* The Most Probable Method (MPN) was used to estimate the *Salmonella* population on liver samples of broiler chickens orally inoculated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: $P = 0.5639$. No Vaccine (NV); Nanoparticle Vaccine (NPV); Commercial Vaccine (CV).

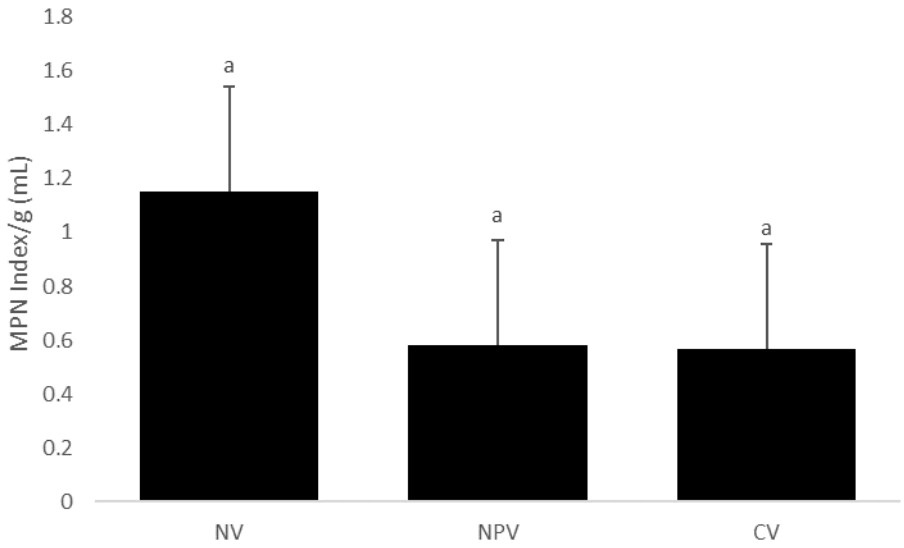


Figure 31. *Effect of CNP–Salmonella vaccine on Salmonella population in the spleen 4d post-Salmonella enterica serovar heidelberg challenge.* The Most Probable Method (MPN) was used to estimate the *Salmonella* population on liver samples of broiler chickens orally inoculated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Bars (+ SE) with no common superscript differ ($P \leq 0.05$). P values: $P = 0.4988$. No Vaccine (NV); Nanoparticle Vaccine (NPV); Commercial Vaccine (CV).

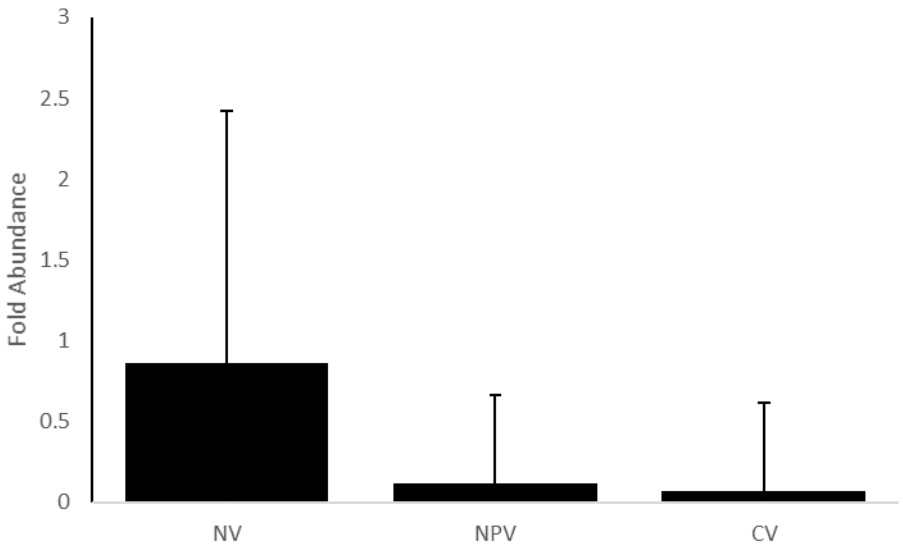


Figure 32. *Real-time PCR quantification of S. heidelberg on cecal content colonization 2d post challenge.* To evaluate the relative proportion of bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: $P=0.865$. No Vaccine (NV); Nanoparticle Vaccine (NPV); Commercial Vaccine (CV).

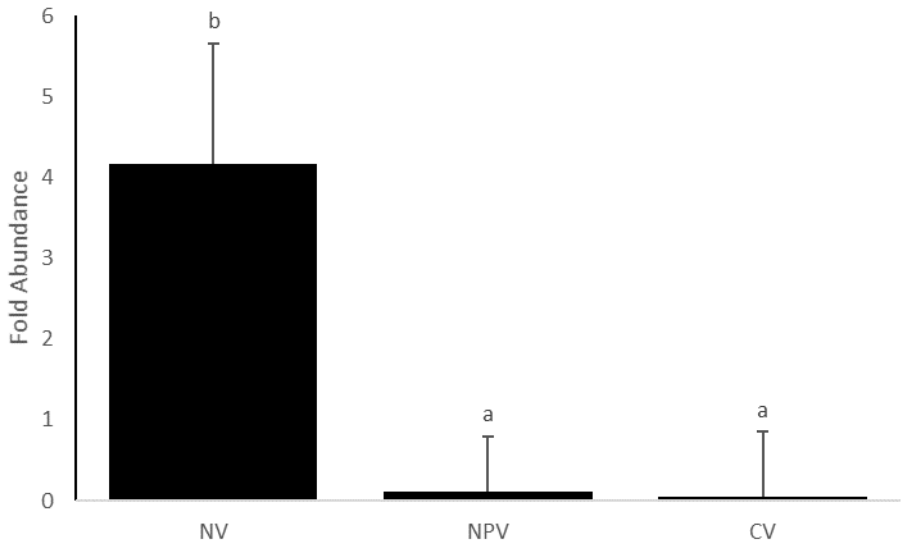
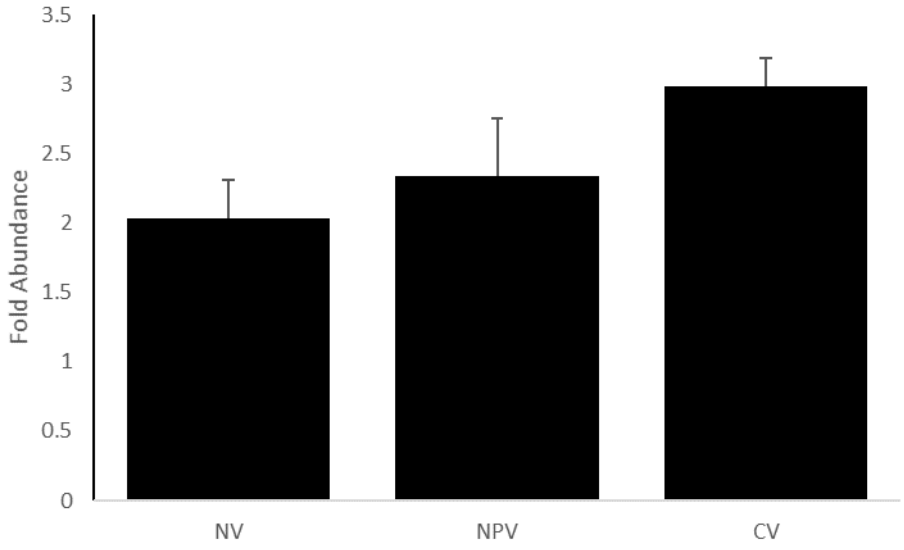


Figure 33. *Real-time PCR quantification of S. enteritidis on cecal content colonization 2d post challenge*. To evaluate the relative proportion of bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100%. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: $P=0.011$. No Vaccine (NV); Nanoparticle Vaccine (NPV); Commercial Vaccine (CV).

A.



B.

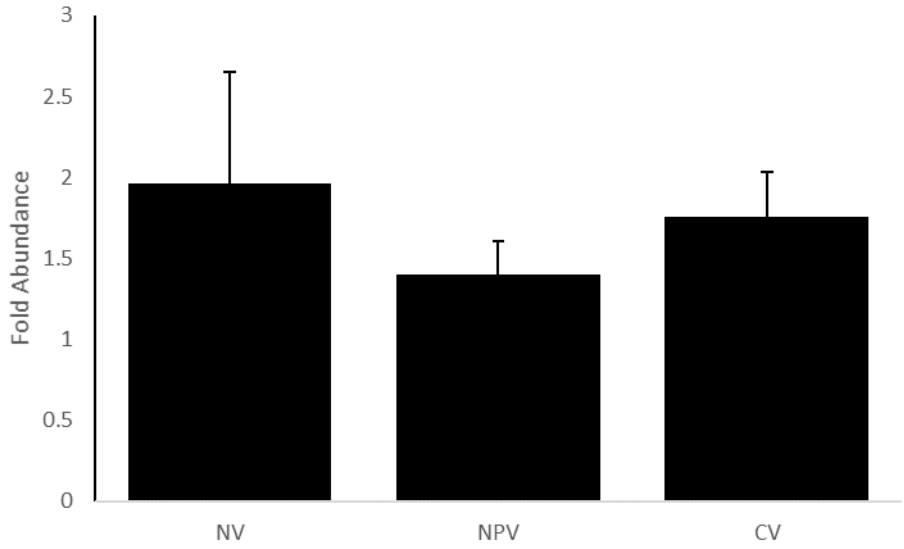
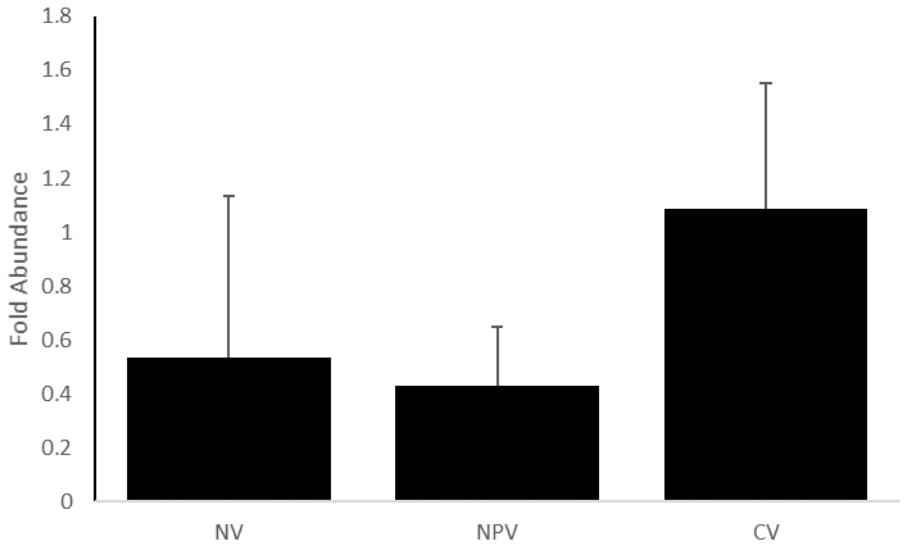


Figure 34. *Effect of CNP- Salmonella vaccine on IL-10 mRNA transcription in the cecal tonsils.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis or heidelberg (10^5 CFU/mL). Cecal tonsil samples were

collected at 4 d post-*Salmonella* challenge and analyzed for mRNA content after correcting for GAPDH mRNA content and normalizing to the mRNA content of the mock group. A – *S.* Enteritidis effect on IL10 mRNA content. B - *S.* Heidelberg effect on IL10 mRNA content. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P = 0.3016$; Panel B, $P = 0.6764$.

A.



B.

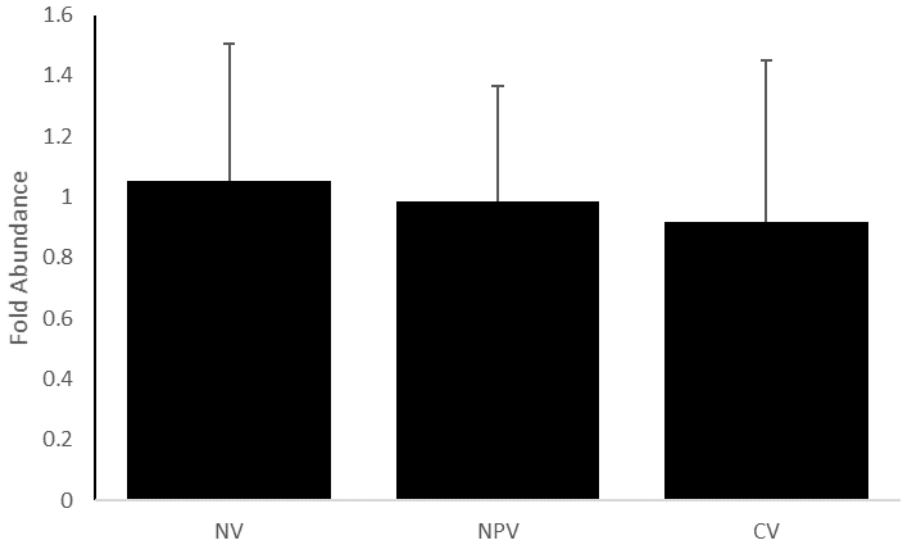


Figure 35. *Effect of CNP- Salmonella vaccine on IL-1 mRNA transcription in the cecal tonsils.* At 0d of age, chickens were orally vaccinated with either PBS (NV), 1000 µg OMPs + flagellin proteins loaded into CNP vaccine (NPV) or a live commercial vaccine (CV). Same route of delivery and doses was repeated at 7d of age. At 14d of age birds were challenged with live *Salmonella* enterica serovar enteritidis or heidelberg (10^5 CFU/mL). Cecal tonsil samples were

collected at 4 d post-*Salmonella* challenge and analyzed for mRNA content after correcting for GAPDH mRNA content and normalizing to the mRNA content of the mock group. A – *S.* Enteritidis effect on IL1 mRNA content. B - *S.* Heidelberg effect on IL1 mRNA content. Bars (SEM) with no common superscript differ ($P \leq 0.05$). P value: Panel A, $P = 0.1307$; Panel B, $P = 0.8753$.

SUPPLEMENTAL MATERIALS

Table 4. MPN table results for liver samples in response to *S.* heidelberg challenge

Sample	*100µl*	*10µl*	*1µl*	*colonies counted (10^{-5})*	*MPN*	*Log10*
NV	2	1	0	0	0	0
NV	3	0	2	63	64	1.8
NV	3	0	0	0	0	0
NV	2	2	1	0	0	0
NV	2	2	2	0	0	0
NV	3	0	1	37	38	1.6
CNP	1	2	1	13	15	1.2
CNP	2	0	0	0	0	0
CNP	1	0	0	0	0	0
CNP	1	1	0	0	0	0
CNP	2	0	2	0	0	0
CNP	3	0	0	0	0	0
CV	1	1	0	0	0	0
CV	0	1	0	0	0	0
CV	1	1	0	0	0	0
CV	1	0	0	0	0	0
CV	1	0	0	0	0	0
CV	2	0	2	20	20	1.3

No Vaccine (NV); Chitosan Nanoparticle (CNP); Commercial Vaccine (CV); Most Probable Number (MPN). Samples were bacterial growth was not seen were discarded as false positives and given an MPN of 0 value.

Table 5. MPN table results for liver samples in response to *S.* enteritidis challenge

Sample	*100µl*	*10µl*	*1µl*	*colonies counted (10^{-5})*	*MPN*
NV	3	0	0	0	0
NV	0	0	0	0	0
NV	0	0	1	0	0
NV	1	1	0	0	0
NV	0	0	0	0	0
NV	0	1	0	0	0
CNP	0	0	1	0	0
CNP	0	0	0	0	0
CNP	0	0	1	0	0
CNP	0	0	0	0	0
CNP	1	0	0	0	0
CNP	3	0	0	0	0
CV	0	0	1	0	0
CV	3	0	0	0	0
CV	0	0	0	0	0
CV	0	0	0	0	0
CV	1	0	0	0	0
CV	0	0	0	0	0

No Vaccine (NV); Chitosan Nanoparticle (CNP); Commercial Vaccine (CV); Most Probable Number (MPN). Samples were bacterial growth was not seen were discarded as false positives and given an MPN of 0 value.

Table 6. MPN table results for spleen samples in response to *S.* heidelberg challenge

Sample	*100µl*	*10µl*	*1µl*	*colonies counted (10^{-5})*	*MPN*	*Log10*
NV	3	3	1	0	0	0
NV	3	1	2	25	120	2.1
NV	3	1	1	4	75	1.9
NV	2	2	2	15	35	1.5
NV	2	2	2	0	0	0
NV	3	0	0	14	23	1.4
CNP	3	2	1	9	150	2.2
CNP	2	2	0	4	21	1.3
CNP	1	0	0	0	0	0
CNP	3	2	1	0	0	0
CNP	2	2	2	0	0	0
CNP	2	0	0	0	0	0
CV	1	1	1	2	11	1.0
CV	2	1	0	0	0	0
CV	1	1	0	0	0	0
CV	1	0	0	0	0	0
CV	3	3	0	12	240	2.4
CV	2	0	0	0	0	0

No Vaccine (NV); Chitosan Nanoparticle (CNP); Commercial Vaccine (CV); Most Probable Number (MPN). Samples were bacterial growth was not seen were discarded as false positives and given an MPN of 0 value.

Table 7. MPN table results for spleen samples in response to *S.* enteritis challenge

Sample	*100µl*	*10µl*	*1µl*	*colonies counted (10^{-5})*	*MPN*
NV	3	1	0	0	0
NV	0	0	0	0	0
NV	0	2	0	0	0
NV	0	1	0	0	0
NV	1	0	0	0	0
NV	0	1	0	0	0
CNP	1	1	1	0	0
CNP	1	0	0	0	0
CNP	0	2	0	0	0
CNP	0	0	0	0	0
CNP	1	0	0	0	0
CNP	1	1	0	0	0
CV	1	0	0	0	0
CV	0	1	0	0	0
CV	0	0	0	0	0
CV	0	0	0	0	0
CV	0	0	0	0	0
CV	1	1	1	0	0

No Vaccine (NV); Chitosan Nanoparticle (CNP); Commercial Vaccine (CV); Most Probable Number (MPN). Samples were bacterial growth was not seen were discarded as false positives and given an MPN of 0 value.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Salmonella were named after the pathologist Salmon over a century ago (Crum-Cianflone 2008). It was first described in farm animals, but soon after *Salmonella* was recognized as a spectrum of disease-causing pathogen among humans. This intracellular bacterium is the primary etiologic agent of salmonellosis, a zoonotic infection in humans (Pires *et al.* 2014). Salmonellosis symptoms include stomach irritation accompanied by vomiting, diarrhea, and high fever (Crum-Cianflone 2008). This disease can often lead to death in humans of an immunocompromised state, especially those who are very young or old (Crum-Cianflone 2008). Approximately 1.3 billion cases of human gastroenteritis due to *Salmonella* are reported annually worldwide, resulting in 3 million deaths (Gong *et al.* 2014). In the United States of America (USA), *Salmonella* is the leading foodborne pathogen, causing the largest number of deaths and has the highest cost burden (Batz *et al.* 2012). From the past decade, the greatest numbers of *Salmonella* outbreaks are related to land animals, with more than 70% of human salmonellosis cases in the USA attributed to the consumption of contaminated chicken or eggs (Braden 2006, Pires *et al.* 2014, Andino and Hanning 2015). The increasing status of *Salmonella* infections is a result of continuous outbreaks and the evolution of multi-resistant *Salmonella* strains (Crum-Cianflone 2008). *Salmonella* enteritidis, *Salmonella* typhimurium and *Salmonella* heidelberg are the three most frequent serotypes recovered from humans each year (Gong *et al.* 2014).

Salmonella spp. possesses effective acid tolerance mechanisms and upon ingestion it will survive passage through the low-pH conditions of the stomach (Hallstrom and McCormick 2011, Higginson *et al.* 2016), stimulate macrophages, evade killing by the host immune system (Pilonieta

et al. 2014), and potentially transition to a systemic infection (Acheson and Hohmann 2001). Previous studies have shown that *S.* enteritidis can suppress nitric oxide (NO) production in infected chicken macrophage HD11 cells, while dead *S.* enteritidis stimulates a high level of NO production(He *et al.* 2013); compromising the cellular and downstream humoral immunity of the host. In addition, *Salmonella*e are susceptible to antibodies following primary invasion, when initially entering the circulation, and when transiting between phagocytes via the blood or extracellular fluids (MacLennan 2014). Thus, antibodies have a vital role in killing this extracellular pathogen and the clearance of *Salmonella* in poultry requires a strong humoral and cell-mediated immune responses (Van Immerseel *et al.* 2002, Raybourne *et al.* 2003, Neto *et al.* 2008, Markazi 2018). Reducing *S.* enteritidis colonization in poultry may lead to a decrease of its transfer to humans, resulting in fewer cases of salmonellosis(Greig and Ravel 2009, Markazi 2018).

Vaccines early protective efficacy is primarily conferred by the induction of antigen-specific antibodies (Siegrist 2008). Live attenuated vaccines produce both a strong humoral and cell-mediated response (Lalsiamthara *et al.* 2016). Still, killed *Salmonella* vaccines are preferred as opposed to live ones due to the ability of the live strain to regain its virulence (Lauring *et al.* 2010, Kollaritsch and Rendi-Wagner 2012, Renu *et al.* 2018b). In addition, the route of administration for commercially available *Salmonella* killed vaccines in poultry poses a high disadvantage as they are injected manually in the breast muscle, which is time consuming, impractical for big poultry flocks, and decreases breast meat quality. Conversely, oral administration is typically referred to as the "ideal route" (Revolledo and Ferreira 2012, Gong *et al.* 2014). This is because it mimics natural infection, stimulates the mucosal and systemic immune responses, and decreases the vaccination cost factor (Revolledo and Ferreira 2012). However, there

are no current oral killed vaccines commercially available for broilers due to the challenging acidic nature of the gastrointestinal tract (GIT) (Shaji and Patole 2008, Vela Ramirez *et al.* 2017, Renu *et al.* 2018b). Instead, oral protein and peptide delivery carrier systems, such as nanoparticles, have been heavily explored over the past few decades to overcome this problem(Shaji and Patole 2008, Salman *et al.* 2009a, Renu *et al.* 2018b).

Nanoparticle vaccines consist of a polymer coating that surrounds the vaccine antigen (Zhao *et al.* 2014) and protects the vaccine against chemical, enzymatic or immunological degradation(Tiwari *et al.* 2012, Sahdev *et al.* 2014, Zhao *et al.* 2014). The prolonged survivability of the vaccine within the GIT results in reducing the dosing frequency and the need for adjuvants, as they can act as adjuvants themselves (Tiwari *et al.* 2012), and also facilitating the presentation of the vaccine antigens to specific immune sites of the mucosal immune system(Cheung *et al.* 2015). In addition, ligands can be conjugated to the surface of the nanoparticle to increase the presentation of the nanoparticle vaccine to a specific site within the GIT (Salman *et al.* 2009a, Renu *et al.* 2018b). As a result, nanoparticles are advantageous for use as an oral vaccine, with an easier administration and a more effective local and intestinal immune responses.

Chitosan is a natural biodegradable copolymer derived from the partial deacetylation of chitin (Cheung *et al.* 2015). Previous research indicates that biodegradable chitosan nanoparticle (CNP) vaccines have ideal traits for delivering vaccine antigen loads orally (Wang *et al.* 2011b, Binnebose *et al.* 2015, Cheung *et al.* 2015). The amino and carboxyl groups in the chitosan molecule can be combined with glycoprotein in mucus to form a hydrogen bond, leading to an adhesive effect (Wang *et al.* 2011b, Cheung *et al.* 2015, Prajakta K.Khobragade 2015, Mohammed *et al.* 2017). This enables it to be internalized by M cells (Hallstrom and McCormick 2011) in the intestinal Peyer's patch and presented to underlying APCs for efficient uptake, processing and

presentation of vaccine antigens. It has been demonstrated that Copper-loaded chitosan nanoparticles (CNP-Cu) can enhance immunity in rats (Du 2008, Wang *et al.* 2011a), and have also shown to improve growth performance in broiler birds, increase the concentrations of immunoglobulins complements and lysozyme in serum; thus enhancing their immunological capacity (Wang *et al.* 2011a).

This project examined the protective effects of a targeted mucoadhesive chitosan-based *Salmonella* nano-vaccine for oral delivery in poultry. This subunit nanoparticle vaccine was engineered containing the immunogenic outer membrane proteins (OMPs), flagellar (F) protein of *Salmonella*, and surface decorated with F-protein of *Salmonella* enterica serovar Enteritis (Fig. 36; Fig. 37; Fig. 38 A, B; Fig. 39 A, B). The vaccine was initially tested using layer chickens, which showed significantly higher OMPs- specific mucosal antibody response. The study demonstrated the capability of this nano-vaccine to target ileal Peyer's patches and induce specific local intestinal immunity by *ex vivo* and *in vivo* studies (Renu *et al.* 2018). All results indicated that the candidate oral *Salmonella* nanoparticle vaccine has the potential to lessen salmonellosis in poultry. Based on previous pilot trials, we hypothesize that the oral delivery of this chitosan nanoparticle will induce anti-*Salmonella* IgG and IgA in serum, fecal swabs and bile, and decrease *Salmonella* shedding/load in broiler birds. Other parameters were also monitored to determine the immunological effects of our vaccine.

Chapter 3 analyzed the protective effects of an oral chitosan-based *Salmonella* nanoparticle vaccine (CNP) loaded with *Salmonella* outer membrane proteins (OMPs) and flagellin proteins. Results demonstrated that the chitosan nanoparticle (CNP) vaccine has no adverse effects on bird's production performance. The 1000µg CNP *Salmonella* vaccine dose showed optimum protection from *S.* enteritis with the release of substantial and moderate NO levels to prevent any potential

NO-dependent tissue injury in broiler birds. More importantly, broiler birds vaccinated orally with 1000µg CNPs showed substantially higher anti-*Salmonella* OMPs-specific serum IgG and IgA titers on serum. Similar results were observed for cloacal anti-*Salmonella* OMP IgA titers in the 1000µg treatment group higher. At 25d-of-age, birds that were vaccinated with 1000µg CNPs had significantly higher bile anti-*Salmonella* OMPs and flagellin-specific IgA titers. Results showed that CNP vaccine has the potential to decrease *Salmonella* Enteritidis population on cecal content as displayed by a numerical decrease of the bacterial population in birds treated 1000µg CNP vaccine dose, when compared to control. Results showed that the CNP 1000µg vaccine dose was the only one that induced substantial IL-10 mRNA levels. However, all CNP vaccine doses induced protective Th1 and Th2 cytokine mRNA expression levels. In conclusion, this pilot study demonstrated that the CNP vaccine has no adverse effects on bird's immunological health, and that vaccinating birds with 1000 µg of CNP can provide optimal protection from *S.* enteritidis infection.

Chapter 4 further explored the protective effects our 1000µg CNP vaccine dose, selected as a result of the prior study. This study tested the potential of our oral chitosan-based *Salmonella* nanoparticle vaccine (CNP) in protecting broiler birds against live *S.* enteritidis (SE) or *S.* heidelberg (SH) oral challenge. Results showed that the 1000µg CNP vaccine dose has no adverse effects on bird's production performance parameters. Broiler birds vaccinated orally with CNP or CV showed significantly higher anti-*Salmonella* OMPs-specific serum IgG titers at 8h-post-challenge in response to SH and SE challenge. CNP and CV numerically increased antibody titers in response to booster, showing potential to induce a protective immune response against SE and SH. At 4d post-SH challenge, birds that were vaccinated with CNP and CV had significantly higher bile anti-*Salmonella* flagellin-specific IgA titers. Both, CNP vaccine and CV substantially

decreased anti-*Salmonella* OMP-specific IgG titers on serum at either 2d-post-SE challenge or 4d-post-SH and SE challenge. These showed the vaccines potential to induce a humoral immune response, as a "typical adaptative response" involves the development of a high concentration of antibodies against the antigen which will decline with time after each immunization (Abbas Abul K. *et al.* 2015). In this study, CNP vaccine displayed potential to mitigate cecal colonization of *Salmonella* enteritidis in broiler birds. Estimates for SH population in liver and spleen showed CNP and CV numerically decreased bacterial population when compared to control. Further research needs to be done to further explore the vaccines capacity to lessen SH population in liver and spleen. The CNP vaccine had no effect on cecal tonsils IL-1 and IL-10 cytokine levels; hence it does not affect bird's health status. In conclusion, results demonstrate that CNP can induce a humoral immune response, similar to that of the CV, against SH and SE infections. It can be concluded that vaccinating birds with 1000 µg of CNP can provide protection from SE and SH infections.

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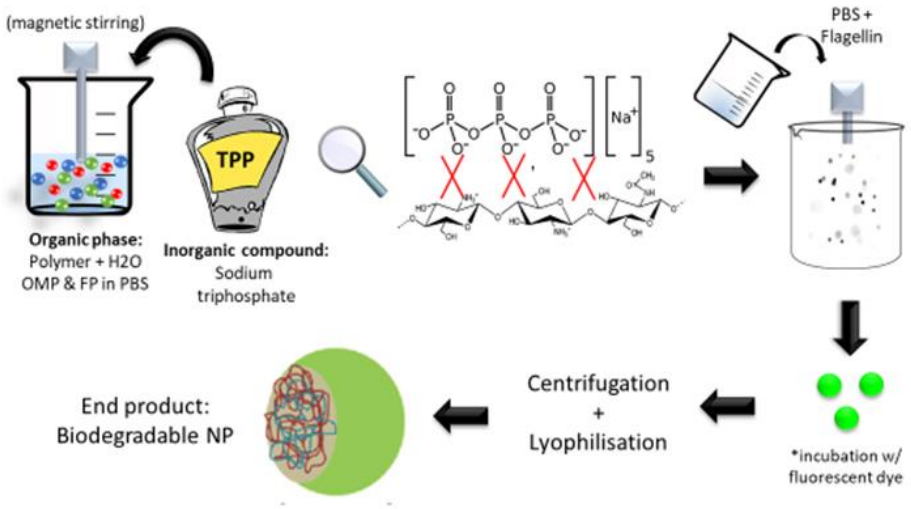


Fig 36. *Diagram for synthesis of Chitosan Nanoparticle-based vaccine for Salmonella by ionic gelation method.* **In depth details of CNP vaccine are described in its patent by corresponding authors (10336-342WO1 2017):** Briefly, nanoparticles were formed by intra- and inter-molecular crosslinking between positively charged chitosan and negatively charged sodium tripolyphosphate (TPP). The 1% (w/v) low molecular weight chitosan (Sigma, MO) solution was prepared by slowly dissolving chitosan in an aqueous solution of 4% acetic acid under magnetic stirring until the 30 solution became transparent. The solution was sonicated, and the pH was adjusted to 4.3 and filtered through a 0.44 μm syringe filter. To prepare chitosan nanoparticles, 5 mL of 1% chitosan solution was added to 5 mL of deionized water and incubated with 2.5 mg each of both OMPs and flagellar in 1 mL of PBS pH 7.4. Subsequently, 2.5 mL of 1% (w/v) TPP (Sigma, MO) in 2.5 mL deionized water was added into the solution and subjected to magnetic stirring at room temperature. For surface conjugation, 2.5 mg of flagellar protein in PBS was added to the particles and the electrostatic interaction helps in surface labeling of flagellar on CNPs and centrifuged at 10,000 rpm for 10 min to collect final NPs.

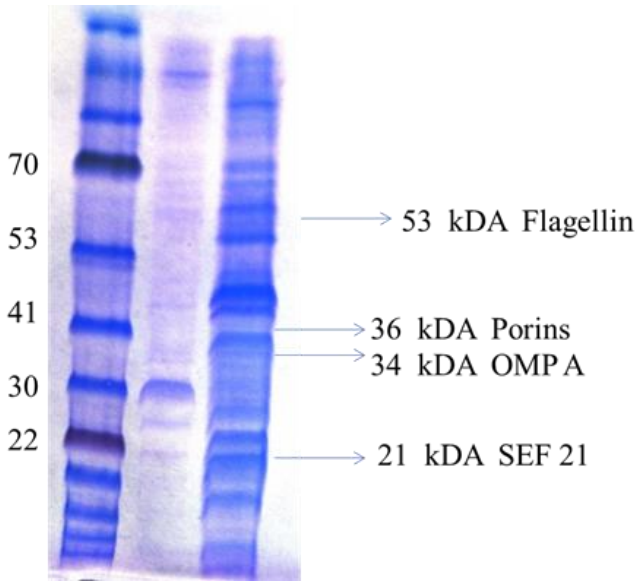
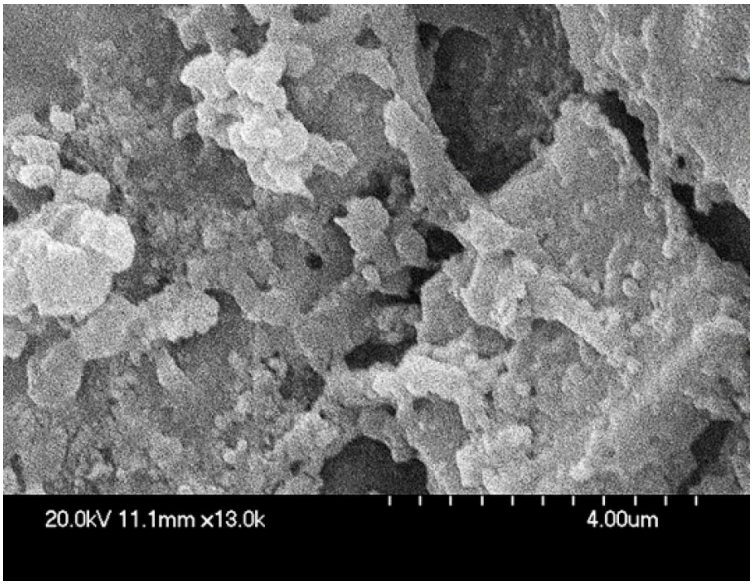


Fig 37. *SDS- PAGE analyses - Lane 1: Standard protein marker; Lane 2: Flagellar; Lane 3: OMPs (position of some known proteins bands are shown by arrows).* **In depth details of CNP vaccine are described in its patent by corresponding authors (10336-342WO1 2017):** Briefly, SDS-PAGE analysis of isolated OMPs revealed a complex electrophoretic profile containing greater than 12 different proteins ranging from 14 to 70 kDa. In some embodiments, the OMP protein is an OMP protein extract from *S.* enteritidis comprising at least one protein selected from the major well characterized antigenic proteins are having the molecular weight 22, 23, 28, 34, 36, 45, 46, 55, 65, 68 and 70 kDa, as shown in Figure 36 (lane 3). In some embodiments, the flagellar protein (flagellin) comprises at least one protein selected from FlgD 28 kDa, FlgL 35 kDa, FlgE 42 kDa, FlgD 50 kDa, FlgK 58 kDa and/or the surface appendages protein SEF21 21 kDa, as shown in Figure 36 (lane 2).

A.



B.

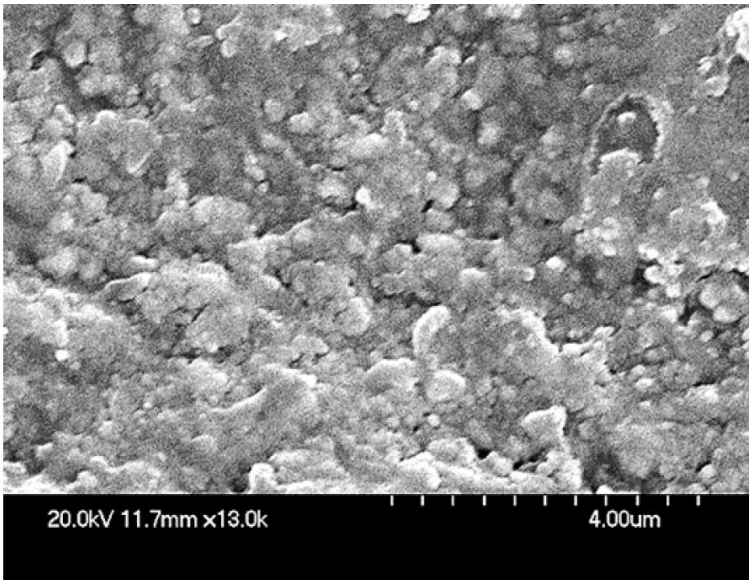
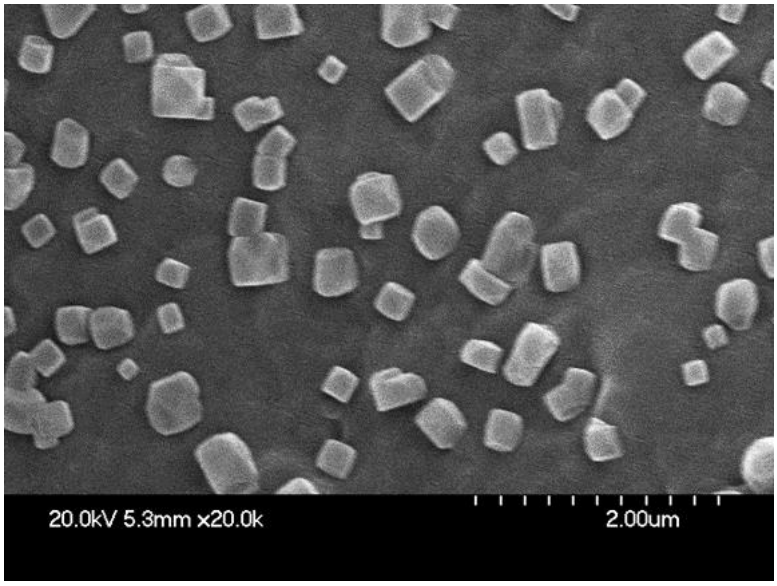


Figure 38. *Confirmation of S. enteritidis extracted proteins. SEM analysis of OMPs (Fig. 37, A) and Flagellar protein (Fig. 37, B), (Scale bar: 13 Kx and 4 um).* Both OMPs and flagellin/flagellar protein can be in spherical and irregular shapes and aggregated in the form of a matrix.

A.



B.

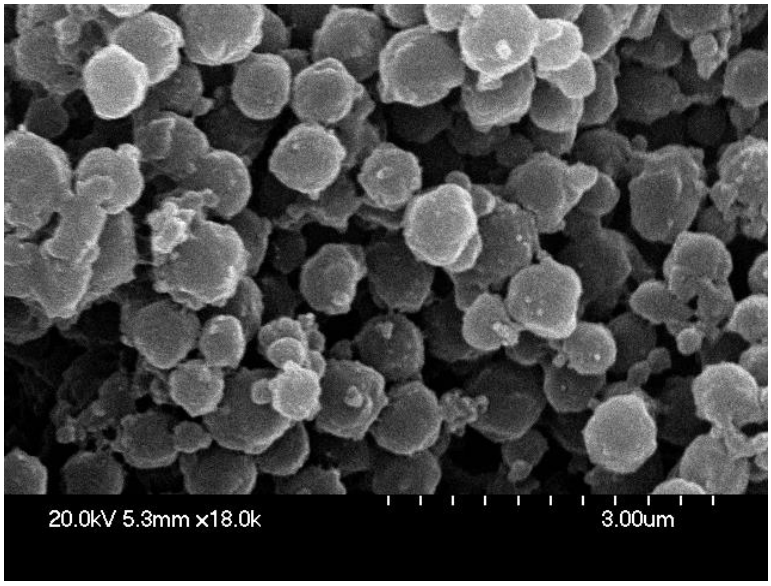


Fig 39. *Physicochemical characterization of chitosan nanoparticles (CNPs). SEM analysis of empty CNPs (Fig. 38, A) and loaded CNPs (Fig. 38, B).* Loaded CNPs displayed a spherical in shape and were evenly distributed in the colloidal matrix without any aggregation (10336-342WO1 2017).