THE EFFECTS OF FEED ADDITIVES, HOUSING SYSTEMS AND STRESS ON *SALMONELLA* SHEDDING IN SINGLE COMB WHITE AND BROWN LAYING HENS

by

Dana L. Hahn

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Animal Science

Under the Supervision of Professor Sheila E. Purdum

Lincoln, Nebraska

May 2014

THE EFFECTS OF FEED ADDITIVES, HOUSING SYSTEMS AND STRESS ON *SALMONELLA* SHEDDING IN SINGLE COMB WHITE AND BROWN LAYING HENS

Dana Leigh Hahn, Ph.D.

University of Nebraska, 2014

Adviser: Sheila E. Purdum

A series of studies were conducted examining feed additives, housing systems and stress on *Salmonella* shedding. Alternative feed additives such as prebiotics, probiotics and essential oils have been shown to reduce pathogenic bacteria colonization. Furthermore, stressors such as movement have been shown to increase *Salmonella* shedding. The goal of the studies was to examine if alternative ingredients reduce *Salmonella* shedding in alternative housing systems and through movement stress. Study 1 examined cage and cage-free housing with mannan oligosaccharide (MOS) supplementation. Treatments were arranged in a 2x2 factorial design: cage or cage free; MOS (0% or 0.08%). There was no effect on housing system or MOS for *Salmonella*. *E. coli* fecal counts increased at 73 wks of age for MOS diets. *E. coli* and coliforms were three times more likely to be found on eggshells from cage free pens then cage. MOS reduced *E. coli* colonization in duodenum. Study 2 examined the effect of transportation stress at 16 wks of age on *S. enteritidis* (SE) shedding through peak lay (33 wks). Incidence of SE positive increased leading up to peak lay. Study 3 examined *Salmonella* vaccination, movement stress and feed additives in laying hens (43-50 wks of age). Treatments were arranged in a 3x2 factorial design: vaccination (yes or no), feed additive (control, 0.03% MOS or 0.15% synergistic). Feed additives did not have a significant

effect on production parameters or *Salmonella*. Vaccinated hens fed MOS had the highest egg wt. Study 4 examined feed additives on pullets (1 day-22 wks), gut microbiome and SE prevalence (12-22 wks of age). Six treatments were arranged in a completely randomized design: control, 0.01% $1x10^{10}$ *P. acidilactici* , 0.01% $2x10^{10}$ live *S. cerevisiae boulardii*, 0.1% MOS, .01% $1x10^{10}$ *P. acidilactici*+ 0.1% MOS, 0.01% $2x10^{10}$ live *S. cerevisiae boulardii* + 0.1% MOS. Treatments did not have an impact on *Salmonella* fecal counts, *E. coli*, coliform fecal and ceca counts or *Enterobacteriacea* fecal counts. No *Salmonella* was found in the ceca. All treatments saw a decrease in *Enterobacteriacea* except for MOS. Current vaccination programs are reducing the risk for *Salmonella*.

Keywords: Laying hens, *Salmonella*, prebiotic, probiotic, MOS

DEDICATION

This dissertation is dedicated to my loving and supportive family and husband. Without you, none of this would be possible.

AKNOWLEDGEMENTS

There are many people that I need to thank, first of which are the members of my committee. I have thoroughly enjoyed learning from all of you.

Dr. Sheila Purdum, working with you these past 5 years has been quite a journey. It is very difficult to put into words my gratitude to you, but I will try. I've grown leaps and bounds under your patient and watchful eye. You taught me so many lessons and instilled confidence in me that I will never forget. Thank you for allowing me to learn and grow, thank you for seeing something in me that I didn't realize I had. You have been a wonderful mentor, I'm forever greatful for this experience.

Dr. Kathy Hanford, thank you for working with me these past 5 years. You taught me so much, words cannot express my gratitude towards you. Thank you for serving not only on my M.S. committee, but also on my Ph.D. committee.

Dr. Samodha Fernando, thank you for your guidance and expertise while allowing me to learn from you and members in your lab. I'm truly thankful for the experience.

Dr. Julie Albrecht, thank you for serving on this committee. You have offered a unique persepective and I have learned a great amount from you.

Thank you to the University poutry group, Brett Kriefles, Pamela Eusebio, Anna Stiener and Leo Sweet for all of your support. Special thank you to David Giraud, Chris Anderson, and Nirosh Aluthge for all of your assistance with lab work and answering all of my questions.

I had the unique opportunity to leave early and work for Smart Chicken. Many thanks go to Bill Schellpeper for taking a chance on me while also allowing me to finish my degree. The entire live operations team: Mandy Wood, Trish Weber, Chris Thompson, Bertus Byleveld, Derek Kraayenbrink, Dave Smith and Steve Helser, thank you for your support.

This section would not be complete without giving thanks to my close circle of friends I made here: Adria Bhatnager, Katie Lucot, and Kim Varnold. You all were my anchor through this crazy journey called grad school. I cannot put into words how much your support ment to me, so a simple thank you will have to do.

I would like to extend my deepest gratitude to my parents, Mark and Sue Hahn, Sister, Laura Hahn and my newest extended family: Michael and Debra Didde and Sarah Didde. You all offered so much support throughout this whole process, even if you didn't completely understand what I was doing, you offered unconditional and unwavering support, for that, thank you. To all of my close friends down in KC and Nashville, thank you, I love you all.

Last, but most definitely not least, my deepest thanks to my loving husband, Mark Didde. You were with me every step of the way from day one of my Master's to now. You supported me through everything; I'm so excited to share this with you. This dissertation is as much your accomplishment as it is mine. Thank you and I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Each year in the US there are approximately 42,000 cases of salmonellosis reported, with 400 being fatal in 2012 (CDC, 2012). In an analysis conducted in 1998, estimated that salmonellosis costs the US nearly 2.3 billion dollars (Frenzen et al., 1999). A majority of these costs are due to of medical care (Frenzen et al., 1999). Overall, incidence of salmonellosis has declined since 1987, but still remain at high levels (Cogan and Humphrey, 2003). There are 2,400 different *Salmonella* serovars associated with human salmonellosis cases with, *S. Typhimurium* and *S. Enteritidis* being the two most frequently serotypes reported (Cogan and Humphrey, 2003). Of the two, *S. Enteritidis* is to be closely associated with poultry and eggs (Cogan and Humphrey, 2003).

As a result of the profound health and economic impact salmonellosis has in the US and worldwide, it is imperative that precautions be taken to reduce the amount of *Salmonella* in poultry environments and eggs. One of the strategies being examined currently is the use of feed additives such as prebiotics, probiotics, essential oils, and organic acids. Each of these feed additives has different properties that have been shown to be effective against *Salmonella*. Prebiotics are nondigestible food ingredients for the host that selectively stimulates the growth or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995; Gaggia et al., 2010). Probiotics are live microorganisms that when administered exhibit health benefits to the host, which include: regulation of bacterial homeostasis, stabilization of gastrointestinal barrier function (Salminen et al., 1996; Gaggia et al., 2010), expression of bacteriocins

(Mazmanian et al., 2008; Gaggia et al., 2010), immunomodulatory effects (Salzman et al., 2003; Gaggia et al., 2010). The hypothesis for the use of prebiotics and probiotics in animal industry is similar, attempting to modulate the animal's gut microbiota of the animal for better gut health and a reduction of pathogen invasion.

The use of both essential oils and organic acids has gained recent attention due to the public pressure to stop antibiotics as growth promoters and also because of their antimicrobial properties (Van Immerseel et al., 2006). Essential oils are complex mixtures of plant metabolites consisting of low-boiling-phenylpropenes and terpenes (Brenes and Roura, 2010). Essential oils can be extracted from plant material such as: flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Brenes and Roura, 2010). Essential oils offer a potential natural method of reducing pathogens in the gastrointestinal tract of poultry. Organic acids are short chain or medium chain fatty acids that have a long history of being utilized in food production as an antimicrobial. Organic acids can either be directly added to feed or are the result of fermentation of starter cultures. Both essential oils and organic acids have shown promising results in reducing *Salmonella* in vitro, however more studies need to be conducted in vivo to evaluate their efficacy.

Adding to food safety concerns, alternative systems such as cage-free, aviary and enriched cages are becoming more prevalent in the US. In California, due to Proposition 2, laying hens will not be allowed to be housed in conventional cages. As a result, United Egg Producers (UEP) and the Humane Society of the US (HSUS) have come forth with a bill that will require all hens in the US to be housed in enriched colony cages by 2029 (O'Keefe, 2011; Green and Cowan, 2012). As a result, studies are being conducted

examining the food safety aspect of eggs produced in conventional systems versus alternative systems. Results have been varied, with some studies finding no differences between conventional and alternative systems (Jones et al., 2012), some finding large differences in favor of conventional cages (Methner et al., 2006; Wales et al., 2007; Snow et al., 2009). As a result, more work needs to be conducted examining the impact alternative systems have on egg food safety.

1.2 INTRODUCTION TO *SALMONELLA*

Salmonella is a gram negative, facultative, mobile bacteria of the phyla enterobacteriacea (Brenner et al., 2000; Grimont et al., 2000; Kim et al., 2006; Park et al., 2009; Park et al., 2013). *Salmonella* are further divided taxonomically into *Salmonella enterica* and *Salmonella bongori* (Park et al., 2013). *Salmonella* enterica cause a variety of diseases, which are commonly referred to as salmonellosis in humans and other mammals (Grimont et al., 2000; D'Aoust and Maurer, 2007; Park et al., 2013). *Salmonella* enterica species are composed of more than 2,500 serotypes, which are determined by the somatic (O), flagellar (H) and capsular (K) antigens (Grimont and Weill, 2007; Park et al., 2013).

In humans, *Salmonella* infection can be divided into two categories: typhoid fever which is caused by *Salmonella Typhi* and *Paratyphi* or gastroenteritis (Kim et al., 2006; Nester et al., 2006; Park et al., 2013). Gastroenteritis occurs in animals as well as humans and is caused by other *S. enterica* serovars, for the purpose of this review the focus will be on gastroenteritis. The symptoms of gastroenteritis in humans are characterized by nausea, headache, diarrhea and fever (Park et al., 2013). In the US

alone, approximately 40,000 cases are reported each year which result in approximately 1% mortality level (Park et al., 2013). Most cases resolve themselves within a few days, in some individuals such as the very young and old, the infections may become serious.

Most *Salmonella* infections in both humans and animals are transmitted in a fecal to oral route. This is by ingesting food or water that has been contaminated with fecal matter. For a human to exhibit symptoms of salmonellosis, at least 10^6 to 10^9 cells need to be ingested (Nester et al., 2009; Park et al., 2013). *Salmonella* are sensitive to acidic conditions, but if introduced to slightly acidic conditions, they can express acid shock proteins during log or stationary growth (Foster, 1991; Foster and Spector, 1995; Park et al., 2013). Through this mechanism, they survive the stomach and enter the small intestine where they adhere to epithelial cells by the type III secretion system, and from there can access different tissues within the body such as liver, bile, bloodstream and spleen (Raskin et al., 1997; Lamont, 2004; Nester et al., 2009; Park et al., 2013).

In poultry, it has been speculated that more than 200 serovars of *Salmonella* have the ability to colonize the gastrointestinal (GI) tract (Gast, 2007; Foley et al., 2011). The outcomes of these infections can range from a subclinical infection that is not noticed by the producer to death (Park et al., 2013). None the less, because of the number of *Salmonella* serovars that are capable of colonizing the chicken GI tract, poultry serve as an important vector for *Salmonella* in humans (Ricke et al., 2001; Ricke 2003b; Howard et al., 2012; Park et al., 2013). It is most commonly passed from poultry to humans through meat and egg products, posing a food safety risk for consumers and a challenge to keep *Salmonella* at reasonable levels for producers.

1.3 HISTORY OF *SALMONELLA* IN POULTRY PRODUCTION

Salmonella enteritidis has been a constant battle for the poultry industry reportedly since the mid-1970's (Baumer et al., 2000; Guard-Petter, 2001). However, it is interesting to note that it has not always been on the forefront. There is evidence that *S. enteritidis* gained a foothold in poultry production as early as the mid 1960's (Baumer et al., 2000). This is because of the interplay between *S. pullorum*, *S. gallinarum* and the possibility of competitive exclusion of *S. enteritidis*.

In the 1930's in both Britain and the US, pullorum disease, caused by *S. pullorum* had a serious economic threat on the poultry industry (Bullis, 1977; Baumer et al., 2000). In response, the National Poultry Improvement Plan (NPIP) was established in the US in 1935 and voluntary testing of poultry flocks began (Baumer et al., 2000). The testing was done by a whole blood agglutination assay of a stained antigen; in 1956 the testing was expanded to include *S. gallinarum* (Baumer et al., 2000). The test expanded to both types of *Salmonella* as both were part of the O9 serotype group (Baumer et al. 2000). As a result of the testing, any birds testing positive were culled, thus by the mid-1970's *S. pullorum* and *S. gallinarum* were eliminated from poultry flocks in the US (Baumer et al., 2000).

Before the 1930's, *S. enteritidis* was primarily found in rodents, as they are the animal reservoir for this pathogen (Baumer et al., 2000; Rabsch et al., 2000; Guard-Petter, 2001). However; the exclusion of *S. pullorum* and *S. gallinarum* likely led to an open niche that was quickly filled by *S. enteritidis*. This is exhibited by the frequency of *S. enteritidis* infections in the US, where it moved from the sixth most causative serotype

in 1963 to the third most common in 1967 (Aserkoff et al., 1970; Baumer et al., 2000). One hypothesis as to why and how *S. enteritidis* gained its foothold in poultry houses is proposed by Baumer et al. (2000), "…the elimination of *S. pullorum* "reactors" may have increased the ability of *S.enteritidis* to gain a foothold in poultry flocks. The O antigen of *S. enteritidis*, *S. pullorum* and *S. gallinarum* consists of the O12 antigen (a sugar backbone composed of O-polysaccharide repeating units) and the O9 antigen (a tyvelose sugar chain). Chickens infected with *S.gallinarum* or *S. pullorum* develop O9 antibody titers that are > 10 fold higher than O12 antibody titers (Barrow et al., 1992) suggesting that the O9 antigen is immunodominant." In short, this explains that how chickens exposed to *S. gallinarum* and *S. pullorum*, develop a very effective immune response to *S.enteritidis*, which also explains why it was not seen in high numbers in chickens until the mid 1960's. *S. enteritidis* incidence continued to rise and by 1990, displaced *S.typhimurium* as the primary cause of salmonellosis in the world (Guard-Petter, 2001; Baumer et al., 2000). With this rise, epidemiological studies confirmed that eggs were the food most commonly associated with *S.enteritidis* (Guard-Petter 2001; Anonymous, 1999; 2001; St Louis et al., 1988; Ullmann and Scholtze, 1989). As a result of the unique method *S. enteritidis* gained its foothold in poultry, it is the only serovar of *Salmonella* routinely found in eggs, thus creating a unique food safety challenge (Guard-Petter, 2001).

1.4 INFECTION BIOLOGY OF AVIAN SALMONELLOSIS

Laying hens are housed in a variety of environments ranging from conventional cages to cage free with outdoor access. As a result of this range in housing, along with pest inhabitation such as rodents, insects and in some cases, wild birds, a wide variety of

niches are provided are provided to bacteria to fill within the environment (Guard-Petter, 2001). *S. enteritidis* is most commonly passed from rodents and insects to the laying hen, which is why it cannot be eliminated as easily as *S. pulloreum* and *S. gallinarum* (Baumler et al., 2000; Guard-Petter, 2001). *S.enteritidis* is continually being reintroduced into the environment as pests gain access to the hen house, reproduce and allow *S.enteritidis* to continue to colonize. Hens gain access to *S.enteritidis* by ingesting contaminated insects, feed or water; from there it is most likely to colonize in the ileum and ceca (Chappell et al., 2009). One interesting characteristic of *S.enteritidis* is that it is not likely to cause illness in hens (Guard-Petter, 2001). This is in distinct contrast to *S.enteritidis* predecessors *S. gallinarum* and *S. pullorum*, which caused mortality, weight loss, and a distinct decrease in egg production (Guard-Petter, 2001; Shivaprasad, 2000; Snoeyenbos, 1991).

A systemic infection of *Salmonella* has three distinct phases (Chappell et al., 2009). During each of these phases, the immune system is heavily involved. The first phase is invasion of the gastrointestinal tract; second establishment of systemic infection by intracellular infection of macrophages (Chappell et al., 2009); third, there are three possible end results: infection is cleared by immune response, bird succumbs to the infection or a carrier state develops (Chappell et al., 2009).

Beginning with intestinal invasion, in mammals *Salmonella* invades the Peyer's patches and M cells in the ileum (Chappell et al. 2009). A gastrointestinal invasion results in enteritis through a combination of secretion effectors of *Salmonella* pathogenicity island 1 type III secretion system and recognition of flagella and lipopolysaccharide (LPS) through toll like and other pattern recognition receptors (Wallis

et al., 1999; Shivaprasad, 2000; Gerwirtz et al., 2001; Zeng et al., 2003; Chappell et al., 2009;). This leads to a pro-inflammatory cytokine and chemokine response (Chappell et al., 2009).

The avian gastrointestinal tract is much different than the mammalian species, with noted differences being the crop, gizzard and lymph system. Outside infecting bacteria pass through the crop prior to entering the proventriculus and gizzard. The crop has a pH of 4-5, which induces acid adaptation mechanisms of *Salmonella* that help with passage through the proventriculus and gizzard (Chappell et al., 2009). Entering the small intestine, which is the main infection site in mammals, the chicken does not have distinct lymph nodes and Peyer's patches but more diffuse lymphoid aggregates and Peyer's patches (Chappell et al., 2009). The main site of *Salmonella* colonization is the ceca. Cecal tonsils are the largest secondary lymphoid organs of the chicken gastrointestinal tract and located at the ileo-cecal junction (Chappell et al., 2009).

Following intestinal invasion, it is believed that *Salmonella* are taken up by macrophages or dendritic cells and transported to the spleen and liver (Mastroeni and Menager, 2003; Chappell et al., 2009) It is there that *Salmonella* develops a key relationship with macrophages, as it is crucial to its survival in the host (Barrow et al., 1994; Chappell et al., 2009). The pathogencity island 2 (SPI-2) type III secretion system has a key role in this relationship. Once *Salmonella* is within the phagocyte vacuole of a macrophage, there is an injection of effector proteins that prevents fusion of phagosomes with lysosomes (Cheminay et al., 2005; Chappell et al., 2009). Lysosomes contain degenerative enzymes and other antimicrobial compounds that would destroy *Salmonella*

encapsulated in the phagosomes. In order for *Salmonella* to survive in its host, it must be able to survive and multiply in the macrophage, which will lead to a systemic infection.

After establishment of a systemic infection, three scenarios will play out. First, the chicken may clear or control the replication of bacteria then clear the infection through adaptive (learned) immunity (Chappell et al., 2009). If replication is not controlled by innate (first response) immunity, *Salmonella* replicates in the spleen and liver leading to lesions and can shed back into the gastrointestinal tract (Chappell et al., 2009). If this occurs, it will result in death of the bird within 6 to 10 days following infection (Shivaprasad, 2000; Wigley et al., 2002a; Chappell et al., 2009;). Carrier state infections frequently occur in birds more than a few days old; the majority of bacteria appear to be cleared by the immune response, with small numbers persisting within intracellular niches (Chappell et al., 2009).

1.5 THE CARRIER STATE AND SHEDDING

Although there have not been many studies examining the carrier state and shedding, especially in older laying hens, there are some inferences that can be made. The consensus among researchers is that the bird develops the carrier state when infection occur early post hatch (Cox et al., 1996; Gast and Holt, 1998). Furthermore, Cox et al., (1996) conducted a series of studies in which day old broiler chicks were subjected to *S. typhimurium* inoculation through a variety of methods: naval, oral, and aerosol. The findings showed regardless of the source, exposure to low levels of *Salmonella* in newly hatched chicks leads to gut colonization (Milner and Shaffer, 1952;

Cox et al., 1991; Cox et al., 1996). This is attributed to the chick lacking a mature gut microbiome to compete with *Salmonella*, allowing for colonization (Cox et al., 1996).

To further expand on the above study, Gast and Holt (1998) examined the shedding of *S. enteritidis* in experimentally inoculated one day old layer chicks until maturity. Their findings were that after 4 weeks of age, there were no positives in spleen or liver samples, however 40% of cecal samples tested positive through 16 weeks of age (Gast and Holt, 1998). In addition, colonization persisted into early stages of lay as 58% of hens shed *S. enteritidis* into feces from 18-24 weeks of age. In addition to the positive hens, there were 448 egg pools conducted during the study, with only 2 egg pools testing positive (Gast and Holt, 1998). Conclusions drawn were although there were high colonization rates of cecum, liver and spleen initially, only the intestinal tract was colonized after 4 weeks postinoculation (Gast and Holt, 1998). In addition, neither immunological maturation nor the development of microflora could dislodge *S. enteritidis* from the ceca (Gast and Holt, 1998).

The above studies focused on experimental colonization, where the results are more dramatic and dosage levels are much higher than what they would be in production systems. To account for this, there have been a few studies conducted which examine the dynamics of shedding within layer flocks in production. One study conducted by Schulz et al. (2011) monitored 41 flocks throughout their layer cycle in countries throughout Europe. Flocks were sampled either three or four times at different intervals. Findings were that hens early in the lay cycle or just reaching sexual maturity, approximately 11-20 weeks of age, had higher shedding rates than when they were older (51-60 weeks of age) (Schulz et al., 2011). These findings show that the previous thinking that hens that

are older have an increase in shedding does not hold true for infected flocks (Schulz et al., 2011). To further confirm these assertions, Johnston et al. (2012) found that as the hen approaches point-of-lay there is a sharp decrease in T lymphocyte and particularly CD4+ cells. In addition, there have been substantial changes in the organization of lymphocytes in the reproductive tract which was likely to increase susceptibility of reproductive tract infection (Johnston et al., 2012). Finally, due to the immunosuppression that occurs during point-of-lay the efficacy of the vaccine is substantially reduced (Johnston et al., 2012). Possibly leading to an increase in fecal shedding and egg contamination.

Immunosuppression, while a large factor in early shedding of *S. enteritidis* was most likely not the only factor. Stressors such as transport, feed and climate changes, stocking density and housing can all have a negative effect on the hen's gastrointestinal tract which most likely results in shedding. A study conducted by Nakamura et al. (1993) found that stressors such as introduction of new hens to the flock and removal of feed and water for 48 hours results in an increase in shedding that lasts several days. Studies have found that any type of feed withdrawal will result in an increase in corticosterone levels (Freeman et al., 1981; Harvey and Klandorf, 1983) . In addition to these findings, the above stressors including molt had a profound effect on cell-mediated immunity resulting in a decrease in the number of CD4 T-lymphocytes (Holt, 1993). This confirms that at key times in the laying hen's productive life, shedding occurs through immunosuppression due to an induction of stressors.