IMPACT OF EGG HANDLING PRACTICES ON *SALMONELLA* RISK IN CAGE-FREE EGGS.

by

Garrett E. Ward

(Under the Direction of Harshavardhan Thippareddi)

ABSTRACT

Food Safety is considered an utmost priority for shell egg producers when providing table eggs for consumers. In the United States, commitments by producers have shifted to cage-free systems due to increased concerns for laying hen welfare. In cage-free systems, floor egg production has become a concern. Floor eggs are susceptible to pathogen contamination from exposure to fecal material and substrate. Concerns about the microbiological safety of floor eggs entering the human food supply are based on an individual consuming potentially contaminated eggs. Currently, regulations pertaining to floor eggs and their use are lacking. This research evaluated *Salmonella* penetration from the contaminated feces on egg shells prior to egg washing. Evidence from this research concludes that *Salmonella* spp. are capable of surviving extended refrigerated storage. However, challenged *Salmonella* Enteritidis was not detected within contents between the timeframe of 48 h after lay in any challenged egg for the study duration.

INDEX WORDS**:** *Salmonella*, eggshell penetration, cage-free, fecal contamination

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# CHAPTER 1

# INTRODUCTION

## Purpose of the Study

The shell egg industry heavily focuses on providing sustainable and safe shell eggs and egg products for consumers. Public opinion has a prominent role in shaping how shell eggs and egg products are produced. Recently, the shell egg industry has begun to transition from conventional cage to extensive housing systems. The concern for animal welfare of laying hens was the determining factor in this transition. With laying hens exposed to a more extensive environment that may have access to litter material and fresh feces, the probability of *Salmonella* to spread within a flock increases. Historically, *Salmonella* contamination of shell eggs has been researched due to a high risk of foodborne illness from *Salmonella* that is linked to the consumption of eggs. The shift to new laying hen housing systems and the history of *Salmonella* foodborne illness linked to eggs has drawn the attention for this research. Specifically, eggs that have been laid on the floor in cage-free housing systems are susceptible to *Salmonella* contamination. Shell egg producers in the United States are able to process and send floor eggs into the human food supply. The risk of salmonellosis could increase if surface *Salmonella* from feces contamination of the shell penetrates to the egg contents..

To understand the current situation, the literature review covers current preventative measures in the shell egg industry, highlighting the definition of a cage-free housing system and how cage-free housing systems impact laying hen behavior and

welfare, shell egg quality, flock management, alternatives flock molting, biosecurity measures, regulations, vaccination programs, refrigeration techniques, shell egg washing practices, as well as how alternatives to shell egg processing can reduce the risk of shell egg contamination from a farm-to-table perspective. The experimental research chapter evaluated the potential risk of *Salmonella* transfer/penetration from the shell of the egg to the egg contents under current regulatory requirements and industry practices.

The objective of the research was to evaluate penetration of *Salmonella* from the egg surface (shell) to the interior (egg contents) of floor eggs that were exposed before *Salmonella*-contaminated feces during simulated egg handling practices. This results will provide information on the potential penetration of *Salmonella* from the shell surface to the egg contents and aid in development of guidance material to mitigate the risk.

# CHAPTER 2

# PREVENTATIVE MEASURES TO REDUCE THE RISK OF *SALMONELLA* CONTAMINATION IN SHELL EGGS: A LITURATURE REVIEW[1]

[1] Garrett Ward, Deana R. Jones, Richard K. Gast, Kenneth E. Anderson, Manpreet Singh, and Harshavardhan Thippareddi. To be submitted to *Poultry Science.*

## INTRODUCTION

*Salmonella* Enteritidis has been linked worldwide to the consumption of contaminated shell eggs (Jackson et al., 2013; Pires et al., 2014). In addition, evidence of commercial layer flocks that have *Salmonella* Enteritidis prevalence throughout the duration of the flocks' life have been connected to human salmonellosis cases (Arnold et al., 2014). According to the Centers for Disease Control and Prevention (CDC; 2011), *Salmonella* Enteritidis related illnesses have increased since the 1990s. Efforts have been made through research and legislation to mitigate the risk of *Salmonella* contamination during shell egg production and processing within the United States. The United States egg industry strives to reduce the risk of salmonellosis from consumption of eggs and egg products.

Retailers and consumers in the United States play a major role in shaping the transition of hens being housed in conventional housing systems to cage-free housing systems. Despite this transition being beneficial to the overall health of the hens, access to litter material in cage-free housing system increases the likelihood that freshly laid eggs are exposed to fecal and possible *Salmonella* contamination. As a result, contaminated floor eggs may enter the human food supply. This literature review will specifically discuss and evaluate the risk factors associated with *Salmonella* contamination during the production and processing of shell eggs in the United States.

### Cage-Free Housing Systems

Commercial egg production systems within the United States vary between egg producers. Historically, conventional cages were the predominant housing system for laying hens. Public concern about animal welfare over the previous decade has resulted in

the fast food restaurant industry, as well as retailers, to demand that eggs be sourced from hens that are housed in cage-free production systems. In response to public opinion, recent regulations have required the egg industry to transition to cage-free housing production systems. Cage-free housing systems, as defined by the United Egg Producers (2017), allow hens to roam vertically and horizontally within indoor houses, which enables the birds to exhibit natural behaviors with available enrichments. This definition encompasses all cage-free housing types because cage-free systems may vary from farm-to-farm, with the focus on the promotion of bird welfare.

To help identify different cage-free housings styles, the Coalition for Sustainable Egg Supply (2016) classified cage-free housing systems as an aviary, enriched colony cage, or floor housing system. Cage-free aviary systems allow for a more spacious environment for hens, while ensuring full mobility, increased movement, exercise, and flight, of hens in the environment. Enriched colony cages provides perches, scratch pads (optional), and nest-boxes and a greater number of hens in a larger enclosed space compared to conventional cages. Cage-free floor systems are similar to cage-free aviary systems in that they allow hens to have full access to litter material and movement throughout the system. However, cage-free floor systems do not have vertical tiers as found in aviaries. Variations of all these generalized housing systems exist through different designs and between egg producers. The type of housing system that is chosen presents different unique, such as farm management, microbiological, and egg quality, and production.

Cage-free housing systems require an understanding of management practices for layer hen populations Karcher et al. (2015) evaluated laying hen performance and egg quality parameters within commercial housing systems. Specifically the performance of

Lohmann LSL White laying hens in conventional, aviary, and enriched colony cage housing systems over the course of a single production period was evaluated. The hen day egg production was similar in all three housing systems (conventional, aviary, and enriched colony cage) until the hens were 45 weeks of age. The hens housed in enriched colony cage systems had greater than 95% egg production and maintained a higher level of production until the end of the egg laying period. The hens housed in aviary systems had a rapidly increasing mortality after 45 weeks of age, which reached above 4% cumulative mortality, as compared to hens in the enriched colony cage and conventional cage systems, which fell below 4% cumulative mortality until 70 weeks of age. The high mortality rate in aviary systems could be due to the following reasons: the pecking and crowding of the large hen group in a given space or the rearing environments, in which the hens were reared. Additionally, Karcher et al. (2015) reported that the feed intake of hens in aviary systems was higher even though the hen's body weight was similar to the body weight of hens that were housed in either enriched colony cage or conventional cage systems. The higher feed intake could be due to higher temperatures within the house or the hen's activity level, which requires a greater energy demand. In addition, the disappearance of feed may be contributed to the hens playing in the fed.

Jones et al. (2014) reported differences in egg weight and better albumen quality during the four egg collections (26, 41, 57, and 70 weeks of age) over the course of a single production period at aviary, enriched colony cage and conventional cage systems. Although the differences were numerically minimal (<1 g), the authors reported higher mean egg weight from aviary (58.69 g) and enriched colony cage systems (58.88 g) as compared to conventional cage systems (57.97 g; $P < 0.05$). The Haugh unit and albumen height were

greater in conventional cage systems (84.62 and 7.21 mm). The differences in albumen quality were less than a fraction in millimeters of the average albumen height between systems. Static compression shell strength was greater in enriched colony cage systems and conventional cage systems (39.57 N and 39.36 N, respectively) rather than in aviary systems (38.53 N). Differences in vitelline membrane strength, vitelline membrane elasticity, yolk index, shell dynamic stiffness, and whole egg percent total solids were minimal.

Although egg production and egg quality parameters are critical for marketing eggs for egg producers, microbiological safety is equally important. Research conducted by Jones et al. (2015, 2016), in conjunction with Karcher et al. (2015), evaluated the microbiological impact of conventional cage, enriched colony cage, and cage-free aviary systems in a commercial setting.

Regardless of the housing system, laying hens can harbor *Salmonella* in their gastrointestinal tract and shed it in their feces. As a result, the environment's contamination by the organism can be spread by the hen's activity, as well as equipment used (Gast, 2007). Jones et al. (2015) reported that hens from all housing systems (conventional cage, enriched colony cage, and cage-free aviary) shed *Salmonella* (89-100% of manure belt scraper blade swabs), with lower prevalence of *Campylobacter* (0-41%). However, Campylobacter prevalence in the aviary forage area drag swabs (100%) and enriched colony cage scratch pads (93%) were higher compared to the manure belt scraper swabs. This is possibly due to greater moisture levels found in the environments compared to the dry belt scraper. The *Salmonella* prevalence in egg shell emulsion pools from conventional system wire, enriched system wire and aviary system wire was 7.5%, 0% and

4.8%, respectively. *Salmonella* prevalence in egg shell emulsion pools from enriched nest boxes (7.5%) was similar to the conventional system wire (7.5%), but lower than the aviary nest box (1.3%) system. However, the *Salmonella* prevalence of egg shell pools from aviary floor (7.8%) was similar to the conventional system wire (7.5%). The *Salmonella* prevalence from egg shell pools is reflective of the prevalence of the organism in the laying hen, as well as in the environment and potential contamination subsequent to lay. Thus, eggs collected from aviary floors present as much risk as conventional cage housing systems or more. As such, extreme care must be exercised when floor eggs from cage-free housing systems are destined for human consumption.

## Laying Hen Behavior

Animal welfare is comprised of numerous factors including: disease, skeletal health, pest control, foot health, parasite prevention, nutrition, genetics, stress levels, and behavior. Considering all of these factors within a conventional housing system, the overall welfare of laying hens is scrutinized due to the hen's inability to express typical behavior patterns, which can lead to the emergence of harmful behaviors, such as feather pecking and hysteria (Lay et al., 2011). As a result, consumer and retailer pressure for greater animal welfare standards has pushed the egg industry to transition into cage-free housing systems.

Unlike conventional cage environments, cage-free housing systems provide grmoreeater space for hens to move in their environment. Since the hens exhibit more natural behaviors, potential instances of bone fractures may increase. However, the risk of osteoporosis decreases (Webster, 2004). Lay et al. (2011) summarized the predicted behavioral expression based on different housing systems, which include: greater perching,

foraging, dust bathing, nesting, wing flapping, stretching and perching with less feather pecking, and social aggression for cage-free housing systems. Behaviorial patterns expressed by hens are not only dependent on the current housing type, but also on prior exposure to different rearing environments, as well as environmental conditions from embryonic development (Janczak et al., 2007; Lindqvist et al., 2007).

Spacious housing and rearing environments may promote better animal welfare and increased natural hen behavior, but access to floor litter material may cause floor egg production. In addition, excessive floor eggs can cause broodiness within hens. To resolve this issue, lights within the nest-boxes can be used to attract the hens; however, this practice has been linked to a potential increased risk of cannibalism within the flock (Zimmerman et al., 2006). Perch access, while rearing chicks has been an effective measure to reduce floor egg production, which reduces cannibalism and social aggression (Appleby et al., 1988; Gunnarsson et al., 1999; Cordiner and Savory, 2001). The incorporation of different stimuli throughout the rearing and housing environments of laying hens provides a combination approach to not only improving natural behaviors within a cage-free environment but a reduction of floor eggs being laid (Cordiner and Savory, 2001).

## Flock Management

*Salmonella* can be widespread in the poultry house environment. Implementation of good on-farm practices such as cleaning and disinfection of the hen's environment and controlling pests have been shown to reduce the risk of *Salmonella* (Holt et al., 2011). Environmental factors such as stocking density, presence of litter, manure, dust, mice, flies, and the surfaces in the hen's environment can influence *Salmonella* prevalence (Van Hoorebeke et al., 2011). *Salmonella* survival within the laying hen environment is extensive

and *Salmonella* has been reported to survive up to 26 months post depopulation period of a flock (Davies and Breslin, 2003b).

Flock management is critical to meet production goals, as well as for the prevention of *Salmonella* infections. Holt et al. (2011) reported on the effect of variations in stocking density on *Salmonella* prevalence within a flock. High stocking density resulted in the accumulation of large volumes of manure and dust, as well as increased stress within the laying hen's environment and potentially increasing prevalence of *Salmonella* (Davies and Breslin, 2004; Van Hoorebeke et al., 2011). Typically, the group size of hens is larger in a cage-free housing system compared to the conventional cage housing system. To prevent overcrowding in certain sections, the placement of enrichments, such as perches, feeders, waterers, nest-boxes, and available litter spaces, throughout the hen's environment should be taken into consideration (Van Hoorebeke et al., 2011).

Hens within a cage-free housing system have access to substrate material within their environment to allow for natural hen behavior, such as foraging and dust bathing. Reports of higher water activity and moisture levels within a poultry house correlate to increased prevalence of *Salmonella* within the litter material (Eriksson de Rezende et al., 2001). Good ventilation throughout a poultry house, as well as regular maintenance of waterers can reduce wet litter and therefore reduce *Salmonella* prevalence in the litter. The litter pH also affects *Salmonella* prevalence in the environment. As the ammonia generated from uric acid hydrolyses from microbial conversion, it dissolves in the available moisture within the litter. As a result the pH of the litter increases. Bennett et al. (2003) focused on altering the litter pH by adding hydrated lime within a poultry house and reported an increase in pH to 12.5. As a result, *Salmonella* prevalence was reduced within the poultry

litter. The utilization of hydrated lime can be a cost effective measure for an egg producer when applied frequently to substrate in a cage-free system.

Dust within a poultry house is a common vector for *Salmonella* transmission. The organism can survive in dust from the floor and stagnate feed troughs for up to 26 months (Davies and Wray, 1996). Several other reports have indicated the recovery of *Salmonella* from airborne dust particles from poultry exit vents (Gast et al., 1998; Davis and Morishita, 2005). Davis and Morishita (2005) found that the average dust outside of the poultry house had an average concentration of 2 mg/$m^3$ from ventilation fans. Also, Davis and Morishita (2005) discovered that *Salmonella* was isolated from dust inside the poultry house and up to 13.3 m outside of the ventilation fans from all five evaluated facilities. Proper ventilation within a poultry facility is critical in preventing the spread of *Salmonella* throughout a poultry house via litter material or dust particles.

Rodents in or on the poultry farms and their vicinity have been implicated as vectors for *Salmonella* in poultry. Henzler and Opitz (1992) reported survival of *Salmonella* in rodent feces for up to 10 months. Rodent droppings have been shown to be a source of *Salmonella* infections in poultry (Davies and Wray, 1995). Umali et al. (2012) reported that *Salmonella* Enteritidis was more frequently isolated from the rodents' spleens and livers than fecal droppings. Thus, *Salmonella* is capable of serving as a potential long-term issue through contaminated vectors on a farm. Since rodents are potential vectors for flock contamination, pest control programs on the farm should be regularly practiced under the Egg Rule (FDA, 2018b) in and around poultry housing facilities.

## Feed Restriction Molting and Alternatives

Molting, a method of nutrient withdrawal to increase egg production after molt and reduce hen mortality has been shown to increase in the prevalence of *Salmonella* within a flock (Alodan and Mashaly, 1999; Holt, 1999; Golden et al., 2008). Extensive efforts have been made to understand the relationship of *Salmonella* Enteritidis fecal shedding and molting in laying hens. Nakamura et al. (2004) evaluated this association between *Salmonella* fecal shedding during a molting flock and reported a reduction of *Salmonella* prevalence in fecal during the molting process. Also, Molting increases lymphocytes, corticosterone, and thyroid hormone production influencing potential infections (Holt, 1995; Holt et al., 1998; Davis et al., 2000; Berry, 2003). In addition, feed deprivation causes crop environment changes, including an increase in pH from 4 to 6, that results in an increase in *Salmonella* colonization in molted hens (Durant et al., 1999). In the egg industry, molting practices do not completely remove available feed for hens (Durant et al., 1999; Davis et al., 2002; Golden et al., 2008).

Non-fed withdrawal methods of molting are now in use to minimize the risk of pathogen prevalence and shedding, while maintaining post-molt performance of egg lay. Alternative feedstuffs, such as alfalfa, wheat, and whole cottonseed meal, have been incorporated into poultry diets to feed birds a low nutritive feed value that will induce bird molting. Incorporation of alfalfa into a hen's diet resulted in a significant decrease in *Salmonella* prevalence within the ceca (Willis et al., 2008). Wheat middlings have proven to be effective at reducing *Salmonella* prevalence, but resulted in reduced egg production that lasted for several days (Seo et al., 2001). Similarly, incorporation of cottonseed meal induced molt by voluntary reducing feed intake by the hens. However, this method

appeared to control feed consumption by natural hen aversion to the feed, and thus is similar to feed deprivation in terms of its impact (Davis et al., 2002; Keshavarz and Quimby, 2002).

Like other organic material, poultry feed is susceptible to contamination by *Salmonella* during all stages of production (Maciorowski et al., 2006; Gast 2007). Li et al. (2012) reported that the *Salmonella* contamination rate for feedstuffs imported into the United States and European countries was 1.1% to 41.7%, respectively. Proteins from animal sources destined for animal feed have historically been implicated as sources for *Salmonella* contamination (Mackenzie and Bains, 1975; Hacking et al., 1978; Nabbut 1978; Davies et al., 2004). The animal feed industry has implemented *Salmonella* control programs, such as monitoring and heat treating raw products, maintaining appropriate climate controlled ingredient and feed storage, and ensuring sanitation standard operating procedures in the feed mill, that would reduce the likelihood of *Salmonella* contamination. From a feed manufacturer's perspective, a primary preventative measure is to mitigate the risk of *Salmonella* prevalence in poultry feed by utilizing *Salmonella*-free feed ingredients from suppliers (Jones, 2008). Secondary environmental control measures, such as regulating dust accumulation, personnel restriction in certain areas of the plant, removal of fat accumulation on processing equipment, rodent and wild bird control, and sanitation of transportation vehicles, are important factors in a preventative feed programs (Fedorka-Cray et al., 1997; Whyte et al., 2003; Jones and Richardson, 2004; EFSA, 2008).

## Biosecurity and Regulatory Flock Testing

Regulatory flock testing and monitoring for *Salmonella* prevalence within the poultry industry have been instrumental in evaluating potential *Salmonella* contamination

in the environment, finding effective methods to control the organism in the environment. Current regulatory testing is limited to *Salmonella* Enteritidis, whereas other serotypes are increasingly being implicated for their egg-related foodborne illness outbreaks. Research has shown that the trace-back and trace-forward labeling of a given product from the supply chain is not an efficient strategy on a national level to identify the source of *Salmonella* contamination in eggs (Hogue et al., 1997; Gast, 2007; Gast and Guard, 2011).

Currently in the United States, regulatory monitoring of *Salmonella* Enteritidis in a poultry house is conducted using a drag-swab method (FDA, 2009). The use of drag-swab sampling method for *Salmonella* in a poultry environment (floors, nest-boxes, egg belts, manure belts, scrapers, fan blades, and dust collections) is sensitive and effective (Davies and Breslin, 2001; Kinde et al., 2005; Gast, 2007). Poultry environmental sampling from *Salmonella* fecal shedding has been linked to *Salmonella* contamination of eggs (Arnold et al., 2010; Gast and Guard, 2011). Arnold et al. (2010) found that the most sensitive method for sampling *Salmonella* Enteritidis in a commercial poultry house setting was environmental sampling as compared to a random individual bird sampling. Gast and Guard (2011) emphasized environmental and egg related testing as a critical strategy to monitor the *Salmonella* prevalence in a flock. However, they propose that by identifying genetic differences between egg-associated and non-egg associated *Salmonella* strains, greater insight for improving testing methods for egg-associated *Salmonella* strains can be achieved.

*Salmonella* Enteritidis infected tissues, including gastrointestinal and reproductive tissues, have been linked to shell egg contamination (Gast and Beard, 1990; Gast, 2007). Gast and Beard (1990) evaluated *Salmonella* contamination of shell eggs from infected

hens. They recovered *Salmonella* Enteritidis from the intestinal tracts of hens that were from three different age groups (27, 37, and 62 weeks after of age) after one week post-inoculation. Gastrointestinal tract samples after one week post-inoculation resulted in *Salmonella* Enteritidis prevalence for 91% of the infected hens at 62 weeks of age, 80% of the infected hens at 37 weeks of age, and 73% of the infected hens at 27 weeks of age. In addition, 50% of egg yolk samples from infected hens were positive for *Salmonella* at 62 weeks of age during the first week after inoculation. Infected hens at 37 weeks of age had 38% positive egg yolk contamination in the first week. In contrast, infected hens at 27 weeks of age had 20% positive egg yolk contamination for the first two weeks of egg collection. Determination of true prevalence of *Salmonella* Enteritidis within the eggs is challenging due to the low prevalence of *Salmonella* in the eggs and low initial populations of Salmonella in freshly laid eggs (Gast, 2007).

According to the FDA Egg Rule, once *Salmonella* Enteritidis is isolated from the layer environment, the farm management is required to clean and disinfect the poultry house before allowing a new flock into the positive house (FDA, 2009). This includes the removal of any visible poultry manure, removal of dust, feathers, and old feed, which is followed by disinfection of the environment through sprays, aerosols, or fumigation (FDA, 2009). Any moveable equipment should be relocated to ensure a thorough cleaning of the space. Rodent baits should be removed prior to cleaning, as well as any visible rodent site of entry must be repaired. Water lines should be sanitized and feed lines should be cleaned and sanitized prior to the placement of a new flock. Lastly, the disinfection of the areas in and around the perimeter of the infected poultry house must be implemented (FDA, 2009).

An effective biosecurity program should be enacted to prevent flock contamination. FDA specified that limiting visitors to the farm, having good standard operating procedures of equipment and personnel, monitoring wildlife prevention, restricting personnel ownership of poultry to reduce the probability of *Salmonella* contamination (FDA, 2009a). Dengagamage et al. (2015) reported that the risk factors strongly associated with *Salmonella* contamination of eggs includes a high level of manure *Salmonella* contamination, hens that are in middle to late phase production, contamination of egg handling equipment, and a flock size ≥ 30,000 hens with a high (≥ 96%) egg production rate. In regards to biosecurity and flock monitoring, all activities conducted on the farm should provide information on a given flock's *Salmonella* status, as well as a plan for decontamination or sanitation immediately after the detection of *Salmonella* in the flock.

**Vaccination Programs**

Vaccinations are one of the most common methods used to prevent *Salmonella* infections within a flock (Van Immerseel et al., 2005b). The principle of poultry vaccination is to expose a pathogenic agent to hens that stimulates an immune response within the bird to eliminate the pathogen (Goldsby et al., 2003). Vaccines may contain either inactivated or attenuated *Salmonella* strains. Evidence has shown that the attenuated vaccines provide greater protection than inactivated vaccines by reducing *Salmonella* prevalence within the reproductive tract, as well as contamination within egg contents, while providing no residual attenuated vaccination strains within eggs (Van Immerseel et al., 2005b; Gantois et al., 2006). Within the experimental setting, *Salmonella* vaccination has proven to decrease *Salmonella* egg contamination from challenged flocks (Nakamura et al., 1994; Cerquetti and

Gherardi, 2000a, 2000b; Liu et al., 2001; Woodward et al., 2002; Khan et al., 2003; Van Immerseel et al., 2005a).

The reduction in *Salmonella* prevalence in feces, internal organs, and shell eggs have been linked to the use of killed vaccines within poultry in orally challenged birds (Clifton-Hadley et al., 2002; Van Immerseel et al., 2005b; Gast, 2007, 2011). Additionally, intravenous or intramuscular administration of a killed vaccine resulted in a reduction in *Salmonella* clinical symptoms, internal lesions, and mortality within a flock (Timms et al., 1994; Woodward et al., 2002). Davies and Breslin (2003) demonstrated the effectiveness of a killed *Salmonella* vaccine in significantly reducing *Salmonella* prevalence from fecal and environmental samples from a flock. The utilization of the killed vaccines does not present a microbiological safety concern to the general public from food producing animals.

Though killed *Salmonella* vaccinations and live attenuated *Salmonella* vaccines can stimulate a protective immune response in poultry, live attenuated *Salmonella* are developed by gene mutation or deletions in metabolism, virulence, or host survival (Van Immerseel et al., 2005b; Desin et al., 2013). Live attenuated vaccines have been capable of having a lasting reduction in *Salmonella* Enteritidis shedding in feces, organ invasion, and egg contamination in experimentally challenged poultry (Cooper et al., 1993, 1994a, 1996). Live attenuated vaccines have several benefits over killed vaccines. Live vaccines stimulate responses to a spectrum of antigens for both the cell-mediated and humoral immune responses in poultry (Babu et al., 2004; Van Immerseel et al., 2005b). Research has shown that live attenuated *Salmonella* vaccines offer better protection by providing effective protection against *Salmonella* infections in poultry for longer exposure than killed vaccination (Gast, 2007; Desin et al., 2013). One advantage to using live vaccines is the

persistence of the specific strain in a production flock and their environment. Conversely, this probability of persistence presents a risk to human health if the strain enters the food chain or even if the optimal conditions allow for gene mutation in a strain resulting in higher virulence or survival in the environment (Tan et al., 1997; Barbezange et al., 2000a, 2000b; Adraensen et al., 2007). In certain situations, environmental persistence of a live vaccine strain is desirable. The spread of the live vaccine through horizontal transmission via bird-to-bird contact could result in the protection of a bird's health that was not originally vaccinated in the housing system (Desin et al., 2013). Reversion to virulence of a live strain can be easily be avoided through gene mutation by deletion of targeted genes instead mutation of the gene (Van Immerseel et al., 2013). Obstacles associated with the administration of vaccines to poultry include: the cost of vaccine to be administered, the uniformity of delivery to the flock, and effectiveness of the immune response based on bird's age. Oral and aerosolized administrations of live vaccines are most widely used to overcome these obstacles within the industry (Desin et al., 2013). Vaccination programs must be cost effective and efficient in a flock by inducing an immune response from laying hens in order to prevent *Salmonella* infections.

## Shell Egg Refrigeration

Refrigeration of shell eggs immediately after lay can inhibit potential growth of *Salmonella* Enteritidis in egg contents and reduce the risk of salmonellosis from eggs (Lock and Board, 1992; Gast and Holt, 2000; Braun and Fehlhaber, 1995). The Final Egg Rule (FDA, 2009b) requires eggs be stored at 7.2°C or below within 36 h of lay and during transportation. An exception may be made when freshly laid shell eggs are sent directly to processing within 36 hours of lay. *Salmonella* contamination can occur via horizontal

transmission into the shell, as well as egg contents, which promote bacterial growth . (Martelli and Davies, 2012; Chen et al., 2005; Gast and Holt., 2000).

*Salmonella* is capable of growth within shell egg contents at ambient temperatures (20°C; Humphrey and Whitehead, 1993). As a result of contamination of the egg contents, whether during formation or subsequent penetration from shell surface, physical egg quality characteristics such as the color, smell, or consistency of the egg contents, may not be affected. However, the association between *S.* Entertidis growth and yolk membrane alteration can allows the pathogen to penetrate through the vitelline membrane (Humphrey and Whitehead, 1993). Martelli and Davies (2012) observed that *Salmonella* spp. are capable of proliferation in egg contents at 20°C, while unable to proliferate at 10°C. Other research has shown that at higher cell concentrations ($10^2$, $10^4$, and $10^6$), *Salmonella* Enteritidis is capable of growth within egg albumen above 20°C, whereas *Salmonella* Enteritidis growth at lower temperatures (4°C and 10°C) is limited (Chen et al., 2005).

*Salmonella* can grow to higher concentrations within contaminated shell eggs and cause salmonellosis in consumers. Gast and Holt (2000) evaluated the growth capability of *Salmonella* Enteritidis at low concentrations in the egg and determined it's able to grow to dangerous concentrations at different temperatures over a period of several days. The intent of the research was to examine the potential opportunities for *Salmonella* Enteritidis contamination and microbial growth after oviposition of the egg. The infection site in the reproductive organs of *Salmonella* Enteritidis influences the location of egg contamination, such as the yolk, albumen, and whole egg. *Salmonella* Enteritidis rapidly grew to 8.7 log CFU/mL at 25°C within the yolk, while the population slightly decreased during storage in the albumen and at the albumen surface. Eggs that were inoculated at the yolk surface

exhibited increased *Salmonella* Enteritidis prevalence during storage. The site of infection in the reproductive tract of the hen, as well as the contamination site is critical for *Salmonella* Enteritidis growth (Gast and Holt, 2000).

Regardless of the site of contamination in the egg, *Salmonella* migration towards the yolk can occur even if *Salmonella* cells have been deposited within the albumen prior to shell formation (Baron et al., 1997; Gantois et al., 2009). Also, *Salmonella* Enteritidis, as well as other serotypes, can survive within the albumen for up to 3 weeks (Messens et al., 2004). *Salmonella* Enteritidis is capable of regulating its metabolism, as well as gene regulation of the cell wall's structure and function (Clavijo et al., 2006). The importance of the cooling rate of the contents of a shell egg can be directly correlated to the inhibition of *Salmonella* growth (Messens et al., 2004).

The yolk within the shell egg is nutritious and can sustain microbial growth. However, a lag period was observed when *Salmonella* Enteritidis was deposited on the vitelline membrane (Gast and Holt, 2001; Gast et al., 2007). A major factor for initial growth of *Salmonella* Enteritidis is available iron content within the immediate environment at the deposition site. When the iron reserves have been depleted within albumen, *Salmonella* Enteritidis enters a lag phase until nutrients are available through migration towards the vitelline membrane and the yolk (Gantois et al., 2009). The temperature of the egg contents plays an important role in potential growth of *Salmonella* Enteritidis. Once temperature begins to fluctuate, the integrity of the vitelline membrane is compromised and nutrients leech into the albumen, enhancing *Salmonella* Enteritidis migration and rapid growth upon entry into the yolk (Humphrey and Whitehead, 1993). To ensure the safety of eggs,

refrigeration prior to washing can slow or even inhibit *Salmonella* Enteritidis migration and growth.

## Shell Egg Processing

In the United States, shell egg processing consists of washing, rinsing, sanitizer application, forced air drying and grading. This process removes the cuticle from the outer most part of the shell, which increases moisture migration and $CO_2$ gas exchange (USDA, 2000). All detergents in official (USDA) plants are approved for use. Regulatory guidelines specify the water temperature used during washing (USDA, 2018a; 9 CFR 590.515). Eggs destined for egg product manufacturing need to be washed and the USDA, Food Service and Inspection Service (USDA-FSIS) specifies that chemicals that are on the Generally Regarded As Safe (GRAS; USDA, 2018b) list may be used up to the maximum allowable concentrations.

Unlike the chemicals used in the egg wash water, sanitizers in egg processing are limited to the use of hypochlorites or approved sanitizing solutions. The solution must be equivalent to a maximum of 200 ppm of available chlorine (USDA, 2018c; 9 CFR 590.552). Additional shell egg regulation from CFR Title 21 Section 118.4e states the requirements for shell egg refrigeration after lay. Specifically, if shell eggs are to be processed and have been refrigerated before 36 h after lay, those eggs may be tempered at room temperature for up to 36 h before processing (FDA, 2018a, 2018b).

Other regulations on shell egg operations include USDA, AMS (Title 7, Section 56.76), which defines the minimum shell egg grading, shell egg cleaning, and packing plant operation requirements. The regulations state that the wash water temperature shall be maintained at 32.2°C or 11.1°C warmer than the warmest egg entering the processing line.

These temperatures should be maintained during the entire cleaning operation. Shell egg wash water should be changed every 4 h or as often as needed and at the end of every operational shift so that sanitary conditions are maintained. Used wash water must be discarded after use. This ensures that the chemical compounds being utilized in the washing operation will remain within the specified concentrations. Replacement water must be potable water that may contain chlorine or quaternary compounds, but not iodine sanitizer. The potable water used for shell egg washing must be analyzed for iron content and may not exceed 2 ppm. When selecting the appropriate chemical and water for use in a shell egg processing facility, the type of washing procedure and equipment should not allow eggs being washed to remain stagnant or soak in the wash water. Thus, immersion-type washers are prohibited and egg washers equipped with sprayers are to be utilized. Additional efforts are to be made to avoid foaming during egg washing; the chemical compound used as an antifoaming agent must fall within the GRAS list (FDA 2018a).

Shell egg washing has its benefits when considering the risk of foodborne pathogens and spoilage microorganisms present on the shell of an egg. The main advantages are the reduction of the microbial load on the eggshell surface via dirt and debris through the means of sanitizing rinse techniques. This also reduces the risk of foodborne pathogens that could potentially penetrate through the shell and inner membranes of the egg and contaminate the egg contents. Egg washing reduces the risk of contamination of the egg contents, unless the shell is compromised. The residual sanitizer on the egg after washing can further reduce the microbial prevalence on the surface of the eggshell during refrigerated storage. The primary disadvantage to egg washing is the potential damage to the eggshell during processing. If the integrity of the shell is compromised then an

increased risk of contamination of the contents can occur. In addition, the cuticle of an egg may be damaged or even completely removed during the washing process, which has historically been seen as the first line of defense in regards to antimicrobial properties of the egg (Board and Halls, 1973; EFSA 2005).

Egg washing procedures may vary by processors but the principle of decontamination of shell eggs remains apparent. Evidence suggests different chemical compounds could potentially interact with the physical barrier components of the eggshell. Kim and Slavik (1996) reported that the cleaning chemicals used in the wash water affect the microstructures of the shell. The more damaged the eggshell surfaces found in their study, the greater the bacterial penetration. Wang and Slavik (1998) reported that the pH of egg washing sanitizer impacted eggshell characteristics, such as the cuticle and calcification of the shell. The authors reported that the quaternary ammonium compound and HOCl, both at pH 7.5, effectively reduced microbial penetration without damaging the eggshell, whereas a $Na_2CO_3$ with a pH of 12 altered eggshell quality and enabled greater bacterial contamination.

The effects of wash water temperature are important to consider for the disinfection of shell surface. Caudill et al. (2010) reported that egg quality parameters including: Haugh Unit values, albumen height, vitelline membrane strength and aerobic bacterial prevalence within the shell matrix were not affected by a series of wash water temperatures. Conversely, Jones et al. (2005) evaluated 6 different temperatures with an exposure time of 60 s. Each washing series maintained pH between 10.5 and 11.5 with a post-washing sanitizing treatment of 200 ppm chlorine solution at 48.9°C. Washed eggs were stored and evaluated for aerobic bacterial levels assessed over a 9-week storage period. Jones et al.

(2005) found shell eggs that were initially washed at 48.9°C with a second washing either at 23.9°C or 15.6°C reduced aerobic bacteria present on the shell surface and reduced *Salmonella* Enteritidis prevalence by 53.33% to 61.8% on the shell and in the membranes, respectively.

To better understand specific standard washing protocols, Hutchison et al. (2004) evaluated *Salmonella* spp. prevalence on the shell surface after spray washing the eggs under different processing conditions. Under the ideal washing protocol, a 5-log reduction of *Salmonella* was achieved on the shell surface with no egg content contamination. The authors' found that the ideal parameters include: a conveyor belt speed of 111 cm/min, a prewash water temperature of 44°C with a spraying pressure of 138 kPa, the wash water temperature containing 3g/L chlorowash at 44°C with a spraying pressure of 262 kPa, the rinse water temperature at 48°C with 2.5 mL/L Quat 800 and a spraying pressure of 262 kPa, and eggs being air dried for 2 min at 42°C. Authors' reported that deviations from the ideal time and/or temperature parameters allowed *Salmonella* contaminant migration through the eggshell to the egg contents.

## Alternative Egg Decontamination and Sanitation Techniques

United States regulations for shell egg processing do not dictate specific egg washing chemicals. However, the chemical that is used should be found on the GRAS list (USDA, 2018a). The federal regulations leave the terms "washing" and "the equipment used for washing" open for interpretation by the processor, as long as operation and equipment are maintained under sanitary conditions (USDA, 2018b). This allows the processor to utilize innovative washing technologies to efficiently decontaminate shell eggs.

Electrolyzed oxidizing water (EOW) is created by electrolyzing weak salt water solution to produce either an acidic (pH2.6) or alkaline (pH 11.4) EOW washing solution. The use of EOW has proven to inactivate bacteria within solutions, in food matrices, and on solid surfaces of equipment in experimental settings (Venkitanarayanan et al., 1999; Kim et al., 2000; Park et al., 2002). Evidence suggests that EOW can reduce *Salmonella* Enteritidis in the washing process for shell eggs by utilizing an alkaline EOW since there is a high pH equivalent to high pH detergents. An acidic EOW solution could be used as a good sanitizer that has a high oxidative reduction potential (Bialka et al., 2004). Implications of EOW during egg washing result in the vaporization of chlorine in the acidic EOW wash water. This not only is a health hazard to operators and corrosive to processing equipment, but also it decreases the wash water's capability to effectively reduce pathogens that are present on the surface of the egg over time (Len et al., 2000). From a food safety standpoint, this technique may be an adequate sanitization method. However, this technique is costly in the sense of damaging equipment and being hazardous to employee health. In the future, safer and more cost efficient improvements will need to be made to this technique in commercial settings.

Pasteurization utilizes heat to destroy bacteria that are present in solutions. Elimination of *Salmonella* risk through pasteurization has proven to be an effective process for shell eggs and egg products (Bermudez-Aguirre and Corradini, 2012; Silva and Giibs, 2012; Jarvis et al., 2016). In addition, pasteurization is approved as a viable heat treatment option by the Food and Drug Administration (FDA, 2011). Hou et al. (1996) evaluated the effectiveness of hot air pasteurization of intact shell eggs to reduce *Salmonella* populations and achieved a 5-log reduction by processing eggs for 180 min at 55°C. An even greater

*Salmonella* reduction was accomplished by using a combination treatment, which is a heated water-bath at 57°C for 25 min followed by hot-air heating at 55°C for 60 min, that resulted in a 7-log reduction. They reported that there was no degradation in shell egg quality. Although these are promising results, the time to achieve a 5-log reduction of *Salmonella* is too long to be implemented in a processing setting. In addition, the immersion of shell eggs into wash water is prohibited in shell egg washing practices due to the potential cross contamination of *Salmonella* from the wash water (USDA 2018b). Exposing inoculated shell eggs to blasts of hot air at 600°C for 8 s intervals significantly reduced the *Salmonella* on the surface of the eggshell with minimal impact on egg quality (Manfreda et al., 2010). Research has shown that hot water pasteurization of shell eggs is capable of reducing *Salmonella* with negative impacts on the functionality of egg contents, such as foaming, volume, and stability (Cunningham, 1995; Herald and Smith, 1989; Day, 2010). Hot water pasteurization may provide an effective decontamination technique, but the damage to the albumen proteins affects the volume and stability of egg contents when incorporated into baked good products.

Hydrogen peroxide is known to have bactericidal properties on aerobic and facultative microorganisms by generating free radical hydroxyl, which is an effective antimicrobial agent (Juven and Pierson, 1996). Hydrogen peroxide, when used as a decontaminating agent, was able to reduce *Salmonella* contamination on the surface of the shell by using a dip treatment method (Padron, 1995). Similar effects have been observed in the work of Cox et al. (2000). They reported that treating *S.* Typhimurium contaminated shell eggs with hydrogen peroxide (1.4%) via dip immersion, followed by application of vacuum pressure for 4 min at 12-13 Hg (0.4 bar), resulted in the elimination of *Salmonella*

without any adverse effects on the hatchability of eggs or chick mortality. Hydrogen peroxide usage has produced promising results when used as a wash water solution for shell eggs. Thus, the dip immersion application of hydrogen peroxide has produced substantial results in preventing *Salmonella* contamination, as compared to a spray washing sanitizer.

Ozone is an effective antimicrobial agent at low concentrations and at low temperatures, while providing potential decontamination treatments in the food industry (Khadre et al., 1999, 2001, 2003). Rodriguez-Romo and Yousef (2005) reported that when challenging *Salmonella* Enteritidis contaminated shell eggs for an exposure time of 3 min of ozone treatment, the *Salmonella* reductions of 5-logs were achieved. The authors reported that ozone is capable of penetrating the eggshell to inactivate *Salmonella* within the egg contents (Rodriguez-Romo et al., 2007). Interactions of ozone with food products may affect the odor and develop off-flavors due to the oxidation of high fat content foods (Kim et al., 2003). To assess the effects of ozone and the high lipid content found within egg yolks, Kamotani et al. (2010) compared the yolk and albumen from eggs treated with ozone versus heat. Visual appearance of the yolk and albumen treated with heat treatment appeared to be cloudier than the ozone treated eggs. In their experiment, cooked eggs were evaluated using the Hedonic and just-about-right scales to assess the overall appearance, aroma, color, flavor, liking, and texture of heat versus ozone treated shell eggs. The results from both the scales showed that there were no significant differences between the ozone or heat treatments. Ozone may provide an effective measure to reduce *Salmonella* on the surface of the shell, but it is not a widely used application throughout the egg industry due to cost effectiveness.

In 2000, the FDA approved the use of ultra violet irradiation for shell egg processing. The maximum allowable dosage for ionizing radiation is up to 3 kGy for shell eggs (FDA, 2015). Serrano et al. (1997) evaluated the sensitivity of gamma irradiation of several *Salmonella* strains that were inoculated either on the surface of the eggshell or within the contents of the eggs. The doses of irradiation used on the eggs were 0, 0.5, 1.0, and 1.5 kGy. Treatment of shell eggs at 0.5 kGy dose resulted in elimination of *Salmonella,* although variations among *Salmonella* strains were observed. Serrano et al. (1997) determined that 1.5 kGy was adequate to reduce *Salmonella* strains by 4-logs on both the surface of the shell and within the egg contents. Irradiation at 1.5 kGy did not affect egg quality, including yolk color and protein quality. Higher irradiation doses of 3 and 4 kGy reduced eliminated bacterial load in the eggs, but resulted in poor egg protein quality, weakened vitelline membranes, and loss of color in the yolk (Wong and Kitts, 2003). More recently, Keklik et al. (2010) achieved a 5.3 log CFU/cm$^2$ reduction of *Salmonella* Enteritidis with a 20 s treatment at 9.5 cm distance between the ultraviolet source and the surface of the egg without any implications for the integrity of the shell. Observations from their research show that temperature of the egg increased with longer exposure times, which could potentially impact internal egg quality. Due to associated health hazards of ultraviolet light, this method of sanitation is approved by USDA Agriculture Marketing Services and is widely implemented within the egg industry.

## Conclusion

Collectively, many risk factors are associated with *Salmonella* contamination with in shell egg production and processing. To minimize the risk factors, a collection of standard operating procedures, sanitation practices, vaccination programs, live operations

management, biosecurity measures, and regulations and federal guidelines, have been developed through extensive research and should be implemented from the farm to the consumer's table. These programs aid shell egg producers and processors in providing safe eggs to the consumer. Researchers continue to strive to improve new live operations management to reduce the risk of *Salmonella* contamination within the flock, whereas others may seek to improve decontamination techniques via egg processing. To ensure that the risk of *Salmonella* contamination may not occur and that safe eggs are produced, the producers and processors are held accountable to adhere to the regulations while trying new and innovative techniques to efficiently produce eggs with minimal risk of pathogen contamination.

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# CHAPTER 3

## IMPACT OF EGG HANDLING PRACTICES ON *SALMONELLA* RISK IN CAGE-FREE EGGS[2]

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**ABSTRACT**

Floor eggs produced in cage-free housing systems are susceptible to pathogen contamination from exposure to fecal material and poultry liter. This study evaluated the penetration of *Salmonella* from contaminated feces on the shell surface of unwashed floor eggs. In the first experiment, two day-old non-washed nestbox eggs from cage-free layers (n = 1,080; 34 - 49 wks of age; stocking density of 0.121 $m^2$/hen) were collected. Four nalidixic acid-resistant (200-ppm) *Salmonella* serotypes: *S.* Enteritidis (SE), *S.* Heidelberg (SH), *S.* Kentucky (SK), and *S.* Typhimurium (ST) were inoculated onto the egg using a (i) fecal paste or a (ii) dip method to achieve a target 5 log CFU/g or mL. Inoculated eggs were stored at 4°C and sampled for 5 wks at weekly intervals. *Salmonella* prevalence on the shell surface, shell and membrane matrix, and the egg contents were evaluated. *Salmonella* spp. was not detected in the contents, regardless of the serotype or the storage period. *Salmonella* prevalence in the shell matrix, across all serotypes, decreased ($P \leq 0.05$) during storage. *Salmonella* prevalence on the shell surface was consistently high during storage, with the exception of SE dip inoculated eggs. ST and SH prevalence in the egg shell matrix was higher during storage as compared to the other serotypes. In the second experiment, SE penetration into the contents of cage-free eggs from SE-contaminated feces was evaluated. Non-washed nestbox eggs (n = 450) were collected from commercial cage-free layers (34, 36, and 38 wks of age) and stored at 25°C. SE-inoculated feces was placed at the equator of the egg to achieve 2, 4, and 6 $\log_{10}$ CFU/g of SE. The egg contents were replaced with either tryptic soy broth (TSB) or TSB supplemented with lysozyme to allow growth of the organism. Inoculated eggs were incubated for 24 h at 37°C and then evaluated for *Salmonella* prevalence. A subsample of eggs were analyzed for any variations in eggshell

quality per replicate. None of the broths were positive for SE, which indicated a lack of penetration of SE into the egg contents during the 24 h storage at 37°C. The current egg handling practices by the industry did not enhance the risk of SE penetration into the cage-free egg contents if exposed to surface contamination from feces.
Key words**:** *Salmonella*, eggshell penetration, cage-free, fecal contamination

## INTRODUCTION

Salmonellosis is a major foodborne illness in the United States. Food attribution studies reported estimates of the relative contributions of salmonellosis from chicken (48%), ground beef (28%), turkey (17%), egg products (6%), intact beef (1%), and pork (<1%) for domestically acquired sporadic human *Salmonella* infections (Guo et al., 2011). The Centers for Disease Control and Prevention (CDC; 2015) reported that nearly 78% of foodborne illnesses from 1990 to 2001 have been linked to *S.* Enteritidis contamination in undercooked or primarily raw shell eggs. In addition, *Salmonella* serotypes, excluding Enteritidis, continue to be a major concern for the shell egg industry and the public health agencies. These serotypes have caused numerous salmonellosis outbreaks. A recent multi-state outbreak of *Salmonella* Braenderup linked to shell egg contamination caused 45 illnesses in 10 states in the U.S. (CDC, 2018). These foodborne illnesses, which relate to enteric pathogens, have steadily increased since 2010, probably due to an increase in clinical laboratory reports these cases as part of the FoodNet system (CDC, 2015).

The United States Food and Drug Administration (US FDA) has published the Final Rule: Prevention of *Salmonella* Enteritidis in Shell Eggs During Production, Storage, and Transportation (Egg Rule). This provides the shell egg industry specific guidelines to follow in order to mitigate the risk of *Salmonella* Enteritidis (SE) from shell eggs. The Egg Rule stipulates that the egg processor should follow SE preventive measures, such as (i) procuring pullets that are SE monitored, (ii) developing biosecurity measures, (iii) controlling rodent, flies and other pests, (iv) cleaning and disinfecting, and (v) regulating refrigeration (FDA, 20018b). Although the Egg Rule requires that eggs be refrigerated by holding and transporting eggs at or below 7.2°C (45°F) within 36 h after the time of lay, it is

difficult to determine the time of lay, especially for floor eggs in a cage-free housing environment.

*Salmonella* can penetrate the internal contents of the egg through deposition during its formation before the addition of the shell in utero (Okamura et al., 2001) and during egg lay via invasion through the pores of the shell (Messens et al., 2005; Holt et al., 2011). The risk of *Salmonella* contamination of the egg contents related to the breaking of the egg from the egg shell and its membranes (shell matrix) has not been elucidated until now. *Salmonella* contamination of the egg contents can occur if *Salmonella* is present in the egg shell matrix. This is possible due to internalization of the organism from the shell surface immediately after lay, secondary to a cooler egg environment as compared to the hen's body temperature. Also, *Salmonella* contamination of the egg contents may occur during refrigeration of the eggs prior to washing, as well as during the processing of the eggs. If *Salmonella* is internalized and deposited in the shell matrix, washing does not eliminate or reduce the populations. Also, certain types of disinfectants or sanitizers may not affect the *Salmonella* organism that is deposited in the egg shell pores during egg washing. This could explain some of the recent non-Enteritidis *Salmonella* outbreaks that have been linked to shell eggs in the U.S. and in Australia.

The egg production environment is replete with reservoirs for *Salmonella*, such as the hen's nesting material, dust, feedstuff, and fecal material, which all in hindsight can potentially contaminate the shell egg surface (Kinde et al., 2005; Snow et al., 2010; Lapuz et al., 2012; Trampel et al., 2014; Wallner-Pendleton et al., 2014). The United States shell egg industry is transitioning laying hen environments from conventional cage systems to cage-free systems because of an increased demand from consumer groups, including hotels and

restaurant industries. These changes have been implemented in the hopes of improving the animal welfare of laying birds.

According to research, housing systems can influence *Salmonella* prevalence within the egg production environment and in laying hens. *Salmonella* contamination has been reported as greater in conventional cage systems as compared to in cage-free housing systems (Methner et al., 2006; Wales et al., 2007; Mahé et al., 2008; Namata et al., 2008; Snow et al., 2010;). Conversely, other research has shown that *Salmonella* contamination is equivalent or even greater in cage-free housing systems than conventional cage systems (Jones et al., 2015, 2016). The risk of fecal contamination on the egg shell surface (dirty eggs) is greater in extensive housing systems. Thus, the risk of *Salmonella* contamination can be greater in cage-free eggs, by either penetration of the organism to the interior contents or deposition in the shell matrix. While shell egg washing may eliminate fecal surface and *Salmonella* contamination, the process does not address the survival of the organisms deposited or translocated into the shell matrix. Since the prevalence of floor eggs from cage-free housing systems is higher, the potential risk for *Salmonella* in these eggs can be greater regardless of shell egg washing and sanitation.

The Egg Rule does not provide any direction on the potential use of floor eggs for human consumption. Since no direction exists, the time lapse between lay and collection of floor eggs is unknown. Therefore, fecal exposure of floor eggs can exacerbate the potential of *Salmonella* penetration from the egg shell surface to the interior egg contents prior to refrigeration and washing. Thus, potentially contaminated floor eggs can be marketed or used for human consumption, as long as they meet the egg grading requirements subsequent to washing.

Currently, the Code of Federal Regulations (FDA, 2018a) requires all shell egg producers to provide instructions on egg cartons, but does not require processors to include an expiration date or a date of lay on the egg carton. Further, the consumer can discern only the processing date from the information provided on the carton and will not be able to distinguish the source of the eggs, as either floor or nest box eggs.

The objective of the research was to evaluate the potential translocation of *Salmonella* from the egg shell surface to the interior egg contents during handling of non-washed eggs and to evaluate the differences between *Salmonella* serotypes. Two experiments were conducted to evaluate *Salmonella* penetration into the interior egg contents from the shell egg surface (i) using different inoculation methods and different serotypes and (ii) by replacing the egg contents with microbiologically rich medium under ideal conditions in the egg interior.

## MATERIALS AND METHODS

### Experiment 1

**Salmonella *Serotypes*.** Four *Salmonella* serotypes (Enteritidis, Heidelberg, Kentucky and Typhimurium) were trained to 200 ppm nalidixic acid (NA) resistance. The serotypes Enteritidis, Heidelberg and Typhimurium were selected based on their implication in various egg-related foodborne illness outbreaks and *Salmonella* Kentucky was selected due to the high prevalence and recovery of this serotype from laying hens (Guard et al., 2015; Jones et al., 2016; Mench et al., 2016). Each trained serotype was resuscitated from -80°C frozen stock by transfer to 10 mL of tryptic soy broth (TSB; Acumedia®, Neogen®, Lansing, MI) with 24 h at 37°C. An aliquot (1 mL) of the culture was

transferred to 200 mL of sterile TSB and incubated for 24 h at 37C and the culture was used for inoculum preparation.

***Egg Collection and Inoculation.*** Non-washed brown shell eggs (n = 1,512) from a single genetic strain, cage-free commercial flock were collected at the North Carolina Department of Agriculture Piedmont Research Station, Salisbury, North Carolina. The flock was managed as described by Anderson (2018). Non-washed eggs (390 per collection cycle) were collected from hens at the age of 32, 34, 36, and 38 wks. The eggs were stored under refrigeration immediately after collection and were packed in an insulated container for transportation to the USDA ARS facility (Athens, GA). The eggs were subdivided into 3 groups and used for inoculation with a specific *Salmonella* serotype. The eggs were stored at 25°C overnight, candled and eggs free of visible cracks were used.

The eggs were assigned to the following inoculation treatments (dip inoculation, feces inoculation, or non-inoculated control). The inoculation methods described by Brant et al. (1965) or Berrang et al. (1991) were followed for dip and fecal paste inoculation. Briefly, the dip inoculation method consisted of preparing a fresh 24 h culture of each *Salmonella* and inoculating the sterile TSB (Acumedia®, Neogen®, Lansing, MI) to obtain ca. 5 log CFU/mL. The eggs (n = 108) were dipped in prepared 25°C inoculum for 10 s, transferred to UV-sanitized flats (5x6 Plastic Flats, Eggboxes.com, Deerfield Beach, Florida) in a biological safety cabinet (BSC) for 1 min to allow the eggs to dry. After drying, the eggs were placed in UV-sanitized egg cartons (12 ct. Foam Carton, Delco Packaging Products Inc, Lawrenceville, Georgia) and stored at 4°C until the appropriate sampling time.

For the fecal paste inoculation method, fresh feces was collected from manure belts from Specific Pathogen Free (SPF) layer flock housed in conventional cages and sterilized

at 121°C for 60 min. Care was exercised to maintain the moisture content of 65% of the fecal material with the addition of sterile water to replicate the natural fecal contamination on the eggshell surface of eggs. The sterilized feces was inoculated with 24 h culture of *Salmonella* (10 µL/g) to obtain the target population in the fecal paste. Inoculated fecal paste (250 mg) was dispensed onto the equator of each shell egg. Fecal paste inoculated eggs (n = 108) were aseptically placed on UV-sanitized flats to dry in a BSC for 1 min, similar to the dip-inoculated eggs and subsequently stored as described for the dip-inoculated eggs. For each of the inoculation methods, a control group (54 eggs) was used, and was treated the similarly, but without the *Salmonella*.

Three separate inoculum cultures were prepared individually and served as individual replications. Serial dilutions were prepared in sterile phosphate buffered saline (PBS; BP665-1PBS; Fisher Scientific, Hampton, NH) and plated in duplicate on Bismuth Green Sulfa agar ($BGS^{NA}$;Acumedia®, Neogen®, Lansing, MI) supplemented with NA (200 ppm). The plates were incubated for 24 h at 37°C and typical colonies were enumerated. The *Salmonella* populations in the dip solution and inoculated feces were similar ($P \geq 0.05$), with mean population of 5.4 ± log CFU/mL or g (Table 1). Control, non-*Salmonella* inoculated eggs were negative for Nalidixic acid resistant-*Salmonella* for each sampling period.

***Determination of* Salmonella *spp. Prevalence***. *Salmonella* penetration of the eggs (external shell rinse, shell matrix, and egg contents) was determined for each egg. Six inoculated and 3 control, non-inoculated eggs were sampled per replicate at weekly for a total of 6 sampling times from refrigerated storage (4°C). *Salmonella* prevalence on/in the eggs was determined using methods described by Gentry and Quarles (1972), Musgrove et

al. (2005) and Jones et al. (2002) to determine the prevalence on egg shell surface, egg shell matrix (egg shell and membranes) and egg contents, respectively. Briefly, egg shell rinse (Gentry and Quarles, 1972) was obtained by shaking the egg in a sterile polyethylene bag (Whirl-Pak, Nasco, Sandy Springs, GA) containing 10 mL of sterile PBS for one min. The egg was aseptically removed, a one mL aliquot of 10x TSB was then added to the rinse sample, massaged for 1 min and incubated for 24 h at 37°C. The incubated medium was streaked on $BGS^{NA}$ (200ppm) for isolation and incubated as described. After rinsing, the egg was sanitized with a 70% ethanol dip for 10 s and dried for 1 min in a BSC before collecting the contents and sampling the shell matrix.

The method described by Musgrove et al. (2005) was followed to obtain the shell matrix sample. The egg contents (albumen and yolk) were removed and the shell interior was rinsed with sterile PBS. The shell was crushed in a sterile gloved hand and placed in a 50 mL conical tube. Sterile PBS (10 mL) was added to the tube, macerated for 1 min using a sterile glass rod, and TSB (1 mL; 10x) was added. The samples were gently shaken, incubated for 24 h at 37°C and prevalence of *Salmonella* was determined by streaking the incubated solution on $BGS^{NA}$ and incubated 24 h at 37°C.

The method described by Jones et al. (2002) was used to determine the prevalence of *Salmonella* in egg contents. Briefly, the egg contents were separated into a sterile polyethylene bag (B01489WA, Whirl-Pak, Nasco), TSB (0.5 mL of 10x) was added and the sample was stomached at for 1 min. The sample was incubated 24 h at 37°C and streaked for isolation on $BGS^{NA}$ (200 ppm) and incubated 24 h at 37°C. Samples (plates) with typical colonies on $BGS^{NA}$ was determined and treated as positive for *Salmonella*.

***Statistical Analysis*.** The *Salmonella* prevalence data was analyzed with JMP 13 software (SAS Institute, 2017) using Pearson's Chi Square test for independence using inoculation method, replication and week of storage as main effects. The results were reported as prevalence percentage.

## Experiment 2

**Salmonella *Serotype*.** Nalidixic acid resistant *Salmonella* Enteritidis was used and the inoculum was prepared similar to the Experiment 1.

***Egg Collection*.** Non-washed brown shell eggs (n = 360) from hen ages of 34, 36, and 38 wk were collected from a single genetic strain flock of commercial cage-free aviary laying hens. Eggs were packaged in insulated containers (812, Thermosafe, Arlington Heights, IL) and transported to USDA ARS facility (Athens, GA) at 25°C. Upon arrival, eggs were candled and eggs free of visible cracks were used in the study. The eggs were stored at 25°C for no longer than 24 h prior to inoculation.

***Egg Preparation and Inoculation*.** Eggs were assigned to either TSB or $TSB^{L}$ (TSB containing lysozyme; 0.12 mg/large egg; J60701; Alfa Aesar; Tewksbury, MA) treatment. Egg contents were removed and replaced with the either TSB or $TSB^{L}$. Briefly, a small hole was punched at the blunt end of the egg using a 16-gauge needle (305198, BD, Franklin Lakes, NJ). The egg was inverted and placed on a glass funnel with rubber grommet on an Erlenmeyer flask (1 L) and vacuum (100 kPa) was applied to each egg to remove contents. The egg internal surface was then rinsed using sterile water and vacuum was applied again to remove any residual egg contents. Eggs were dried on a sterile pulp flat at 25°C (Eggboxes.com; Deerfield Beach, Florida) and the appropriate volume of either TSB or $TSB^{L}$

was transferred to the interior of the egg and sealed using molten glue and was allowed to harden before egg surface inoculation.

The fecal inoculation method, described in Experiment 1 was used (Berrang et al., 1991). Three 24 h individual *Salmonella* Enteritidis cultures were prepared, in a similar manner as in Experiment 1, to inoculate at different concentrations (low, medium, and high). The mean *Salmonella* Enteritidis populations were 2.1, 4.3, and 6.2 log CFU/g.

***Determination of* Salmonella *Enteritidis Prevalence*.** The glue plug on the blunt egg of the egg was removed aseptically and an aliquot (100 µL) was streaked for isolation on $BGS^{NA}$ and incubated for 24 h at 37°C. An additional sample (100 µL) was transferred to Rappaport-Vassiliadis (RV, Acumedia®, Neogen®, Lansing, MI) and incubated for 24 h at 42°C. Samples were then streaked for isolation onto $BGS^{NA}$ and incubated 24 h at 37°C. Plates showing typical *Salmonella* colonies on $BGS^{NA}$ were considered to be positive for the organism.

***Physical Egg Quality***. Egg quality parameters (n = 30) the volume of the shell, shell strength, shell deformation, and shell thickness were assessed from each egg collection following methods described by Jones et al. (2018). The volume of the shell was determined by scanning an intact egg with a laser-imagining device, VolScan Profiler (VSP300, Texture Technologies, Hamilton, MA). Each egg was placed (blunt end up) in the VolScan Profiler using mounting putty to hold the egg steady. The egg volume was measured using scan settings of rotation speed per second with a vertical step of 2 mm. The static compression shell strength was measured with a texture analyzer (TA-XTplus; Texture Technologies, Hamilton, MA). The texture analyzer was equipped with a 10 Kg load cell and 60 mm diameter aluminum compression disc (TA-30, Texture Technologies,

Hamilton, MA). The egg was presented on its side in an egg holder (TA-650, Texture Technologies, Hamilton, MA), exposing the equator of the egg to the compression test. The test speed of 2 mm/s and trigger force of 0.001 Kg was used. Mean shell thickness was determined from three readings along the equator using a shell thickness gauge (Model 25-5; Melrose, MA).

***Statistical Analysis*.** Differences in *Salmonella* Enteritidis prevalence was determined using JMP 13 software (SAS Institute, 2017) using Pearson's Chi Square test, with *Salmonella* Enteritidis concentration in the inoculum and lysozyme treatment as main effects. The data collected from physical egg quality parameters was assessed using One-way ANOVA (JMP 13; SAS Institute, 2017) for mean separation. Hen age was the main effect. Intact eggs (n = 90) was analyzed for physical eggshell parameters.

## RESULTS

### Experiment 1

Differences in *Salmonella* prevalence between inoculation methods (dip vs. fecal paste) and sampling location were observed. *Salmonella* prevalence on the shell surface (Figure 1) remained high during storage for all *Salmonella* serotypes whereas it decreased in the shell matrix (Figure 2). *Salmonella* was not detected in any of the egg contents throughout the duration of storage.

**Salmonella *Enteritidis Prevalence*.** The prevalence of *Salmonella* Enteritidis (SE) decreased considerably during storage overtime when observing both inoculation methods in the shell matrix sampling ($P \leq 0.05$; Figure 3). Up to 50% of the sampled eggs for either inoculation treatment had SE present within the shell matrix at initial sampling. SE

prevalence diminished significantly after 1 week of refrigerated storage. *Salmonella* prevalence decreased to 22% by week 2 and beyond.

**Salmonella *Kentucky Prevalence*.** *Salmonella* Kentucky (SK) prevalence for both inoculation methods in the shell matrix decreased after initial sampling ($P \leq 0.05$; Figure 4). Eggs sampled from both inoculation treatments had up to 44% *Salmonella* prevalence subsequent to inoculation in the shell matrix and reduced to below 23%.

**Salmonella *Heidelberg Prevalence*.** *Salmonella* Heidelberg (SH) prevalence within the shell matrix differed between dip- and feces-inoculated eggs ($P \leq 0.05$; Figure 5). Eggs initially sampled had up to 61% SH prevalence for both methods of inoculation. After 1 week of refrigerated storage, SH prevalence decreased to <23% in the shell matrix. SH prevalence continued to diminish to undetectable levels in dip-inoculated shell matrix throughout refrigerated storage. *Salmonella* prevalence inoculated with fecal paste method showed 22 to 61% prevalence in the shell matrix.

**Salmonella *Typhimurium Prevalence*.** The prevalence of *Salmonella* Typhimurium (ST) within the shell matrix significantly decreased during refrigerated storage ($P \leq 0.05$; Figure 6). Dip-inoculated eggs were all positive for initial sampling in the shell matrix whereas only 50% of feces-inoculated eggs positive for ST in the shell matrix. During refrigerated storage, ST prevalence in the shell matrix decreased but remained above 20% for both treatments of inoculated eggs throughout three weeks of refrigerated storage.

## Experiment 2

Throughout the duration of experiment 2, no differences of monitored eggshell quality parameters existed between the egg collections, which were observed for shell strength, shell deformation, shell thickness, shell volume, and egg weight (Table 2).

Prepared inoculum concentrations were within the desired concentration range for each assigned treatment (Table 3). *Salmonella* Enteritidis was not detected within the contents of inoculated eggs for either assigned enrichment treatments for each replicate. The data from this study shows that *S.* Enteritidis is unable to contaminate egg contents of unwashed eggs laid from cage-free hens (34, 36, 38 weeks of age) via contaminated feces when challenged within 48 h of lay.

## DISCUSSION

The U.S. produced 106 billion eggs in the year 2017, with 92.1 billion produced as table eggs for consumption (NASS, 2018). The majority of these eggs are produced under conventional caged housing system, with some proportion being produced under a variety of cage-free systems. The European Union (EU) passed a directive on welfare of laying hens in 1999 requiring phasing out of conventional cage system for egg production by the year 2012. This was a significant change in the production system and affected both the egg quality and safety as the breeds that are appropriate for cage-free housing systems are often selected based on their behavior and performance. In conventional housing systems, the birds are housed in a an enclosure, with water and feed provided, generally the manure is continuously removed from the environment through use of belt systems and the eggs are collected and transported to collection locations separate from the laying environment. This allows for significant risk mitigation in terms of fecal contamination of the egg shell surface. However, the transition to cage-free environment allows for the birds to lay eggs in a variety of locations, and in some cases on the floor or substrate that may contain freshly laid feces and potentially contaminated with *Salmonella* and other enteric pathogens. Thus, there is a significant risk of egg shell surfaces to be contaminated with the organism in

cage-free housing systems. This is especially true in the case of floor eggs. Published literature indicates that eggs produced from furnished cages, cage-free systems, and aviaries have a higher shell bacterial prevalence compared to those from conventional caged systems (De Reu et al., 2005; Harry, 1963; Quarles et al., 1970; Mallet et al., 2006; Wall et al., 2008; Hannah et al., 2011).

Once the feces is deposited on the shell, regardless of whether during lay or from deposition of the egg on substrate or freshly laid feces, *Salmonella*, if present, can survive for extended periods of time on the shell. Recent *Salmonella*-related outbreaks from low water activity or moisture foods (peanut butter, chocolate, dried vegetable powders, etc.) has brought to realization among the scientific community the ability of the organism to survive for extended periods of time in such food products and environments. Park et al. (2015) reported greater survival of *Salmonella* on shell eggs that were surface inoculated with either inoculated-chicken feces or by spot inoculation with *Salmonella*-inoculated Phosphate Buffered Saline (PBS), with reductions in Salmonella populations by 1-log to 5-log CFU/egg, with greater reductions observed at higher temperatures, and lower humidity values in the storage environment. Regardless, the authors reported survival of *Salmonella* on the egg shell surface during extended storage for 21 days. The current FDA regulations require the eggs to be stored or transported at 7.2°C within 36 h of lay, if they were not processed within that time frame. Thus, *Salmonella* on the egg shell surface can survive for extended periods of time prior to washing of the eggs. However, the authors did not evaluate the potential translocation or penetration of *Salmonella* to the shell matrix or the internal contents of the eggs. Lublin et al. (2015) reported survival of *Salmonella* Infantis (initial population of 3.8 log CFU) on egg shell surfaces subsequent to storage for 10 weeks,

regardless of the storage temperature (6 or 26°C). The authors reported *Salmonella* reductions of up to 2 log CFU were observed on egg shell surface during storage at either of the storage temperatures.

Pasquali et al. (2016) reported differences in survival of *Salmonella* serotypes on egg shell surfaces stored at 4, 8 and 20°C. Greater survival of *Salmonella* serotypes Typhimurium and Tennessee were observed at 4°C compared to Enteritidis. Populations of *Salmonella* Enteritidis decreased by 4-logs during storage at 4°C for 28 days, compared to minimal reductions (≤ 0.5 log CFU) for Typhimurium and Tennessee. In the current study, *Salmonella* survival was observed throughout the storage period regardless of the serotype, although prevalence was evaluated rather than the populations. It is possible that the *Salmonella* populations in the feces may not be similar to the populations observed subsequent to inoculation of the eggs. Determining the *Salmonella* prevalence, as well as populations on surfaces of the shell eggs prior to washing, will provide information on potential survival of the organism during storage prior to washing and sanitizing processes. Regardless of the survival of *Salmonell*a on egg shell surfaces and the *Salmonella* populations, the washing of eggs with detergents with high pH values followed by sanitizing rinses provides adequate reduction in *Salmonella* populations and thus, the risk of *Salmonella* on shell eggs. While eggs for consumption as shell eggs are to be washed and sanitized in the U.S., the same is not true in other parts of the world where egg washing is prohibited. In such situations, *Salmonella* survival on the shell egg surfaces can present significant risk to the consuming public and a potential risk of cross-contamination during handling of eggs in the kitchen.

While numerous studies evaluated the survival of *Salmonella* on the shell surface, as well as penetration to the interior contents, none of the studies determined the prevalence of the organism in the shell matrix (egg shell and the membranes). The risk of *Salmonella* is greater when the organism present on the egg shell can penetrate the shell, the shell membranes, and enter the egg contents. Even if the contamination of egg contents does not occur, the presence of *Salmonella* in the shell matrix can present a significant risk of cross contamination during breaking of the egg in the consumer kitchen. To prevent this potential cross contamination of *Salmonella* organisms secondary to penetration, refrigeration of processed shell eggs is crucial. However, significant risk remains in other countries that do not mandate washing of the eggs and refrigerated storage. In addition, the washing process and refrigeration do not eliminate the risk of *Salmonella* from shell eggs. Preparation of eggs through mild heat treatment (such as sunny side-up) may present a risk as contamination that can occur from the egg shell matrix during breaking of the eggs.

Further, research has shown that *Salmonella* deposited on the surface of the egg can translocate to the interior of the egg subsequent to lay due to shrinkage of egg contents resulting from temperature differential between the hen's body temperature and the ambient temperature (Berrang et al., 1999; Musgrove et al., 2005). In addition, ability of *Salmonella* to penetrate to the interior contents through the shell and the shell membranes has been reported extensively (De Rue et al., 2005, 2006; Gantois et al., 2009; Jones et al., 2015, 2016).

Gole et al. (2014) reported significant penetration of surface inoculated *Salmonella* in non-washed eggs, with 62% and 56% positive in low and high translucent eggs, respectively. Translucency indicates the appearance of lighter colored regions of the shell

that can be observed during candling, probably due to the presence of liquid within the shell due to structural differences in the egg shell (Talbot and Tyler, 1974; Chousalkar et al., 2010). Although egg shell is considered the first barrier to microbial contamination from the egg shell to the interior contents, it is ineffective as microbial penetration has been reported for a variety of organisms, including *Salmonella* (Berrang et al., 1999). However, we did not observe penetration of the egg shell and contamination of the egg contents, regardless of the serotype or the inoculation method. It is possible that the inhospitable conditions of the egg contents, especially the albumen may have contributed to the non-survival or non-detection of the organism in the egg contents. Also, the cage-free eggs are produced from different breeds of birds compared to those of the conventional caged housing systems, with different shell characteristics (Holt et al., 2011). However, egg shell characteristics such as the shell thickness, number of pores, and area of egg shell surface (volume of the egg) were reported to have minimal impact on *Salmonella* penetration of the shell eggs (De Reu et al., 2006; Chousalkar et al., 2010).

The egg shell characteristics of shell strength, shell deformation, shell thickness, volume of the egg shell and the egg weight were similar ($P \geq 0.05$) regardless of the egg collection time (34, 36 and 38 weeks of hen age). In the subsequent experiment (2), the egg contents were replaced with nutritionally rich microbiological medium (TSB) to evaluate the effect of lysozyme, included in a treatment at the same concentration as would normally be present in albumen. Regardless, *Salmonella* did not penetrate the interior contents of the egg, which indicates that the shell characteristics of the eggs that were used in the study were not different between replications. Therefore, *Salmonella* penetration or

the experimental conditions (24 h incubation at 25°C) were not adequate to allow for *Salmonella* penetration into the contents (Jones et al. 2014, 2015).

The current regulatory requirements in the U.S. to refrigerate eggs destined for consumption as shell eggs and the time limit (36 h) prior to refrigeration do not present significant risks in terms of *Salmonella* prevalence on the egg shell surface or the penetration of surface *Salmonella* to the interior of the egg contents. However, the presence of *Salmonella* or higher rates of surface contamination due to deposition of eggs in manure, subsrtate or freshly laid feces may allow *Salmonella* penetration into the shell matrix. Differences in shell matrix penetration were observed between *Salmonella* serotypes, with Typhimurium and Heidelberg showing greater penetration. Organisms deposited in the shell matrix present greater risk as the organisms are protected from egg washing and sanitizing chemicals and can potentially result in cross contamination during breaking of the eggs in consumer kitchen or at restaurants.

Eggs produced from cage-free housing systems, especially the floor eggs, could present a greater risk of *Salmonella* contamination. Future research should focus on the prevalence and concentrations of *Salmonella* on the egg shell and shell matrix of floor eggs produced from cage-free housing systems. Under current processing conditions, floor eggs from cage-free housing systems should be prevented from entering the human food chain, especially the shell egg market, until further research can elucidate the food safety risks.

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Table 1: *Salmonella* spp. inoculation concentrations per replicate for extended storage experiment 1.

| **Serotype** | **Rep 1** | | **Rep 2** | | **Rep 3** | |
|---|---|---|---|---|---|---|
| | Dip ($Log_{10}$ CFU/mL) | Fecal ($Log_{10}$ CFU/g) | Dip ($Log_{10}$ CFU/mL) | Fecal ($Log_{10}$ CFU/g) | Dip ($Log_{10}$ CFU/mL) | Fecal ($Log_{10}$ CFU/g) |
| *S.* Enteritidis (32 Weeks) | 7.73 | 7.75 | 7.71 | 7.72 | 7.73 | 7.73 |
| *S.* Typhimurium (34 Weeks) | 7.69 | 7.71 | 7.69 | 7.69 | 7.72 | 7.69 |
| *S.* Heidelberg (36 Weeks) | 7.72 | 7.73 | 7.75 | 7.73 | 7.74 | 7.74 |
| *S.* Kentucky (38 Weeks) | 7.73 | 7.72 | 7.74 | 7.75 | 7.74 | 7.72 |

Table 2: Physical egg quality per replicate of eggs in experiment 2.

| Replicate (Hen Age) | Shell Strength (g*s) ± SE | Shell Deformation (mm) ± SE | Shell Thickness (nm) ± SE | The Volume of the Shell (mL) ± SE | Egg Weight (g) ± SE |
|---|---|---|---|---|---|
| 1 (34 Weeks) | 5144.0 ± 134.65 | 0.44 ± 0.01 | 40.2 ± 0.40 | 53.3 ± 0.67 | 58.7 ± 0.72 |
| 2 (36 Weeks) | 5481.0 ± 139.37 | 0.45 ± 0.01 | 40.7 ± 0.42 | 53.0 ± 0.70 | 58.5 ± 0.74 |
| 3 (38 Weeks) | 5465.7 ± 141.93 | 0.46 ± 0.01 | 41.4 ± 0.42 | 53.7 ± 0.70 | 59.2 ± 0.74 |
| P - value | 0.15 | 0.55 | 0.11 | 0.78 | 0.78 |

Table 3: *Salmonella* Enteritidis inoculum concentrations for feces-inoculated floor eggs.

| *Salmonella* Enteritidis Inoculum Concentrations | Low ($2\ Log_{10}$ CFU/mL) | Medium ($4\ Log_{10}$ CFU/mL) | High ($6\ Log_{10}$ CFU/mL) |
|---|---|---|---|
| Rep 1 ($Log_{10}$ CFU/g) | 2.0 | 4.3 | 6.3 |
| Rep 2 ($Log_{10}$ CFU/g) | 2.2 | 4.4 | 6.3 |
| Rep 3 ($Log_{10}$ CFU/g) | 2.0 | 4.2 | 6.1 |
| Average | 2.1 | 4.3 | 6.2 |

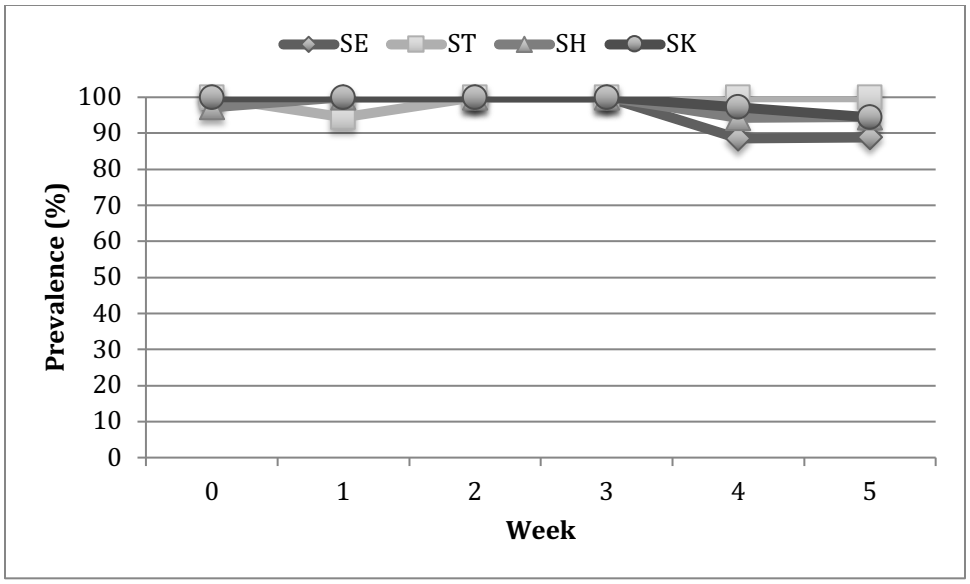

Figure 1: Comparison *Salmonella* prevalence between serotypes for the shell surface throughout refrigerated storage.

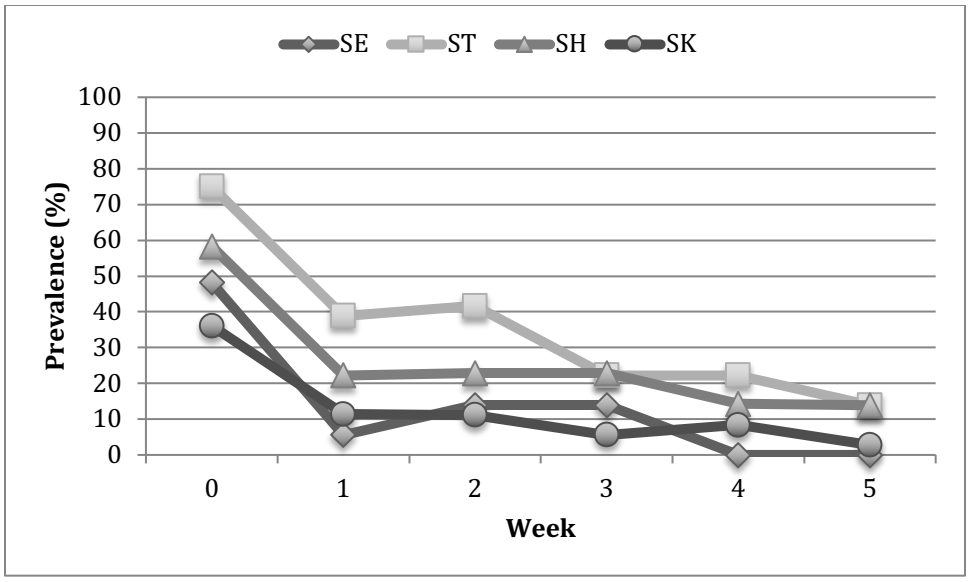

Figure 2: Comparison *Salmonella* prevalence between serotypes for the shell matrix throughout refrigerated storage.

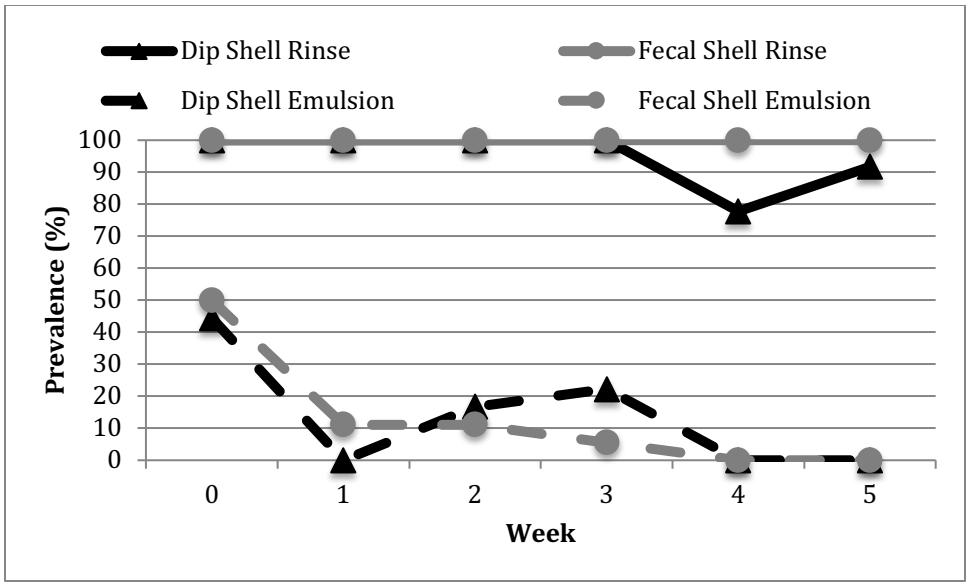


Figure 3: Comparison of *S.* Enteritidis prevalence between dip-inoculated and feces-inoculated treatments for shell surface and shell/membrane prevalence throughout refrigerated storage.

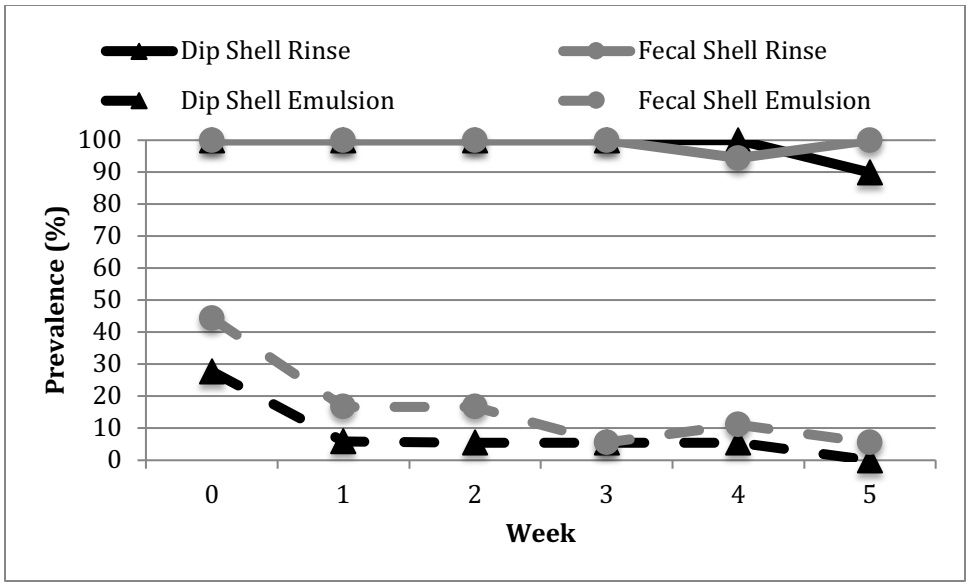

Figure 4: Comparison of *S.* Kentucky prevalence between dip-inoculated and feces-inoculated treatments for shell surface and shell/membrane prevalence overtime

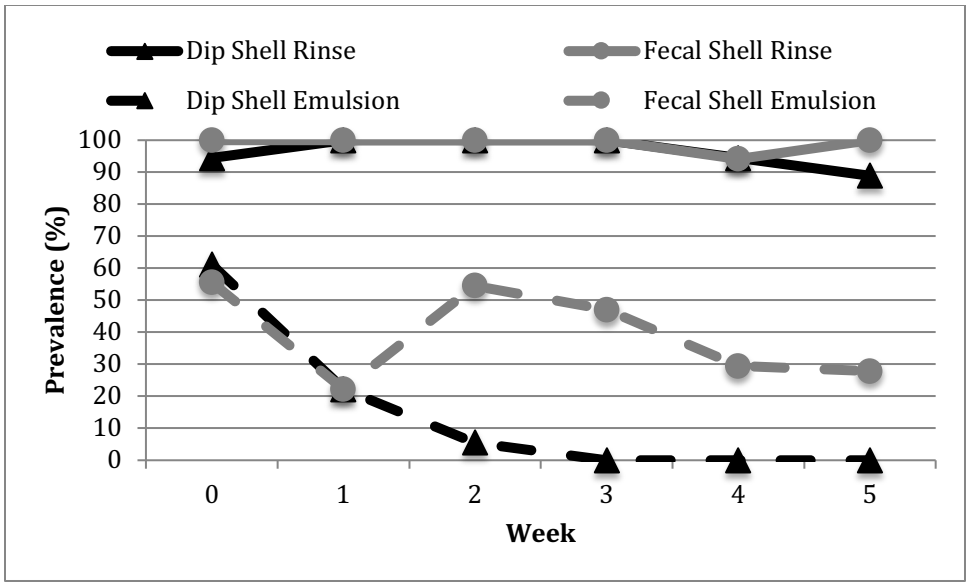


Figure 5: Comparison of *S.* Heidelberg prevalence between dip-inoculated and feces-inoculated treatments for shell surface and shell/membrane prevalence overtime

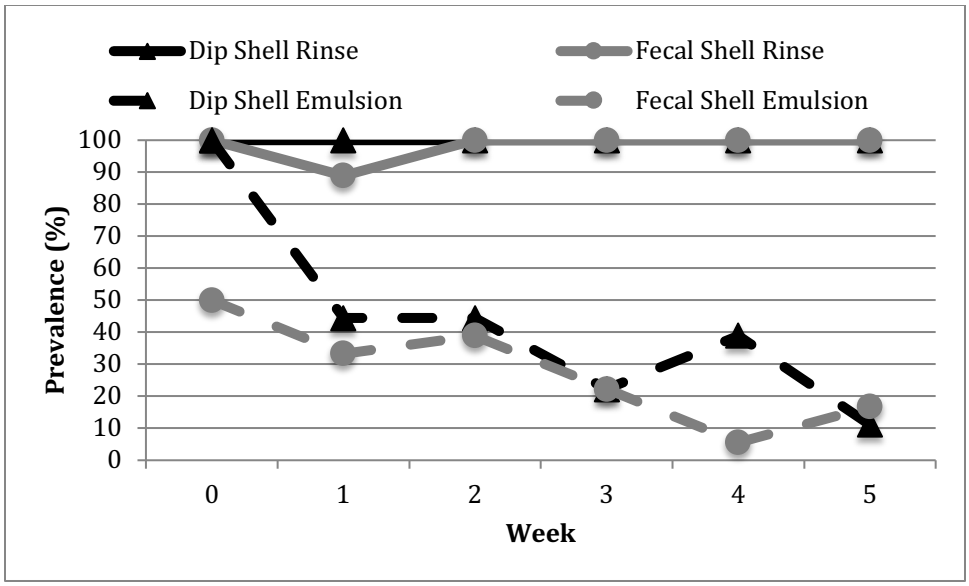


Figure 6: Comparison of *S.* Typhimurium prevalence between dip-inoculated and feces-inoculated treatments for shell surface and shell/membrane prevalence overtime

# CHAPTER 4

## SUMMARY OF THESIS

Current mandated egg-handling practices within the shell egg industry have been developed and enacted from extensive research. The implementation of good on farm management can provide a foundation to reducing the risk of *Salmonella* infection within a laying hen flock. This includes the cleaning, disinfection, and good biosecurity practices of poultry houses, which have proven to reduce naturally occurring *Salmonella* prevalence in the laying hen's environment. From the evidence of this research, *Salmonella* has proven to survive in experimental extended refrigerated storage periods on the shell surface and shell matrix. Other research has shown that *Salmonella* is capable of penetrating the shell and contaminating egg contents under refrigerated conditions. From this research, these findings has shown that when simulated floor eggs that are challenged with *Salmonella* in extend refrigerated storage periods, as well as simulating stagnant floor eggs conditions after lay did not yield *Salmonella* contamination of egg contents. The frequent collection of eggs in a cage-free environment as well as good egg washing practices upon egg collection have can help prevent *Salmonella* contamination of shell eggs during the farm to table process. In addition, safe-handling practices can further prevent cross contamination of *Salmonella* from within the shell matrix to the egg contents during further processing. This research has shown that floor egg contents are not contaminated within 48 h of lay, but to truly evaluate whether or not floor eggs should be allowed within the human food supply,

further research is needed to better understand the current issue within cage-free housing environments.