# INFLUENCE OF HOUSING SYSTEM ON BACTERIAL EGGSHELL CONTAMINATION AND HORIZONTAL TRANSMISSION OF SALMONELLA AND CAMPYLOBACTER AMONG LAYING HENS

by

JACKIE FISHER HANNAH

(Under the Direction of Jeanna L. Wilson)

## ABSTRACT

In the U.S. table egg industry, commercial laying hens are primarily housed in conventional battery cages. Although there are several advantages to cage management, this housing system has been extensively criticized for providing a barren and confined environment that physically restricts laying hens from performing many of their natural behaviors. To address growing hen welfare concerns associated with caged housing and to meet consumer demand for cage-free products, a number of table egg producers have transitioned to alternative, cage-free production systems. A study was conducted to evaluate eggshell bacterial numbers of non-washed and washed eggs from caged and cage-free laying hens housed on all wire slats or all shavings floor systems. Non-washed eggs produced in an all-shavings environment had higher aerobic plate counts (APC, 4.0 $\log_{10}$ cfu/mL of rinsate) than eggs produced on slats (3.6 $\log_{10}$ cfu/mL), which had higher bacterial counts than eggs produced in cages (3.1 $\log_{10}$ cfu/mL). Washing eggs significantly ($P<0.05$) reduced APC levels by 1.6 $\log_{10}$ cfu/mL. The influence of caged and cage-free housing systems on the spread of *Salmonella* and *Campylobacter* among

laying hens was also evaluated. Hens challenged with *Salmonella* (*S.* Typhimurium or *S.* Enteritidis) and *Campylobacter* (*C. coli* or *C. jejuni*) were commingled with non-challenged hens in conventional cages, on all wire slats, or on all shavings floors. There was no significant difference ($P<0.05$) in horizontal transmission of *Salmonella* among non-challenged hens housed in cages (12%), on slats (15%), and on shavings (14%). However, horizontal transmission of *Campylobacter* among non-challenged hens was significantly lower in cages (28%) than on shavings (47%), with slats (36%) being intermediate. The objectives of the final study were to compare the colonization potential of the previously utilized *S.* Enteritidis marker strain to that of a *S.* Enteritidis field strain and the previously utilized *S.* Typhimurium marker strain, and evaluate the effects of a vancomycin pretreatment on *Salmonella* colonization in laying hens. The *S.* Enteritidis field strain and *S.* Typhimurium marker strain colonized the ceca, spleen, and liver/gallbladder at significantly ($P<0.05$) higher rates than the *S.* Enteritidis marker strain. Vancomycin pretreatment had no significant effect on *Salmonella* colonization.

INDEX WORDS: Eggshell bacteria, hen housing system, caged laying hens, cage-free laying hens, horizontal transmission, *Salmonella*, *Campylobacter*, vancomycin

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# DEDICATION

I would like to dedicate this dissertation to my mother, Faith Thomas, who has always been there with unwavering support and encouragement and has made many sacrifices for my successes, and to my daughter Cassidi, who is the absolute joy of my life.

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# CHAPTER 1

# INTRODUCTION

In the United States, commercial laying hens are primarily housed in conventional battery cages as they offer lower production costs and improved egg hygiene compared to cage-free systems (De Reu et al., 2005; Singh et al., 2009), but in recent years, this housing system has been intensively criticized by animal welfare and consumer interest groups for providing a barren and confined environment for laying hens (Singh et al., 2009). Growing concerns regarding hen welfare have prompted changes in the housing of laying hens. Many table egg producers are transitioning from conventional colony cages to alternative, cage-free housing systems, such as aviaries, floor, paddock, and free-range.

The majority of eggs produced by healthy hens are thought to be clean at the time of lay (Mayes and Takeballi, 1983), but despite the type of housing system used, eggs are contaminated to some extent when they come in contact with environmental debris and bacteria after being laid (Harry, 1963; Quarles et al., 1970; Wall et al., 2008). Studies have been conducted to compare the bacteriology of eggs from hens housed in conventional cages to those from hens housed in alternative housing systems. Quarles et al. (1970) found that eggs obtained from hens housed on litter floors have 20 to 30 times more aerobic bacteria on the shell than eggs from hens on wire floors. Furthermore, eggs produced in conventional and furnished cages have been reported to harbor significantly fewer aerobic bacteria on the shell than eggs from aviary and free range systems (De Reu et al., 2005). However, eggs from these three housing systems were reported to have similar levels of Gram-negative bacteria (De Reu et al., 2005). When comparing the

bacteriology of eggs from conventional and furnished cages, studies have shown that eggs from furnished cages have higher bacterial loads on the shell (Mallet et al., 2006; Wall et al., 2008). A small number of studies have evaluated the effects of floor housing systems on eggshell bacteriology, but no studies have evaluated the eggshell bacteriology of hens raised in the same housing system and then placed into caged and cage-free systems with similar environmental conditions before the start of egg production.

Many genera of bacteria, including *Escherichia*, *Micrococcus*, *Salmonella*, *Streptococcus*, and *Staphylococcus*, and have been recovered from the shells of naturally contaminated table eggs (Mayes and Takeballi, 1983; Musgrove et al., 2004). External eggshell contamination can adversely affect the shelf life and safety of eggs. Table eggs are routinely washed in the United States, as well as Australia, Canada, and Japan, to reduce eggshell contamination, thus reducing the potential for egg spoilage and egg associated illnesses (Hutchinson et al., 2004; De Reu et al., 2006). However, washing Class A table eggs is prohibited in the European Union and washed eggs cannot be sold as table eggs (CEC, 2003). This practice is partially due to the historical perception that wetting or washing eggs prior to storage can increase egg spoilage rates (Brooks, 1951; Hutchinson et al., 2003) and more recently, reports that washing can damage the egg's cuticle, which serves as a physical barrier and protects against microbial contamination (Wang and Slavik, 1998).

*Salmonella* and *Campylobacter* have been isolated from commercial laying hens (Camarda et al., 2000; Van de Giessen et al., 2006; Pieskus et al., 2008; Cox et al., 2009), and natural infection occurs by means of the oral route, and following ingestion, *Salmonella* and *Campylobacter* invade and colonize within the intestinal tract (Brownwell et al., 1970; Galan and Curtiss, 1989; Meinersmann et al., 1991). Once intestinal colonization has occurred, both

*Salmonella* and *Campylobacter* can be shed in the feces, thus providing potential for the bacteria to spread within the flock (horizontal transmission) and contaminate the environment. The potential for bacteria to horizontally transmit may be influenced by housing system, as Mollenhurst et al. (2005) identified housing system as a risk factor associated with *Salmonella enterica* serovar Enteritidis infection among laying hens. However, Pieskus et al. (2008) reported no significant difference in *Salmonella* prevalence among hens reared in conventional cages, enriched cages, and aviaries. There are limited data available on the influence of housing system on the transmission of *Salmonella* and *Campylobacter* among laying hens.

After intestinal colonization occurs, *Salmonella* and *Campylobacter* can spread to and colonize within the reproductive tissues (Keller et al., 1997; Camarda et al, 2000) of laying hens and potentially contaminate eggs prior to oviposition. *S.* Enteritidis is an important food safety concern for the table egg industry (Garber et al., 2003; Mollenhorst et al., 2005) as it is the only human pathogen that routinely contaminates eggs (Guard-Petter, 2001). Greig and Ravel (2009) recently analyzed the international food-borne outbreak data reported between 1988 and 2007, and found that 73.7, 15.3, 8.4, and 0.6% of egg associated outbreaks (n=584) were due to *S.* Enteritidis, other *S. enterica, S.* Typhimurium, and *Campylobacter* spp, respectively. The prevalence of *S.* Enteritidis among the internal contents of eggs produced by naturally infected hens has been estimated to be less than 0.01% (Ebel and Schlosser, 2001).

The objectives of this dissertation were to 1) evaluate eggshell bacterial numbers of non-washed and washed eggs from caged and cage-free laying hens, 2) determine the potential for horizontal transmission of *Salmonella* and *Campylobacter* among caged and cage-free laying hens, 3) compare the colonization potential of a *S.* Enteritidis marker strain (original) to that of a *S.* Enteritidis field strain and a *S.* Typhimurium marker strain, and 4) evaluate the effects of a

vancomycin pretreatment on *Salmonella* colonization in laying hens. This dissertation is divided into 6 chapters. Chapter 2 is a literature review in which the U.S. table egg industry, cage and cage-free housing systems, egg formation and contamination, and *Salmonella* and *Campylobacter* colonization are discussed. Chapter 3 describes a study evaluating the effect of housing system and egg washing on bacterial eggshell contamination. Chapter 4 describes a study evaluating the effect of housing system on the horizontal transmission of *Salmonella* and *Campylobacter*. Chapter 5 describes a study conducted to compare colonization potential of three different strains of *Salmonella* in antibiotic pretreated and non-pretreated laying hens. A summary and conclusion of the studies from Chapters 3, 4, and 5 are included in Chapter 6. The appendices are included to provide data from two additionally conducted studies that were not thoroughly discussed. Appendix A describes methods used to obtain (*in vitro*) the growth curves of four different strains of *Salmonella*. Appendix B describes methods used to evaluate the colonization potential of an additional marker strain of *S*. Enteritidis in broilers.

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# CHAPTER 2

# LITERATURE REVIEW

## United States Table Egg Industry

Chicken eggs are the primary focus of the United States table egg industry, and unless otherwise stated, all references in this text will refer to chicken eggs. Being the world's second largest producer of table eggs, the U.S. egg industry is an important part of the national and international food systems (FAO, 2007; Earley, 2009). In 2009, the 284 million laying hens in the U.S. produced 77.7 billion table eggs (NASS, 2010). It has been estimated that 58% of those eggs were sold at the retail level, 31% were further processed for retail, foodservice, or export, 8% were used in the foodservice industry, and 3% were exported (UEP, 2010a).

Per capita consumption of shell eggs and egg products has varied considerably over the past century. During the early 1900s, total consumption fluctuated between 271 and 331 eggs per person (ERS, 2010). By 1945, per capita egg consumption peaked at 404 (ERS, 2010). This substantial increase in consumption was primarily due to a shortage of animal proteins and a strong demand for dried egg products during World War II (Fulmer, 1948; Bell, 1995). After 1945, egg consumption gradually decreased and by 1973, it fell below 300 eggs per person. The downward trend in per capita consumption continued, and hit a record low of 230 eggs in 1995. Since 2000, annual consumption has fluctuated between 244 to 253 eggs per person (ERS, 2010). As overall per capita egg consumption declined, there was a decrease in shell egg consumption and an increase in egg product consumption (ERS, 2010). Changing lifestyles that allowed less time for breakfast preparation and health concerns associated with cholesterol intake and heart disease are regarded as the primary reasons for the decline in shell egg consumption

(Austic and Nesheim, 1990; Putnam and Allshouse, 1999). Egg product consumption has increased as a result of consumers incorporating more egg containing prepared and processed foods into their diet.

Over the past century, the U.S. egg industry has undergone many geographical, structural, technological, and economical changes (Bell, 1995; Bell, 2002a) that have greatly impacted the way eggs are produced today. In the 1890s, egg production was centered in the Corn Belt states where feed was largely available or near highly populated areas to provide eggs for large city markets; therefore, the early egg industry was more dominant in the North Central and North Atlantic states (Bell, 1995). In 1898, the top ten egg producing states were Iowa, Ohio, Illinois, Missouri, Kansas, Indiana, Pennsylvania, New York, Texas, and Michigan. Through 1980, the aforementioned states, along with California, Minnesota, and Wisconsin, continued to lead the nation in egg production. An increase in contract egg production, development of an efficient system for feedstuffs transportation, favorable climate, and lower land and labor costs were all factors that brought the egg industry to the Southeastern region of the U.S. during the 1960s and 1970s (Austic and Nesheim, 1990; Bell, 1995). In 2009, the top ten egg producing (includes table and hatching egg production) states were Iowa, Ohio, Pennsylvania, Indiana, California, Texas, Georgia, North Carolina, Arkansas, and Minnesota (NASS, 2010).

As the egg industry has grown and expanded, the number of egg producing farms has decreased, dropping from 5 million in 1900 to less than 1,000 in 1999 (Bell, 2002a). This trend was principally due the increase in contract production during the 1960s and 1970s (Bell, 1995). Contract production provided growers with more overall investment capital, potentially allowing them to finance larger operations, and reduced the risk of price fluctuation, with a guaranteed

egg price included in the contract. A survey conducted by Watt Publishing Company in 1972 showed that 35% of the eggs in the U.S. were produced under contract. As contracting became more popular and company operations expanded, egg production began to move from family owned farms to company owned farms. From 1994 to today, the percentage of the nation's laying flock that is company owned, has increased from at least 72% to 95% (Bell, 1995; UEP, 2010a).

Another important factor that has changed with the growth of the egg industry is flock size. In the early 1900s, approximately 90% of the commercial eggs in the U.S. were produced on general farms by small, free range flocks with 100-300 hens (Bell, 1995). As specialized farms became more common, flock size increased to 1,000-10,000 hens. A statewide study conducted in California from 1925 to 1965, found that the number of hens per farm progressively increased from 1,651 to 23,158. Flock size continued to increase as egg production shifted toward company operations and ownership became more concentrated. Some of the earlier complexes had 20,000 to 50,000 hens per house. Today, it is common for companies to have 100,000 or more hens per house and over one million hens per complex (Bell, 1995; Bell 2002a). From 1980 to 1995, the number of companies with one million or more laying hens increased from 22 to 43. Currently, there are 61 egg producing companies with flocks of one million laying hens or more, and 13 companies with flocks of five million or more (UEP, 2010a). Over the past century, the nation's flock size and total egg production have increased considerably with the expansion of the egg industry. In 1890, there were approximately 100-125 million hens in the U.S. that produced 9.8 billion eggs, and by 2000 there were 270 million hens producing 71.7 billion table eggs (Bell, 1995; ESMIS, 2006).

Technological advances made in the poultry industry have significantly impacted modern day egg production. Management of hens in cages, perhaps one of the most important technological advances made, became increasingly popular as this type of housing resulted in increased flock size, improved hen and house hygiene, reduced disease transmission, reduced labor requirements, reduced production costs, and improved nutrition management (Bell, 1995; Green et al., 2009; Singh et al., 2009). It has been estimated that 95% of all U.S. table eggs are produced by caged laying hens (UEP, 2010b). Large, multiple bird cages that are either stacked or offset in multiple-tier arrangements are commonly used in the table egg industry. To accommodate large scale egg production, these cages are often equipped with automated systems that deliver feed and water to the hens, and collection belts that gather and transport eggs. The development of automated processing and packing equipment and the incorporation of processing facilities in egg production complexes have also made the egg industry more efficient. With the capability of processing between 72,000 and 144,000 eggs per hour, commercial facilities process eggs from both in-line and off-line operations to increase productivity and keep equipment running at capacity (Bell, 2002a). Prior to farm electrification in the 1930's, egg production was seasonal with hens primarily laying eggs in the spring and the summer. Eggs would be stored during times of peak production and then made available in the winter (Bell, 1995). By using artificial lighting, another important development, producers have successfully extended the laying period and productivity of hens. Production is no longer seasonal and fresh eggs are available to consumers year-round. Other technological advances such as environmentally controlled housing to regulate temperature and adjust ventilation, vaccine development to reduce disease and improve flock health, and genetic selection to

improve productivity and egg quality have also contributed to the growth and development of the egg industry.

In the national and international table egg industry, commercial laying hens are primarily housed in conventional or battery cages (Bell, 1995; Pohle and Cheng, 2009). Of the 77.7 billion table eggs produced in the U.S. in 2009, it is estimated that 95%, or 68.1 billion were produced by hens housed in conventional cages. Although there are several advantages to cage management, this housing system has been extensively criticized for providing a barren and confined environment for the laying hens (Singh et al., 2009; Tactacan et al., 2009). To address growing animal welfare concerns associated with caged housing, a portion of table egg producers have transitioned to alternative, cage-free production systems (Green et al., 2009). Alternative systems include free-range, aviaries, and floor pens. The remaining 5% of U.S. table eggs were produced by hens housed in these alternative, cage-free systems (UEP, 2010b). Furnished cages, which contain nest boxes, dusting areas, and perches, are also an alternative to conventional cages, but eggs produced in these systems are not considered cage-free.

The majority of eggs produced by healthy hens are thought to be clean at the time of lay (Mayes and Takeballi, 1983), but eggs are contaminated to some extent when they come in contact with environmental debris and bacteria after being laid (Harry, 1963; Quarles et al., 1970; Wall et al., 2008). Washing eggs generally removes external eggshell contamination, which can adversely affect the shelf life and food safety of eggs. In the early part of the twentieth century, there was much global debate over the feasibility of washing eggs because it was thought that this practice, especially prior to cold storage, encouraged premature spoilage and diminished overall egg quality. Egg washing was under consideration in the U.S., and by the end of the 1940s, it was a widely accepted practice (Hutchison et al., 2003). Studies have shown

that washing eggs effectively reduces eggshell bacterial levels. As reported by Moats (1981), washing reduced bacterial counts by an average of 1.9 $\log_{10}$ cfu/eggshell. Washing significantly ($P<0.0001$) reduced total surface bacteria from 4.0 to 1.2 $\log_{10}$ cfu/egg (Hutchison et al., 2004) and aerobic bacteria from 4.6 to 1.9 $\log_{10}$ cfu/mL of rinsate (Musgrove et al., 2005). Other countries, such as Japan and Australia, eventually allowed table eggs to be washed. However, the European Union (EU) never adopted this practice and currently prohibits the washing of Class A table eggs (CEC, 2003).

Although table eggs are routinely washed in the U.S. today, federal regulations require that only USDA graded eggs be washed prior to human consumption. The requirements for shell egg washing are detailed in 7 CFR Part 56, entitled 'Regulations governing the voluntary grading of shell eggs' (AMS, 2008). The current commercial egg washing process consists of a wetting, washing, rinsing, and drying stage (Hutchison et al., 2003). Spraying a continuous flow of water on the eggs to pre-wet them is permitted as long as the water is able to drain away from the product. Pre-wetting is done to soften up and loosen any debris on the eggshell. Water that is used to pre-wet and wash the eggs should be 32.2°C or higher or at least 6.7°C warmer than the internal temperature of the eggs being washed (AMS, 2008). Only approved compounds and detergents, which must be GRAS (generally regarded as safe) substances that are in compliance with 21 CFR 178.1010, can be added to the wash water. To control the bacterial load, wash water pH should not fall below 10 (Kinner and Moats, 1981; Musgrove et al., 2005). Rinse water must be the same temperature as the wash water and contain at least 100 but not more than 200 ppm available chlorine or its equivalent. Eggs are dried by passing under an air blower that removes surface water from the eggshell (Hutchison et al., 2003). USDA (AMS, 2008) requires that eggs are adequately dried before they are packaged. Other processing procedures that are

commonly employed in the commercial egg industry are oiling, candling, grading, weighing, sorting, and packaging (AMS, 2000). Eggs are lightly coated with a food grade mineral oil to reduce the rate of carbon dioxide and moisture loss. Candling, a process that uses light to help determine egg quality, allows operators to identify and remove eggs with cracks, irregular shells, blood spots, and meat spots. With automatic weighing equipment, eggs are individually weighed and sorted according to official weight classes. Automatic packaging equipment is used to place eggs into cartons, close the cartons, and stamp the cartons with a production code (AMS, 2000).

Commercial table eggs are produced in either in-line or off-line facilities. In an in-line operation, eggs from multiple layer houses are transported by a common conveyor belt to an on-site processing facility where they are processed, packaged, and shipped (Knape et al. 2002). Automated egg collection begins in the morning and generally continues through one work shift (~ 8 hours). Most modern production facilities are large in-line operations (AMS, 2000). In off-line facilities, eggs are produced in layer houses that may not be fully integrated with the processing facility (Knape et al. 2002). Off-line eggs are collected daily (3 times a day is recommended to maintain quality), placed in flats or on carts, stored in an on-site egg cooler, and transported to a processing facility at a later date (AMS, 2000). Data reported by Knape et al. (2002) show that eggs from various sites within off-line processing facilities have significantly higher counts of aerobic bacteria on their shells (by an average of 1.5 $\log_{10}$ cfu/mL after contact with re-circulated wash water, 1.5 $\log_{10}$ cfu/mL after sanitizer treatment, 1.6 $\log_{10}$ cfu/mL at packaging) than eggs from the same sites within in-line processing facilities. In-line facilities are designed to transport eggs from the layer house to the carton in one continuous operation; therefore, in-line eggs are at least one day fresher than off-line eggs and there is less time for organic material to adhere and become fixed to the eggshell and for microbial populations to

increase (Knape et al., 2002). Eggs from off-line facilities are stored prior to processing and, because organic material has more time to adhere to the shells, these eggs are more difficult to clean. Similarly, Cox et al. (1994) found that after subjecting nest clean and nest dirty broiler hatching eggs to the same sanitation treatment, nest dirty eggs were not cleaned as efficiently as nest clean eggs, and that the level of aerobic bacteria recovered from treated dirty eggs was below that of untreated clean eggs.

**Caged Housing**

Experimentation with housing laying hens in cages began in the mid 1920s, shortly after scientists discovered that indoor confinement was possible with dietary supplementation of vitamin D (Hartman and King, 1956). After a series of tests in 1926 at the Ohio Agricultural Experiment Station, D.C. Kennard found that hens kept in wire cages produced eggs with strong shells and minimal breakage, had a seemingly lower mortality, performed well without roosts or nests, and did not suffer from sore feet on wire (Hartman and King, 1956). Various producers throughout the U.S. began housing laying hens in cages during the early 1930s, and by the mid to late 1940s, caged housing was commonly accepted (Hartman and King, 1956; Bell, 1995). Housing laying hens in cages became increasingly popular around the world in the 1960s and 1970s (Tauson, 1998), and today, the United Egg Producers (UEP) estimate that 90% of the eggs produced worldwide and 95% of the commercial table eggs produced in the U.S. are from caged layers (UEP, 2010b).

Single Comb White Leghorns are the most common type of chicken used in the U.S. commercial table egg industry (Bell, 2002b). The Leghorn breed is a Mediterranean type chicken that was imported to North America from the Italian port of Livorno (Leghorn) during the nineteenth century (Delany, 2003; Kerje et al., 2003). In the early days of the egg industry,

breeders only used pure Leghorn lines to produce commercial pullets. However, it is more common for breeders today to cross two or more Leghorn lines that have been individually bred for superiority in specific production areas so the female offspring will have positive production traits from both parental lines (Bell 2002b). Some of the production traits that Leghorn breeders genetically select for are hen livability, egg weight, egg production, eggshell quality, interior egg quality, and feed-to-egg conversion (Bell, 2002b; Muir, 2003). As a result of these genetic improvements, commercial laying hens are currently able to efficiently produce over 300 eggs per year (Cheng, 2010). In order to survive the domestication process, animals must adapt to their environment, and Leghorns have been highly selected for egg production in battery cage systems (Pohle and Cheng, 2009; Cheng, 2010).

When cages were originally designed to house laying hens, they were single bird units that were built out of wood and had wire floors (Bell, 1995). Single bird cages still exist, but they are rarely used in the commercial egg industry. Modern cages can hold between 5 and 10 hens, depending on the strain of the hen and the size of the cage. To optimize welfare, UEP (2010b) recommends that white and brown hens have a minimum of 67 and 76 square inches of usable space per bird, respectively. Cages are typically made out of welded wire, sheet metal, plastic, or combinations of the three (Bell, 2002c), and have either wire or plastic slatted floors, although plastic floors are not very common (Appleby, 1998).

Modifications in cage design and deployment since the mid 1920s have been driven by the animal behavioral and health benefits, as well as the economic efficiencies they offer the producer. Cages commonly have sloped floors that allow eggs to roll away from the hens, thereby reducing the number of eggs that are damaged or eaten (Appleby, 1998). Cages also prevent hens from laying floor eggs, which is a common problem in alternative housing systems

(Bell, 1995; Appleby, 1998). Another advantage of cages is that hens are efficiently separated from their feces (Duncan, 2001; Wall et al., 2008); an important factor in controlling fecally transmitted diseases (Appleby and Hughes, 1991; Bell, 1995). Reducing the incidence of disease benefits the hens and the producer. The advantages in controlling behavior and health are partially due to the fact that caged laying hens are commonly kept in small groups (Appleby and Hughes, 1991). Although there have been conflicting reports regarding the effect of group size on aggression, some have suggested that aggression toward other hens is less frequent in caged systems because stable hierarchies, based on individual recognition are commonly formed and there is less competition for resources (Appleby et al., 2004; Cooper and Albentosa, 2004; Shinmura et al., 2006). A low stocking density can also reduce the risk of disease transmission by reducing contact with several other hens and their feces (Appleby, 1998).

Modern cage systems are economically efficient because they allow producers to place large numbers of hens per house and increase overall stocking density. When compared to alternative facilities, modern cage systems offer lower production costs and more efficient use of resources as they require less land and energy to house a given number of hens (De Reu et al., 2005; Earley, 2009; Green et al., 2009). Labor requirements are also reduced in caged systems with feed and water being automatically delivered to the hens and eggs being collected by conveyor belts (Tauson, 1998; Pohle and Cheng, 2009). Eggs from modern cage systems ultimately cost less than their cage-free counterparts because producers are able to spread production expenses over more birds and reduce the costs per dozen eggs (Tauson, 1998; Bell, 2002c; Earley, 2009). Cage management also makes it easier for employees to observe the hens, results in cleaner eggs, and minimizes broodiness (Bell, 2002c).

Although conventional cages offer lower production costs and improved hygiene, these systems have been excessively criticized by animal welfare and consumer interest groups for providing a barren and confined environment for laying hens (De Reu et al., 2005; Singh et al., 2009). The major disadvantage associated with conventional cages is that hens are restricted from performing natural behaviors such as nesting, dust bathing, and perching (Keeling, 2004; Hester, 2005). Research has shown that hens are inclined to perform these behaviors, but because they are not required for survival, the resources needed for such behaviors are not included in modern cage systems (Keeling, 2004). The hen's ability to perform other natural or comfort behaviors including wing flapping, limb stretching, body shaking, preening, litter scratching, foraging, and running are also restricted in these systems (Appleby and Hughes, 1991; Appleby, 1998; Keeling, 2004; Hester, 2005). Furthermore, cages can have adverse effects on the physical condition of laying hens. Due to increased pressure from wire floors and lack of natural wear, laying hens housed in cages tend to have feet with more lesions, fissures, and hyperkeratosis and twisted or overgrown toe nails (Tauson, 1980; Abrahamsson and Tauson, 1997; Taylor and Hurnik, 1994; Duncan, 2001). Studies have found that hens housed in conventional cages have lower bone density and strength than hens housed in alternative systems because their opportunities for exercise and movement are more restricted (Moinard et al., 1998; Michel and Huonnic, 2003; Tactacan et al., 2009). Hens housed in battery cages are also more likely to trap body parts such as the head or neck and they tend to lose more feathers (Appleby and Hughes, 1991; Bell, 2002c).

The welfare of animals in husbandry systems was first reviewed in the Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems, the Brambell Report, December 1965 (HMSO London, ISBN 1 10 850286

4). They proposed that all farm animals should have the freedom to 'stand up, lie down, turn around, groom themselves, and stretch their limbs'. As a result of the Brambell Report, the UK Farm Animal Welfare Advisory Committee was formed, and in 1979 the FAWAC released a press notice with provisions that should be made for farm animals in five different categories (FAWC, 2009). These standards became known as the Five Freedoms and they are now defined as follows:

1. **Freedom from Hunger and Thirst**-by ready access to fresh water and a diet to maintain full health and vigour.
2. **Freedom from Discomfort**-by providing an appropriate environment including shelter and a comfortable resting area.
3. **Freedom from Pain, Injury, or Disease**-by prevention or rapid diagnosis and treatment.
4. **Freedom to Express Normal Behavior**-by providing sufficient space, proper facilities and company of the animal's own kind.
5. **Freedom from Fear and Distress**-by ensuring conditions and treatment which avoid mental suffering.

One of the alternative production systems that has been developed to address growing concerns that conventional cages exceedingly compromise hen welfare is the furnished cage. These systems were first developed in the mid 1970s (Bareham, 1976; Elson, 1976), and they have since been thoroughly evaluated through applied research to determine what effects they may have on hen welfare, performance, and behavior (Duncan et al., 1992; Appleby et al., 1993; Appleby and Hughes, 1995; Pohle and Cheng, 2009; Tactacan et al., 2009). Modified to include nest boxes, dusting areas, and perches, furnished cages are meant to maintain some of the

advantages associated with conventional cages, while reducing some of their restricted movement disadvantages (Tauson, 2005; Pohle and Cheng, 2009).

The primary advantage of furnished cages is they enable hens to perform some of the natural behaviors that they are otherwise deprived of in conventional cages. These behaviors include nesting, dust bathing, perching, and litter scratching. Several studies have shown that hens are strongly motivated to lay their eggs in nests (Duncan and Kite, 1989; Cooper and Appleby, 1995, 1996). The incidence of feather picking, which can escalate to cannibalism and adversely affect productivity, is reduced when litter or some type of loose material is made available to the hens in cage systems (Appleby and Hughes, 1991). Providing perches in furnished cages helps improve muscle and bone strength as they allow for vertical movement (Appleby, 1998). Nest boxes and perches also serve as a refuge, allowing hens to escape the potentially aggressive behaviors of cage-mates. Furthermore, furnished cages allow producers to house hens in smaller groups than cage-free facilities, a potentially important factor in minimizing aggression (Appleby et al., 2004). The commonly identified disadvantages of furnished cages are an accumulation of feces in various parts of the cage (Tauson 1998, 2005), an increased number of dirty and cracked eggs (Appleby 1998; Wall et al., 2002; Tactacan et al., 2009), and a higher incidence of sternum-keel deformations associated with perches (Appleby, 1998; Tauson, 2002). Additionally, increased production and labor costs associated with furnished cage management will result in eggs from these systems being priced considerably higher than eggs from battery systems (Appleby, 1998).

The effects of conventional and furnished cages on mortality and egg production have also been extensively studied. Mortality can be largely dependent on management practices, laying hen strain, and hen behavior; therefore it should not be used as the only criterion when

assessing different housing systems (Weitzenburger et al., 2005). However, Abrahamsson and Tuason (1997) and Tactacan et al. (2009) found hen mortality to be similar in conventional (3.9 and 4.0%, respectively) and furnished (5 hens/cage=2.8 and 5.6%, respectively) caged systems. Research has also shown that egg production in both caged systems is comparable (Smith et al., 1993; Appleby et al., 2002; Tactacan et al. 2009).

Increasing public opposition to housing commercial layers in conventional cages has led to the development and implementation of alternative systems intended to improve hen welfare (Earley, 2009; Singh et al., 2009). Animal welfare and consumer interest groups are primarily concerned with the fact that hens housed in conventional cages are physically restricted and unable to perform many of their natural behaviors. These concerns have lead to the development and proposition of legislation in the U.S. and other countries to ban conventional cages and implement more animal-friendly production systems (Singh et al., 2009). In the U.S., animal advocacy groups have presented such legislation in 13 states. Although legislation has failed in 6 states and is still pending in 5 states (Earley 2009), their attempts have been reasonably successful. California voters approved the implementation of Proposition 2 in 2008, which will prohibit housing laying hens in conventional cages, sows in gestational pens, and calves in veal crates beginning in 2015 (California, 2008). The proposition, also known as the Standards for Confining Farm Animals, prevents laying hens from being housed in a manner that would not allow them to lie down, stand up, turn around freely, or fully extend their limbs. Similar legislation was presented to state officials in Michigan, and House Bill 5127, meant to amend the Animal Industry Act, was signed into law in 2009 (Michigan, 2009). This bill requires that laying hens be housed in the same manner outlined in Proposition 2. Banning caged systems may ultimately eliminate egg production in these states. The investments and increased costs

that will be required to replace cage facilities and maintain alternative facilities will be passed on to consumers through higher egg prices. Consumers are then likely to purchase eggs that were produced in other states at lower costs (Earley, 2009).

In 1999 the European Agricultural Commission presented legislation to ban conventional cages and require producers in EU to convert all laying hen facilities to either furnished cages or cage-free systems by January 2012 (European Commission, 1999). Savory (2004) predicts that this ban will adversely impact commercial egg production in the EU and lead to an increase in the importation of low cost eggs.

**Cage-free Housing**

Recent transitions toward cage-free management represent a restoration of early industrial practices and an attempt to return to an agrarian way of housing laying hens (Bell 2002d). Prior to the widespread implementation of battery cages, it is unlikely that the terms 'caged' and 'cage-free' were commonly used with reference to egg production. Laying hens of the early egg industry were kept in houses, under shelters, or on pastures with unrestricted range, and flocks were often moved about a farm to maintain hygienic conditions and provide fresh resources (Bell, 1995). To accommodate the increase in flock size associated with specialized farming, producers increasingly housed hens indoors, often in facilities with littered floors and outdoor access (Elson, 2004). Despite the growing popularity of cage management, some egg producers continued to house laying hens in cage-free facilities, and over the past forty years these systems have been modified to improve hen welfare, reduce disease transmission, and optimize housing density (Elson, 2004). The ideal cage-free system balances hen welfare and health, with consumer preferences and economic productivity (Singh et al., 2009).

While white laying hens derived from the Leghorn breed are primarily used for caged egg production, brown laying hens are primarily used for commercial cage-free egg production. Brown laying hens used in the commercial table egg industry today have been derived from several dual-purpose breeds including the Barred Plymouth Rock, Rhode Island Red, Rhode Island White, Australorp, and New Hampshire (Scott and Siversides, 2000). The shells of eggs produced by brown laying hens are pigmented with biliverdin-IX, zinc chelate, and protoporphyrin-IX (Kennedy and Vevers, 1976; Butcher and Miles, 1995), and are brown in color. Although there is no difference in the nutritional content of white and brown eggs, some consumers prefer brown eggs over white eggs because they are often thought of as being more natural and healthier (Scott and Silversides, 2000). The selection of brown egg lines for egg production has fallen behind that of white egg lines by many years, as white laying hens have been extensively bred for optimal egg production in battery cages (Scott and Silversides, 2000). To accommodate consumer and legislative demands for cage-free egg production, companies have begun to select brown egg laying hens for optimal egg production and their ability to survive in cage-free housing systems (O'Sullivan, 2009).

A variety of alternative, cage-free production systems are used in the table egg industry. Cage-free laying hens can be kept in houses with all litter, wire, or slat floors, or a combination of litter and either slat or wire floors (Bell, 2002d). Fully wired or slatted floors are less common than fully littered floors, and cage-free systems intended for pullets hatched after January 1, 2010 must contain a small amount (15% of total space) of litter (UEP, 2010b). Open floor houses, also known as single tier or barn systems, commonly feature nest boxes and automated egg collection belts, as well as automated feeding and watering systems. The inclusion of perches in these production systems is variable (Earley, 2009). However, UEP

guidelines (2010b) state that cage-free facilities intended for pullets hatched after January 1, 2010 must contain 6 inches of elevated perch space for 20% of the birds. Cage-free laying hens can also be housed in aviaries and free-range systems (Taylor and Hurnik, 1994; Bell, 2002d; Tauson, 2005). Aviaries are traditional open floor systems that have been modified to include multiple tiers of wire or slat platforms. These systems optimize the use of vertical building space and increase overall stocking density (De Reu et al, 2005; Anderson, 2009). Hens generally have access to nest boxes, feed, and water on each tier. Free-range laying hens are kept in cage-free houses and given access to outdoor foraging areas, such as pastures or croplands (Bubier and Bradshaw, 1998; Bell, 2002d; Elson, 2004). This outdoor area should be enclosed to contain the flock and reduce the threat of predators. Large areas may be divided into several paddocks, which are used in rotation to prevent over foraging and fertilization (Zeltner and Hirt, 2003; Anderson, 2009). Free-range systems can be relatively complex and take on several forms as there are currently no U.S. standards defining the size, quality, or availability of the outdoor enclosure.

Cage-free systems have various advantages and are often viewed as systems that optimize laying hen welfare. Much like furnished cages, non-cage systems provide the resources for hens to perform most of their natural behaviors. The majority of cage-free facilities contain nest boxes for laying eggs, loose material for dust bathing and foraging, and perches or platforms for roosting (Appleby and Hughes, 1991; Elson, 2004). The availability of a substrate for scratching or foraging helps prevent overgrown toe nails and reduces the incidence of feather pecking (Appleby and Hughes, 1991). Perches and platforms allow for increased vertical movement, which improves skeletal and muscular strength (Appleby, 1998; Michel and Huonnic, 2003).

Cage-free laying hens also have more freedom of movement with space to walk, run, stretch, and fully extend their wings (Koelkebeck and Cain, 1984; Hester, 2005).

One of the most controversial welfare issues facing the table egg industry today is hen housing, and social criticisms of the matter are often driven by a public opinion that is uninformed, moderately informed, or misinformed of the scientific and industrial evidence regarding cage management (Savory, 2004). Nevertheless, increasing public opposition to the use of battery cages has impacted consumer demand for cage-free products (Savory, 2004). To address the concerns regarding hen welfare, several restaurant chains such as Burger King, Wendy's, Quizno's, and Subway, have agreed to incorporate a certain percentage of cage-free eggs into their food products. Furthermore, some companies, including Wolfgang Puck, Ben and Jerry's, Omni Hotels, and Google have chosen to use only cage-free eggs. At the retail level, the demand for cage-free eggs may be leveling off. According to recently published data from Information Resources Incorporated, a firm that tracks checkout data from 34,000 retail stores throughout the U.S., consumers buy caged or regular eggs over cage-free eggs at a margin of 40 to 1. The data also show that only 2 and 1% of the eggs purchased were cage-free and organic/free-range, respectively (UEP, 2010c).

Housing layers in cage-free production systems may not be as beneficial to hen welfare as commonly perceived by critics (Craig and Swanson, 1994). Compared to caged layers, cage-free hens are more susceptible to bacterial and parasitic infections (Permin et al., 1999; Hester, 2005; Tauson, 2005; Fossum et al., 2009). Access to litter or free-range areas and contact between birds and feces increase the risk of infection (Appleby and Hughes, 1991; Fossum et al., 2009). The incidence of bumble foot, a bacterial infection of the foot pad resulting in inflammation, is higher in cage-free systems (Tauson et al., 1999; Tauson, 2002; Hester, 2005).

Numerous studies have reported higher rates of cannibalism and mortality (Michel and Huonnic, 2003; Fossum et al., 2009, Tauson; 2005; Weitzenburger et al., 2005; Voslarova et al., 2006) among hens housed in cage-free systems. There are many economic disadvantages and added production costs associated with cage-free productions. Hens housed in non-cage systems consume more feed (Taylor and Hurnik, 1994; Tauson et al., 1999, Earley, 2009) and lay fewer eggs (Abrahamsson and Tauson, 1995; Voslarova et al., 2006) than those housed in cage systems. Hens housed in cage-free systems may also lay a portion of their eggs on the floor (Singh et al, 2009), which leads to increased labor cost associated with egg collection. The marketability of these eggs may also be compromised if they are heavily contaminated. Documented egg production may be further reduced when there is a high incidence of floor eggs as these eggs are susceptible to being damaged or consumed by pen mates (Tauson, 2002). The production costs (dollars/dozen eggs) are also higher (estimated 40 %) in cage-free systems because overall expenses are spread over fewer hens (Earley, 2009).

Hen welfare is a multifaceted issue and is largely dependent on system management. Welfare will be compromised to some extent in poorly managed systems. All housing systems have advantages and disadvantages, but despite production efforts, the advantages are not always fulfilled and the disadvantages are not always eliminated (Appleby and Hughes, 1991).

**Egg Formation**

An egg is formed by the reproductive system of a sexually mature hen, and in galliformes the reproductive system consists of the left ovary and its oviduct (Johnson, 2000). The ovary of a pullet contains a mass of small ova, with approximately 2,000 being visible to the unaided eye, and each ovum is enclosed by a follicular membrane that is attached to the ovary by a narrow stalk. Over the life span of the laying hen, 250-500 ova will mature and be ovulated for egg

formation (Johnson, 2000). Within the fully functional ovary, there is a follicular hierarchy that results in sequential development and ovulation of mature ova. The yolk filled follicles grow in diameter and increase in volume with the deposition of yolk lipids and proteins, which are transported to the ovary from the liver through the blood. A mature ovum is approximately 40 mm in diameter and weighs around 19 g (Solomon, 1991; Johnson, 2000). The ovary will generally release an ovum once every 24 hours, but the rate of ovulation is dependent upon the lighting regime that hens are subjected to. During ovulation, the follicle ruptures at the stigma and ovum is released into the infundibulum of the oviduct for initiation of egg formation.

The oviduct is made up of five distinguishable regions, and beginning nearest the ovary, these regions are the infundibulum, magnum, isthmus, shell gland (uterus), and vagina (Johnson, 2000). Following ovulation, the released ovum passes into the infundibulum whose primary function is to capture and direct the ovum into the oviduct. This process can take between 15 and 30 minutes. The ovum then passes into the magnum, the longest region of the oviduct, where albumen is secreted and deposited around the yolk. Around 2 to 3 hours are required to complete this process. Albumen is comprised of water and several types of protein, including ovalbumen, ovotransferrin, ovomucoid, lysozyme, and avidin. Tubular gland cells and goblet cells are responsible for the secretion of the majority of the albumen proteins. As the egg rotates in the shell gland and salts and water are added for plumping, four distinct layers (inner thick, inner thin, outer thick, and outer thin) of albumen are formed (Johnson, 2000). The chalazae, twisted fiber-like strands of ovomucin extending from each end of the yolk, is also formed to suspend the yolk in the center of the egg and reduce yolk displacement.

After albumen deposition, the egg passes into the isthmus where the inner (~60 μm thick) and outer (~20 μm thick) shell membranes are deposited (Johnson, 2000). These semipermeable

fibrous membranes are made up of protein, glycoprotein, and collagen, and it is thought that either epithelial or tubular gland cells are responsible for the secretion of these membranes. The egg remains in the isthmus for 1 to 2 hours. From the isthmus, the egg passes into the shell gland where plumping and shell formation occur. The addition of salts and water during the plumping process creates the outer thin layer of albumen, and in doing so, decreases the albumen's protein content (Solomon, 1991). Calcium carbonate makes up 97-98% of the eggshell, while magnesium carbonate, tricalcium phosphate, and organic compounds make up the remaining 2-3%. The calcium used for egg formation is transported to the oviduct through the blood and is secreted by tubular gland cells in the shell gland. Mammillary cores on the surface of the outer shell membrane serve as the foundation for shell formation, and as these cores are calcified, a mammillary knob layer forms. Columns of crystalline calcium carbonate are deposited over the mammillary knobs to form the porous palisade (~200 μm thick) layer of the eggshell. Shell pigments are also deposited in the shell gland. The egg spends approximately 20 hours in the shell gland. From the shell gland, the egg passes into the vagina where the cuticle, a thin waxy layer of proteins, polysaccharides, and lipids, is deposited over the shell. The pigment-rich cuticle is deposited onto the eggshell at about the same time shell deposition reaches a plateau, about 90 minutes prior to oviposition.

An egg is susceptible to bacterial contamination before and after oviposition and is therefore equipped with various physical and chemical antibacterial defenses to protect the internal contents. There are 7,000 to17,000 pores in an eggshell (Mayes and Takeballi, 1983), depending on the size of the egg, and each pore is a potential route for bacterial contamination. Pores can be found all over the egg, although there are more at the blunt end than at the narrow end (Walden et al., 1956), and pore diameter ranges from 0.3 to 0.9 μm (Johnson, 2000). The

cuticle, the outermost layer of the egg, physically impairs bacterial invasion by covering the mouth of each pore and reducing eggshell permeability (Fromm, 1963; Messens et al., 2005). Several factors including humidity, ambient temperature, storage time and temperature, shell abrasion, fumigation, and egg washing can affect the integrity of the cuticle and reduce its overall effectiveness (Fromm, 1963; Mayes and Takeballi, 1983). Another physical barrier meant to reduce bacterial contamination is the eggshell. Shell thickness can affect bacterial penetration, and Sauter and Petersen (1974) demonstrated that eggs with low specific gravity, an indication of thinner shells, are less resistant to bacterial penetration. Alterations to eggshell integrity can also increase the risk of internal contamination (Sellier et al., 2007), as eggs with cracked shells are more likely to have contaminated contents than those with intact shells (Todd, 1996; Widdicombe et al., 2009). Once through the eggshell, bacteria must penetrate both the outer and inner shell membranes before reaching the albumen. The outer membrane is attached to the eggshell and the inner membrane directly surrounds the albumen. Held firmly together with the exception of air cell location, these two semipermeable membranes act as bacterial filters (Mayes and Takeballi, 1983). With finer and more closely packed fibers, the inner shell membrane is more resistant to bacterial penetration than the outer shell membrane (Lifshitz et al., 1964). These authors also reported that the most difficult physical barriers for bacterial cells to breach are the inner membrane, followed by the shell, and then the outer membrane. The last physical barrier that bacteria will encounter is the albumen. The viscous nature of this gelatinous substance hinders bacterial motility.

When bacteria penetrate through the egg's physical barriers, they enter into the albumen where several of the egg's chemical defenses are found. An important function of the albumen is to prevent bacterial contamination of the yolk (Johnson, 2000). Albumen contains several

proteins with bactericidal properties. Ovotransferrin or conalbumin makes up approximately 13% of the protein content found in albumen, and this protein primarily acts as an iron-chelator (Baron et al., 1997; Johnson, 2000). By rendering the iron ions unavailable, ovotransferrin prevents bacteria from multiplying. Ovomucins, insoluble fiber like proteins, are a key component in maintaining albumen viscosity to help reduce bacterial motility (Omana and Wu, 2009). These proteins make up 2-4% of albumen proteins. Lysozyme, which makes up 3.5% of albumen protein, can be detrimental to Gram-positive bacteria by causing the hydrolysis of the 1,4 beta linkages between N-acetylneuramine and N-acetylglucosamine in the peptidoglycan layer of the cell wall (Mayes and Takeballli, 1983). Avidin, an albumen protein present in low concentrations, also inhibits bacterial growth by decreasing biotin levels (Johnson, 2000). Another factor affecting bacterial growth is the alkaline pH of the albumen (Sellier et al., 2007). The albumen pH of a freshly laid egg is around 7.7, but as an egg ages and it loses carbon dioxide, albumen pH increases. According to Mayes and Takeballi (1983), most bacteria do not grow well in an environment with a pH greater than 9.

**Bacteriology of Eggs**

Eggs can become contaminated prior to oviposition as a result of the reproductive tissues being infected or after oviposition when the eggshell comes in contact with environmental surfaces, and these routes of contamination have been identified as trans-ovarian, oviducal, and trans-shell (Bruce and Drysdale, 1994; Board and Tranter, 1995). Trans-ovarian and oviducal routes of contamination are also referred to as vertical routes of transmission, and the trans-shell route of contamination is also referred to as horizontal route of transmission.

Trans-ovarian and oviducal infection can result in direct contamination of the yolk, vitelline membrane, albumen, shell membranes, or eggshell prior to oviposition and colonization

of the reproductive tissues can be a consequence of systemic infection (Gordon and Tucker, 1965; Okamura et al., 2001a; Gantois et al., 2009). After being ingested, bacteria enter into the intestinal tract, and those that are able to invade the intestinal epithelial cells elicit an immune response. Macrophages, large phagocytic white blood cells, then infiltrate the intestinal epithelium where they engulf and potentially destroy the bacterial cells. However, some bacteria are able to survive and replicate within these immune cells, resulting in the systemic spread of infected macrophages to reproductive and other internal tissues of laying hens (Gantois et al., 2009). Although trans-ovarian transmission may be of minimal concern for most genres of bacteria, it is of significant concern for *Salmonella* spp., especially *S. enterica* serovar Enteritidis (Gast et al., 2004), because of its increased affinity for the reproductive tissues and ability to overcome antibacterial defenses during egg formation and survive within the egg (Keller et al., 1997; Okamura et al., 2001b; Gantois et al., 2009). Bacteria can also enter and contaminate the oviduct through ascending infection from the cloaca (Okamura et al., 2001a; Gantois et al., 2009).

Trans-shell contamination occurs when bacteria gain access to an egg after oviposition by penetrating through the shell and several factors affecting the extent of contamination have been identified (Bruce and Drysdale, 1994). From the time an egg leaves the oviduct, several opportunities for eggshell contamination exist as the egg comes in contact with various environmental surfaces, and the extent of contamination is directly related to the cleanliness of these surfaces and the presence of contaminants in the environment in which eggs are laid (Harry, 1963; Bruce and Drysdale, 1994; Board and Tranter, 1995). Some possible sources for eggshell contamination are feces, dust, cage material, bedding material, conveyer belts, nest boxes, broken eggs, and hands. Eggshell porosity and sweating due to temperature differentials

are other factors affecting the degree of trans-shell contamination. The porous nature of the eggshell makes eggs vulnerable to trans-shell contamination. The temperature of an egg at lay is around 42°C and as a freshly laid egg cools to environmental temperature, the contents contract creating negative pressure within the egg that can pull environmental and surface contaminants into the shell through the pores (Bruce and Drysdale, 1994; Berrang et al. 1999). Moisture also affects trans-shell contamination. Water, either in the liquid or vapor state, is essential for bacterial penetration of the eggshell (Board et al., 1979) and a temperature differential between the egg and the water only enhances bacterial penetration (Berrang et al. 1999). The degree of trans-shell contamination is increased by the depletion of the cuticle (Bruce and Drysdale, 1994; De Reu et al., 2006a).

Eggshell aerobic bacterial levels have been reported to range from 2 to 7 $\log_{10}$ (cfu/per shell, with 5 $\log_{10}$ cfu/shell being the average number of bacteria for unwashed eggs (Board and Tranter, 1995). In egg washing studies, Knape et al (1999, 2002) reported an average initial contamination level of 3.9 and 4.6 $\log_{10}$ cfu/eggshell. With the exception of heavily soiled eggs, the apparent cleanliness of an eggshell is a poor indication of the level of contamination (Board, 1966).

Most of the early studies conducted to determine the genera of bacteria present on eggshells focused on hatching eggs. Mayes and Takeballi (1983) summarized the data collected from a number of these studies and reported, in order of frequency, *Micrococcus*, *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Cytophaga*, *Escherichia*, *Flavobacterium*, *Pseudomonas*, *Staphylococcus*, *Aeromonas*, *Proteus*, *Sarcina*, *Serratia*, and *Streptococcus* as the most common genera of bacteria found on the eggshell. Musgrove et al. (2008) sampled table eggs from three different commercial egg processing facilities and used

biochemical tests to identify isolates recovered from eggshell rinsates. The genera isolated from pre-processed eggs were determined to be *Aeromonas*, *Cedecea*, *Chryseomonas*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Listonella*, *Morganella*, *Proteus*, *Providencia*, *Pseudomonas*, *Rahnella*, *Salmonella*, *Serratia*, *Sphingobacterium*, *Vibrio*, and *Xanthomonas*. Yeasts and molds have also been recovered from the surface of table eggs (Musgrove et al., 2005). Gram-positive bacteria are able to tolerate drier conditions and this characteristic enables them to be better suited for survival on the eggshell. However, bacteria that are Gram-negative, are able to multiply at lower temperatures, and have simple nutritional requirements are more likely to survive and grow within the contents of the egg (Board and Tranter, 1995; De Reu et al., 2008). These bacteria are also more likely to cause egg spoilage. Common egg spoilage bacterial genera include *Aeromonas*, *Alcaligenes*, *Citrobacter*, *Escherichia*, *Hafnia*, *Proteus*, *Pseudomonas*, and *Serratia*.

As with any food of animal origin, eggs may be contaminated with bacteria that are capable of causing food-borne illness in humans (Humphrey, 1994). The most important bacterial pathogen associated with eggs and egg products is *Salmonella*. Eggs can become contaminated with *Salmonella* through the trans-ovarian, oviducal, or trans-shell route of contamination. Before the Egg Products Inspection Act was passed in 1970, the majority of salmonellosis outbreaks were associated with *S. enterica* serovar Typhimurium. However, during the mid1960's *S.* Enteritidis began to emerge as a public health concern and by 1990 displaced *S.* Typhimurium as the leading cause of salmonellosis worldwide (Baumler et al., 2000; Guard-Petter, 2001; Gaintois et al., 2009). *S.* Enteritidis is currently the primary cause of egg associated salmonellosis (De Reu et al., 2006b; Gantois et al., 2008; Greig and Ravel, 2009). Greig and Ravel (2009) recently analyzed the international food-borne outbreak data reported

between 1988 and 2007, and found that 73.7, 15.3, 8.4, 1.2, and 0.6% of egg associated outbreaks (n=584) were due to *S.* Enteritidis, other *S. enterica, S.* Typhimurium, *Staphylococcus aureus*, and *Campylobacter* spp, respectively. *Bacillus cereus*, *Clostridium botulinum*, and other bacteria account for the remaining 0.8% of outbreaks. Although *Campylobacter* is commonly associated with poultry and poultry meat products, it is rarely associated with table eggs. Doyle (1984) sampled eggs produced by laying hens known to be positive for *Campylobacter jejuni* to determine the rate of surface and content contamination and was able to recover *Campylobacter* from only two shell surfaces and no egg content (n=226). *C. jejuni* was also recovered from the inner shell and shell membranes of inoculated eggs subjected to refrigeration (Doyle, 1984).

**Influence of Housing System**

Laying hens used in the commercial table egg industry are housed in either caged or cage-free production facilities, but despite housing system, eggs are contaminated to some degree when they come in contact with various surfaces after being laid (Harry, 1963; Quarles et al., 1970; Wall et al., 2008), and the extent of contamination is directly associated with the presence of contaminants in the environment. Therefore, housing system and husbandry conditions can significantly influence eggshell bacterial contamination levels. Various studies have been conducted to compare the bacteriology of eggs produced by hens housed in conventional cages to those produced by hens housed in alternative or cage-free systems.

Providing hens with nest boxes, dust baths, and perches makes the environment of furnished cages more complex than that of conventional cages (Wall et al., 2008). When comparing eggshell contamination levels of eggs produced in conventional cages to those produced in furnished cages, Mallet et al. (2006) reported significantly higher total bacteria and enterococci counts in furnished cages (2.86 and 1.06 $\log_{10}$ cfu/cm$^2$, respectively) compared to

conventional cages (2.52 and 0.77 $\log_{10}$ cfu/$cm^2$, respectively). The presence of nest boxes in furnished cages seemed to influence eggshell contamination as eggs that were laid outside of the nest had increased surface bacterial loads, while nest laid eggs had contamination levels similar to those produced in conventional cages. Wall et al. (2008) also reported significantly higher aerobic bacteria counts, *Enterococcus* counts, and Enterobacteriaceae prevalence among eggs produced in furnished cages. The location of nest boxes, dust baths, and perches in relation to one another can impact egg hygiene and contamination levels (Appleby et al., 2002; Mallet et al., 2006; Wall and Tauson, 2007). The presence of high moisture excreta in cage systems can increase eggshell contamination, as it is more likely to stick to the cage rather than fall through the wire floor (Smith et al., 2000). Fecal material in the nesting and dust bathing areas of furnished cages can also increase the potential for eggshell contamination. However, in an earlier study, De Reu et al. (2005) sampled the shells of intact eggs produced in conventional and furnished cages and found no significant difference between cage system with regard to aerobic and Gram-negative bacterial levels. In addition, Pieskus et al. (2008) found *Salmonella* prevalence among naturally infected laying hens housed in conventional (33.3%) and furnished (26.8%) cages to be similar.

In early studies carried out to determine the effect of housing system on eggshell contamination, surface bacterial levels of hatching eggs produced on litter and wire floors were compared. Eggs produced by hens housed on litter floor systems had on average 15 times more surface bacteria than those produced by hens housed in battery cages (Harry, 1963). Similarly Quarles et al. (1970) found that eggs produced by hens kept on litter floors had 20 to 30 times more aerobic bacteria on the shell than those produced by hens housed on all wire floors. The concentration of airborne bacteria was also greater in the all litter floor system (Quarles et al.,

1970). In more recently conducted studies, Protais et al. (2003) and De Reu et al. (2005) found that eggs produced in aviaries harbored more aerobic bacteria on their shells than those from cage systems. Increased levels of eggshell contamination were linked to higher concentrations of bacteria in the air of aviaries. Eggs from conventional cages have also been reported as having significantly ($P<0.05$) lower *Escherichia coli* and coliform counts than nest and floor laid eggs produced in a cage-free floor system (Singh et al., 2009). When evaluating the microbial populations of nest clean and floor laid hatching eggs, Berrang et al. (1997) found that floor laid eggs (5.9 to 7.6 $\log_{10}$ cfu/egg) had significantly ($P<0.05$) higher levels of aerobic bacteria than nest clean eggs (4.1 to 5.3 $\log_{10}$ cfu/egg).

There have been conflicting reports regarding the influence of housing system on *Salmonella* prevalence among laying hens. Pieskus et al. (2008) showed no significant difference ($P<0.05$) n the prevalence of *Salmonella* between hens housed in cage and aviary systems. Green et al. (2009) found no differences in *Salmonella* prevalence between caged and cage-free laying hens, and De Vylder et al. (2009) concluded that housing laying hens in alternative production systems would not increase *Salmonella* colonization or shedding. One of the main advantages associated with conventional cages is that, because hens are efficiently separated from their feces, the risk for fecally transmitted diseases is reduced. However, with flock size being typically larger in caged housing systems than in cage-free housing systems, Van Hoorebeke et al. (2010) considered housing laying hens in conventional cages a significant risk factor for the spread of *Salmonella*. Other identified risk factors associated with *Salmonella* transmission and housing system are large flock size, facility condition, rodent control, outdoor access, and contact with wild birds, other animals, or their feces. It has been suggested that hens housed in cage systems with wet manure were less likely to become infected with *Salmonella*

Enteritidis than hens kept in a cage system with dry manure, where the potential for *S*. Enteritidis to be transmitted through the air and spread between cages, or by flies is higher (Mollenhorst et al., 2005).

### *Salmonella* and Laying Hens

*Salmonella* spp. are small (0.7-1.5 µm by 2 to 5 µm), rod shaped, Gram-negative, facultatively anaerobic bacteria that are generally motile with peritrichous flagella (Bell, 2004). The optimum growth temperature for *Salmonella* spp. is 37°C (Holt et al., 1994). *Salmonella* belong to the family Enterobacteriaceae and can be differentiated from other bacteria in the family by the fermentation of glucose with gas production, utilization of thiosulfate with hydrogen sulfide production, and the inability to ferment lactose (Cox et al., 2000). *Salmonella* can also be distinguished by agglutination reactions. Each *Salmonella* serovar has a unique combination of surface antigens (O: somatic or outer membrane antigens, H: flagella antigens, Vi: capsular antigens) and this antigenic formula, in combination with homologous antisera, has led to the current recognition of over 2, 500 *Salmonella* serovars (Bell, 2004; CDC, 2006). Phage typing, determined by the sensitivity of bacterial cells to the lytic activity of certain bacteriophages, is another important tool used to further identify *Salmonella* (Ward et al., 1987). This method is often used to trace *Salmonella* in epidemiological studies of food-borne outbreaks (Patrick et al., 2004).

Over the past century, *Salmonella* has been recognized as a causative agent for human illnesses such as food poisoning, typhoid, paratyphoid, septicemia, and sequelae. Although a significant amount of research has been conducted to further the understanding of *Salmonella* ecology and pathogenicity in humans, the microorganism constantly presents new food safety challenges and continues to be one of the most important food-borne pathogens throughout the

world (Bell, 2004; Humphrey, 2004). *Salmonella* spp. cause disease by route of infection and most individuals with salmonellosis develop food poisoning or gastroenteritis (Bell, 2004). Ingested bacteria will multiply in the small intestine, colonize and invade intestinal tissues, and provoke an inflammatory response. Following a 12 to 72 hour incubation period, infected individuals may experience abdominal pains, vomiting, diarrhea, and fever. Healthy individuals usually recover from salmonellosis within 2 to 7 days. The disease may cause more severe symptoms in young children, the elderly, pregnant women, and immunocompromised individuals.

Food producing animals are reservoirs for many zoonotic pathogens, including *Salmonella* (Korsgaard et al., 2008; Santos et al., 2008), and this is partially due to their ability to either persist in the animal intestine or translocate to and invade other internal tissues (Humphrey, 2004). Invasive disease poses a great threat to food safety as contamination can spread to the interior part of a food source, and an important example of this is the continuing pandemic of egg associated salmonellosis caused by *S*. Enteritidis infection (Humphrey, 2004). Egg associated salmonellosis is primarily caused by the consumption of eggs, especially those that are raw or undercooked, (St. Louis et al, 1988; Palmer et al., 2000; De Buck et al., 2004a; Lynch et al., 2006) that have been contaminated with *S*. Enteritidis (Guard-Petter, 2001; De Reu et al., 2006b; Gantois et al., 2008; Greig and Ravel, 2009). Egg contents can become contaminated with *S*. Enteritidis through the trans-shell or trans-ovarian route of contamination, and although it is unclear which route is most important (Humphrey, 1994; Gantois et al., 2009), it is believed that infection of the reproductive tissues (ovary and oviduct) often is the underlying cause for the production of contaminated eggs (Keller et al., 1995; Miyamoto et al., 1997; De Buck et al., 2004a). Prior to the colonization of reproductive tissues, laying hens are likely

infected with *S.* Enteritidis by oral ingestion from an environmental source (Kinde et al., 1996; Gast et al., 2007). Following intestinal colonization, the bacteria can contaminate the reproductive tissues through systemic infection or ascending infection from the cloaca. Although *Salmonella* has been recovered from the reproductive tissues of the laying hen, little is known about the exact site and mechanism of bacterial colonization (De Buck et al., 2004a; Gantois et al., 2009). *Salmonella* has been recovered from the ovaries of experimentally infected hens in several studies (Miyamoto et al., 1997; Howard et al., 2005; Gast et al., 2007) and it has been suggested that the permeability of the capillary endothelia in the ovary contribute to bacterial colonization (Griffin et al., 1984). Thiagarajan et al. (1994) also suggested that *S.* Enteritidis is capable of colonizing preovulatory ovarian follicles by interacting with granulosa cells. *S.* Enteritidis has been recovered from tubular gland cells of the magnum and isthmus (De Buck et al, 2004b).

*S.* Enteritidis is an important food safety concern for the table egg industry (Garber et al., 2003; Mollenhorst et al., 2005) as it is the only human pathogen that routinely contaminates eggs (Guard-Petter, 2001), and the link between egg contamination and hen infection is well established. However, controlling *S.* Enteritidis to reduce the risk of hen infection is a vexing issue for producers because there are several sources for environmental (initial) contamination, niches for bacterial proliferation, and opportunities for horizontal transmission within a laying facility (Guard-Petter, 2001; Mollenhorst et al., 2005). Their efforts are only confounded by the fact that laying hens infected with *S.* Enteritidis typically show no clinical signs of illness to indicate to producers that their eggs may be contaminated (Guard-Petter, 2001). The infection route of *S.* Enteritidis to humans begins with environmental contamination of the housing facility. Flies (Olsen and Hammock, 2000; Holt et al., 2007), rodents (Garber et al., 2003;

Meerburg and Kijlstra, 2007) humans (Guard-Petter, 2001), and wild birds (Craven et al., 2000) have all been identified as possible sources for *Salmonella* transmission. Once inside the housing facility, *S*. Enteritidis must adapt to and multiply within the environment. It has been suggested that the survival of *Salmonella* through the food chain is partially due to its ability to, through a complex regulatory system, respond effectively to environmental changes (Humphrey, 2004). Laying hens can then ingest the bacteria, and upon bacterial proliferation and colonization, become infected with *S*. Enteritidis. Following colonization, *S*. Enteritidis can be shed through the feces and spread throughout the flock. Risk factors associated with horizontal transmission of *S*. Enteritidis transmission include housing system, large flock size, and airborne transmission (Mollenhorst et al., 2005). Research has suggested that during induced molting, a practice commonly used in the table egg industry to rejuvenate the reproductive system and improve eggshell quality and egg production, hens subjected to feed withdrawal are more susceptible to *S*. Enteritidis infection (Holt, 1993, 1995; Ricke, 2003; Dunkley et al., 2007). However, when evaluating the populations and prevalence of *Salmonella* during the production and molting cycle of commercial layers, Li et al. (2007) found no significant difference in *Salmonella* populations, and prevalence was lowest among molted hens. The National Animal Health Monitoring System conducted the Layer ’99 study to estimate the prevalence of *S*. Enteritidis among commercial laying houses in the U.S., and of the houses surveyed, 7% were environmentally positive for *S*. Enteritidis (Garber et al., 2003).

Over the past 20 years, *S*. Enteritidis has been leading cause of salmonellosis worldwide, and eggs are an important vector for transmission of this pathogen to humans. Egg associated salmonellosis is mainly due to the consumption of contaminated egg content. The prevalence of *S*. Enteritidis among eggs produced by naturally infected hens is low. Humphrey et al. (1991)

and Poppe et al. (1992) reported overall *S*. Enteritidis prevalence among egg content as 0.55 and less than 0.06%, respectively. Using available data on the occurrence of *S*. Enteritidis in U.S. laying hens and eggs, Ebel and Schlosser (2000) estimated that one in every 20,000 (0.005%) eggs produced annually would be contaminated with *S*. Enteritidis. This estimated frequency seems low, but it may be misleading. Of the 77.7 billion table eggs produced in the U.S. in 2009, when using the estimation, approximately 3.88 million eggs would have been contaminated with *S*. Enteritidis, and this is a substantial number of potential human exposures. There were 997 outbreaks of *S*. Enteritidis reported in the U.S. from 1985-2003, which resulted in 33,687 illnesses, 3,281 hospitalizations, and 82 deaths (Braden, 2006). Of the 439 (44%) cases where a food vehicle was confirmed, 329 (75%) were egg based or contained egg ingredients. One of the largest recorded outbreaks of salmonellosis in the U.S. was included in the aforementioned study. In 1994, an estimated 224,000 humans in at least 41 states were infected with *S*. Enteritidis after consuming contaminated ice cream (Hennessy et al., 1996). A premix that was used to make the ice cream was transported in tankers that had previously hauled raw, unpasteurized eggs contaminated with *S*. Enteritidis. Failure to properly clean and disinfect the tankers after transporting the eggs resulted in the aforementioned outbreak, emphasizing that the manner in which humans handle eggs is a key factor in reducing egg associated salmonellosis (Braden, 2006).

*S*. Enteritidis is not the only serovar known to colonize the ovaries and oviducts of laying hens and contaminate the internal content of eggs. *S*. Typhimurium, S. *enterica* Hadar, *S. enterica* serovar Gallinarum biovar Gallinarium (*S*. Gallinarum), and *S. enterica* serovar Gallinarum biovar Pullorum (*S*. Pullorum) have also been recovered from the reproductive tissues and eggs of infected hens (Snoeyenbos et al., 1969; Keller et al., 1997; Okamura et al,

2001a, 2001b). Based on gene content, *S*. Enteritidis is closely related to the serovar S. Gallinarum (Porwollik et al., 2004). Unlike *S*. Typhimurium and *S*. Enteritidis, the most common serovars associated with human salmonellosis in the U.S. (CDC, 2006), *S*. Gallinarum and *S*. Pullorum rarely cause illness in humans (Braden, 2006). *S*. Gallinarum and *S*. Pullorum are host specific avian pathogens that produce clinical disease in infected chickens (Barrow, 1994), causing Fowl Typhoid and Pullorum Disease, respectively (Chappell et al., 2009). These diseases have largely been eradicated in the U.S.

Being the predominant serovar associated with laying hens and eggs, *S*. Enteritidis is presumed to possess certain characteristics and capabilities that give it a selective advantage over other serotypes in its capacity to colonize reproductive tissues (Okamura et al., 2001a), and although the exact reason for this preferential association has not been defined, several theories have been considered (De Buck et al., 2004a; Gantois et al., 2009). It has been suggested that *S*. Enteritidis, particularly phage type 4, has a higher affinity for reproductive tissues than other serovars. When laying hens were intravenously inoculated with *S*. Enteritidis, *S*. Typhimurium, *S*. Infantis, *S*. Hadar, *S*. Heidelberg, and *S*. Montevideo, *S*. Enteritidis was recovered from the ovary and preovulatory follicles (7 days post-inoculation) at significantly higher ($P<0.05$) rates than *S*. Typhimurium and *S*. Hadar, the other serovars isolated from these tissue types (Okamura et al, 2001b). *S*. Enteritidis also persisted longer in the blood than any other serovar, suggesting that the bacteria would be more likely to spread to the ovary or oviduct. Research has indicated that *S*. Enteritidis strains producing large amounts of high molecular weight lipopolysaccharides contaminate eggs more efficiently (Guard-Petter et al., 1995; Guard-Petter, 1998). The presence of type 1 fimbriae may also influence colonization, as De Buck et al. (2004c) reported a significantly higher contamination rate among eggs produced by hens infected with a wild type

*S*. Enteritidis compared that of eggs produced by hens inoculated with a mutant strain (lacking type 1 fimbriae) of *S*. Enteritidis. Additionally, this serovar possesses the *yaf*D gene, a gene that is thought to play a significant role in repairing DNA damage caused by albumen. This gene makes *S*. Enteritidis more resistant to the antibacterial properties of albumen than other serovars (Lu et al., 2003). Understanding *S*. Enteritidis specific factors involved in the egg contamination process may be useful in minimizing egg associated salmonellosis (Okamura et al, 2001a).

**_Campylobacter_ and Laying Hens**

*Campylobacter* spp. are slender (0.2-0.9 µm wide by 0.2-5.0 µm long), non-spore forming, spiral shaped, Gram-negative, microarerophilic bacteria that are motile by means of unipolar or bipolar flagellae and often characterized by their corkscrew-like darting motility (Park, 2002; Humphrey et al., 2007). *Campylobacter* can grow at temperatures ranging from 30 and 46°C, and pathogenic species grow optimally at 42°C (Park, 2002). The four thermophilic campylobacters *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are most often associated with human illness (McClure and Blackburn, 2004; Snelling et al., 2005). The genus *Campylobacter* belongs to the family Campylobacteraceae and there are 18 species within the genus (Humphrey et al., 2007). *Campylobacter* spp. are able to reduce nitrate and nitrite, but lack the ability to ferment carbohydrates (McClure and Blackburn, 2004).

*Campylobacter* was first recognized as a human pathogen in 1972 (Dekeyser et al., 1972), and with an increasing incidence of disease, has since become the most common cause of bacterial food-borne diarrheal worldwide (Park, 2002; Butzler, 2004; Humphrey et al., 2007). Most infections are caused by *C. jejuni* and *C. coli*; however, *C. upsaliensis* has become an important pathogen in the developing world (Humphrey et al., 2007). *Campylobacter* spp. also cause disease by infection and most individuals with campylobacteriosis develop acute

gastroenteritis within 12 to 72 hours of ingestion. Although the mechanism by which campylobacters induce disease is not well understood (Nachamkin, 2002; Snelling et al., 2005), it has been suggested that the bacteria disrupt epithelial cell function by invading and colonizing intestinal mucosa or by adhering to intestinal surfaces and producing toxins (Ketley, 1997; Park, 2002). Symptoms of campylobacteriosis include diarrhea, which may be inflammatory with bloody stools, abdominal pain, fever, malaise, nausea, and on rare occasion, vomiting (Humphrey et al, 2007). Infections are generally self-limiting and symptoms subside within 10 days. On rare occasions, patients infected with *C. jejuni* develop reactive arthritis, bacteremia, Guillain-Barre syndrome (GBS), or Miller Fisher Syndrome (MFS), a rare variant of GBS (Nachamkin, 2002).

Various types of domestic livestock and wild animals, such as birds, cattle, deer, dogs, pigs, and sheep, are natural reservoirs for the zoonotic pathogen, *Campylobacter* (Humphrey et al., 2007). Commercial poultry have been identified as a significant reservoir for *Campylobacter* infection in humans as *C. jejuni*, the species most often associated with campylobacteriosis, has seemingly evolved to preferentially colonize the avian digestive tract (Snelling et al., 2005; Pope et al., 2007) and become a commensal organism in poultry. Campylobacters are ubiquitous in the poultry farm environment (Camarda et al., 2000; Newell and Fearnley, 2003), and horizontal transmission of *Campylobacter* from an environmental source is an important route for flock infection. Risk factors for *Campylobacter* transmission and colonization include presence of rodents, insects, and other animals on the farm, contaminated water supply, human traffic (i.e. staff, service personnel, visitors), poor facility maintenance, substandard hygiene practices, and inadequate disinfection between flocks (Newell and Fearnley, 2003). When infection has occurred, *Campylobacter* can spread rapidly among birds and become difficult to control.

Although there is potential for all commercial poultry species to become infected with *Campylobacter*, the risk is greatest for the broiler industry because of the large quantities of broiler meat consumed by humans (Humphrey et al., 2007). *Campylobacter* can be found on processed carcasses and consuming raw or inadequately cooked chicken or foods cross-contaminated by chicken increases the risk of human infection (Skirrow, 1991; Altekruse et al., 1999; Humphrey et al., 2007).

One of the most contentious issues regarding *Campylobacter* and poultry is the possibility of infection through vertical transmission. For many years, it has been thought that horizontal transmission from environmental sources is the primary route of *Campylobacter* infection and that vertical transmission is unlikely (Sahin et al., 2003; Cox et al., 2005). Studies have suggested that *Campylobacter* does not survive well within the contents of the egg. When sampling eggs produced by laying hens known to be C*ampylobacter* positive, Doyle et al. (1984) could not recover *C. jejuni* from egg content. Neill et al. (1985) did not recover *C. jejuni* from egg content. Neither Sahin et al. (2003) nor Fonseca et al. (2006) could recover *Campylobacter* from eggs produced by *Campylobacter* positive broiler breeder hens. While results from these studies suggest that vertical transmission is an unlikely route of infection, accumulating evidence from other studies suggests that vertical transmission may occur. Sahin et al. (2003) found that *C. jejuni* could survive in egg yolk for up to 14 days when eggs were stored at below optimal growth temperatures at 18°C. *Campylobacter* has also been isolated from the ovarian follicles (Cox et al., 2005) and reproductive tracts (Camarda et al., 2000; Buhr et al., 2002; Cox et al., 2009) of laying and broiler breeder hens, suggesting that egg contents can become contaminated during egg formation. Cox et al. (2002) recovered *C. jejuni* isolates from breeder hens and their progeny that had identical ribotype patterns and *fla*A short variable region DNA sequences. By

suggesting that breeder hens are a source of *Campylobacter* contamination, these results further support the possibility of vertical transmission. Additionally, Byrd et al. (2007) isolated *Campylobacter* from commercial hatchery trayliners, and Idris et al. (2006) detected *C. coli* DNA in ileal, cecal, and yolk content of day old chicks, although the bacteria may have been on the surface of the eggs at the time of hatching.

Although studies have shown *Campylobacter* spp. can colonize internal and reproductive tissues of laying hens (Camarda et al., 2000; Cox et al., 2009), table eggs have not been identified as a significant source of *Campylobacter* infection in humans thus far. The growth characteristics (thermophilic and microaerophilic) of *Campylobacter* place severe restrictions on its ability to survive outside of the host (Park, 2002; McClure and Blackburn, 2004), and unlike *Salmonella* spp., *Campylobacter* spp. are generally not capable of multiplying in foods during processing or storage (Park, 2002). Studies have shown that the prevalence of *Campylobacter* among table eggs is low (Doyle, 1984; Izat and Gardner, 1988; Adesiyun et al., 2005; Sulonen et al., 2007).

**Factors Affecting *Salmonella* Colonization**

Numerous factors are involved in determining how susceptible chickens are to intestinal colonization with *Salmonella* spp. (Bailey, 1987). An important factor is the age of the chicken. Newly hatched chicks are thought to be most susceptible to *Salmonella* colonization because they lack mature gut microflora (Cox et al., 1996). Milner and Shaffer (1952) found that day-old chicks could be readily infected with very small doses (51% colonization achieved with 10 cells) of *Salmonella* and that their susceptibility to infection decreased with age. Cox et al. (1990) found that intracloacally inoculated day-old chicks could be colonized with as few as two cells of *Salmonella*, and through oral and intracloacal inoculation, the number of cells required to

colonize half (colonization dose $CD_{50}$ value) of the day-old chicks was 100 times fewer than that of 3 day old chicks. Gast and Holt (1998) challenged day old chicks to evaluate the persistence of *Salmonella* through maturity. Of the ceca samples collected at 1, 4, 8, 12, and 16 weeks of age, *Salmonella* was isolated from 100, 100, 65, 45, and 40%, respectively. Chickens generally become more resistant to *Salmonella* colonization with age and the establishment of intestinal microflora. One approach used to help control *Salmonella* colonization in chicks, which lack mature intestinal microflora, is competitive exclusion. First reported by Nurmi and Rantala (1973), competitive exclusion as a treatment involves the oral administration of intestinal microflora from healthy, salmonellae-free adult chickens to newly hatched chicks. The microflora used in competitive exclusion cultures accelerates the intestinal maturation process and increases resistance to *Salmonella* colonization. The concept behind the use of probiotics and prebiotics is similar to that of competitive exclusion.

Colonization is dependent upon the ability of *Salmonella* to survive passage through the gastrointestinal tract. Natural infection occurs through the oral route and following ingestion, *Salmonella* first encounter the acidic (pH ~4.5-5) environment of the crop. *Lactobacillus* present in the crop help maintain the low pH, but upon feed withdrawal, the lactobacilli population decreases and crop pH increases (Durant et al., 1999). As a result, the crop becomes a more suitable environment for *Salmonella* survival. The proventriculus and gizzard are also acidic environments in which *Salmonella* must survive. In an *in vitro* study, Cox et al. (1972) reported decreased survivability of *Salmonella* spp. at a pH corresponding to the proventriculus, and limited survivability at a pH corresponding to that of the gizzard. The pH of the small intestine (5.8) and large intestine (6.3) is closer to neutrality, and is therefore more suited for *Salmonella* growth and survival. Research has suggested that hens subjected to feed withdrawal are more

susceptible *Salmonella* colonization (Holt, 1993, 1995; Durant et al., 1999; Ricke, 2003; Dunkley et al., 2007). Antimicrobial or anticoccidial feed additives may also influence *Salmonella* colonization by altering or reducing normal intestinal microflora (Bailey, 1987). Alterations in protective gut flora can increase a hen's susceptibility to *Salmonella* colonization.

Intestinal colonization can be affected by the dose level and strain of *Salmonella* chickens are subjected to (Bailey, 1987), and the ability of *Salmonella* to attach, colonize, and invade intestinal tissues (D'Aoust et al., 1991). Chickens that are exposed to higher doses of *Salmonella* are more likely to become colonized and some *Salmonella* spp. colonize the avian intestinal tract more efficiently than others (Barrow et al., 1988). Bacterial cells must first attach themselves to the host epithelial cells to initiate the processes of colonization and invasion (Finlay and Flakow, 1989; Khan et al., 2003). Attachment is mediated by proteins known as adhesins, and *Salmonella enterica* possess several fimbrial and nonfimbrial adhesins that bind to intestinal epithelial cells (Korhonen, 2007). *Salmonella* Pathogenicity Island (SPI) 1 contributes to cecal colonization, while SPI2, in the absence of SPI1, inhibits cecal colonization (Dieye et al., 2009). Bacterial invasion is mediated by genes located on SPI1 (Bohez et al., 2006). Several studies have shown that mutations in these SPI1 specific genes can affect colonization (Porter and Curtiss, 1997; Turner et al. 1998; Morgan et al., 2004).

Other factors known to affect *Salmonella* colonization include stressors, either environmental or physiological, host health and disease status, medication effects, nature of diet, and host genetic background (Bailey, 1987; 1993). Bacterial colonization and invasion are influenced by parameters specific to *Salmonella* and the effects of environmental stimuli (avian gastrointestinal tract) on gene expression (Dunkley et al., 2008). Scientists continue to research

serovar and host specific colonization factors and mechanisms to further the understanding of *Salmonella* ecology within laying hens.

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# CHAPTER 3

# BACTERIA COMPARISON OF NON-WASHED AND WASHED TABLE EGGS HARVESTED FROM CAGED LAYING HENS AND CAGE-FREE FLOOR HOUSED LAYING HENS[1]

[1]J. F. Hannah, J.L. Wilson, N.A. Cox, J.A. Cason, D.V. Bourassa, M.T. Musgrove, L.J. Richardson, L.L. Rigsby, and R.J. Buhr. Submitted to Poultry Science.

**Abstract**

These studies evaluated the eggshell bacteria of non-washed and washed eggs from caged and cage-free laying hens housed on all wire slats or all shavings floor systems using Hy-Line W-36 White and Hy-Line Brown laying hens. On sampling days for Experiment 1, 2, and 3, twenty eggs were collected from each pen for bacterial analyses. Ten of the eggs collected from each pen were washed for 1 min with a commercial egg washing solution, while the remaining 10 eggs were unwashed prior to sampling the eggshell and shell membranes for aerobic bacteria (APC) and coliforms (Experiment 1 only). In Experiment 1, the aerobic bacterial counts (APC) of non-washed eggs produced in the shavings, slats, and caged housing systems were 4.0, 3.6, and 3.1 $\log_{10}$cfu/mL of rinsate, respectively. Washing eggs significantly ($P<0.05$) reduced APC by 1.6 $\log_{10}$cfu/mL and reduced coliform prevalence by 12%. In Experiment 2, non-washed eggs produced by hens in triple-deck cages from 57 to 62 wk (previously housed on shavings, slats, and cages) did not differ with APC ranging from 0.6 to 0.8 $\log_{10}$cfu/mL. Washing eggs continued to significantly reduce APC to below 0.2 $\log_{10}$cfu/mL. In Experiment 3, the levels of APC for non-washed eggs were within 0.4 log below the APC values attained for non-washed eggs in Experiment 1, although hen density was 28% of that used in Experiment 1. Washing eggs further lowered APC levels to 0.4-0.7 $\log_{10}$cfu/mL, a 2.7 log reduction. These results indicate that eggshell bacteria levels are similar following washing for eggs from hens housed in these cage and cage-free environments. However, housing hens in cages with manure removal belts resulted in lower APC for both non-washed and washed eggs (compared to eggs from hens housed in a room with shavings, slats, and cages).

**Keywords:** eggshell bacteria, egg washing, hen housing system, caged, cage-free

## Introduction

In the United States, commercial laying hens are primarily housed in conventional colony or battery wire cages as they offer lower production costs, improved egg hygiene, and greater hen livability compared to cage-free systems (De Reu et al., 2005; Singh et al., 2009). However, recently colony cage housing systems have been criticized by animal welfare and consumer groups for providing a barren, crowded, and confined environment for laying hens (Singh et al., 2009). Rising concerns regarding hen welfare have prompted changes in the housing systems for table egg laying hens. Many table egg producers are transitioning from conventional colony cages to either enriched environmental housing systems (cage system that includes a perch, nest, and shavings/litter area) or to alternative cage-free housing systems, such as aviary, wood shavings covered floor, paddock, or free-range. California voters approved the implementation of Proposition 2, which will prohibit housing egg laying hens in conventional colony cages beginning in 2015 (California, 2008). Conventional colony cages for laying hens will be banned in the European Union by 2012 and replaced with either enriched environmental housing systems or alternative cage-free systems (European Commission, 1999). Increased consumer aversion to the use of conventional colony cages has also lead to an increase in demand for cage-free table eggs, although presently at less than 8 percent of the U.S. table egg market (Savory, 2004; United Egg Producers, 2010).

The vast majority of eggs produced by healthy hens are clean at oviposition when passing through the vent (Mayes and Takeballi, 1983). However, regardless of the housing system, eggs are contaminated to some extent when they come in contact with environmental bacteria after being laid (Harry, 1963; Quarles et al., 1970; Wall et al., 2008). Studies have been conducted to compare the shell bacteria of eggs from hens housed in conventional colony cages to those from

hens housed in alternative housing systems. Quarles et al. (1970) found that eggs obtained from hens housed on shavings covered floors have 20 to 30 times more aerobic bacteria on the shell than eggs from hens on wire (eggs collected daily and held for up to 14 d). Furthermore, eggs produced in conventional and furnished cages have been reported to harbor significantly fewer aerobic bacteria on the shell than eggs from aviary and free range systems (De Reu et al., 2005; 2006a). However, eggs from these housing systems were reported to have similar levels of Gram-negative bacteria (most human food borne pathogens; De Reu et al., 2008). When comparing eggs from conventional and furnished cages, studies have shown that those from furnished cages have higher bacterial numbers on the eggshell (Mallet et al., 2006; Wall et al., 2008). A small number of studies have evaluated the effects of floor housing systems on eggshell bacterial contamination. However, no studies have evaluated the shell bacteria of eggs produced by pullets raised in the same housing system and then placed into caged and cage-free systems within the same environmental conditions (temperature and humidity ranges, photoperiod, and ventilation) during rearing and egg production.

Many genera of bacteria, including *Escherichia*, *Micrococcus*, *Salmonella*, *Streptococcus*, and *Staphylococcus*, and have been recovered from the shells of naturally contaminated table eggs (Mayes and Takeballi, 1983; Musgrove et al., 2004). External eggshell contamination can adversely affect the shelf life and food safety of eggs. In an eggshell penetration study, De Reu et al. (2006b) reported a significant positive relationship between the level of eggshell contamination and the resulting internal egg contamination. Table eggs are routinely washed in the United States, Australia, Canada, and Japan to reduce eggshell contamination, thus reducing the potential for egg spoilage and human illnesses associated with the consumption of raw or undercooked eggs (Hutchinson et al., 2004; De Reu et al., 2006c).

However, washing Class A table eggs is prohibited in the European Union and washed eggs cannot be sold as table eggs (European Commission, 2003 and 2007). This practice is partially due to the historical perception that wetting or washing eggs prior to storage can increase egg spoilage rates (Brooks, 1951; Bagley and Christensen, 1991; Wang and Slavik, 1998; Hutchinson et al., 2003) and reports that washing can damage the egg's cuticle, a natural but temporary physical barrier that impedes bacterial penetration by covering the opening of each pore and reducing eggshell permeability.

With an increasing number of laying hens being housed in cage-free systems, shell bacterial levels of eggs from alternative housing systems will be a significant issue potentially affecting food safety policies. There is limited published work available on the shell bacteria of table eggs from cage-free hens, so additional research is needed to compare the eggshell bacterial numbers of eggs produced by conventionally caged hens to those produced by cage-free hens. The objective of this study was to evaluate the eggshell bacterial numbers of non-washed and washed eggs from caged and cage-free laying hens housed either on all wire slats or all wood shavings floor systems. A single commingled flock of Hy-Line W-36 (White) and Hy-Line Brown (Brown) layer strains, reared and housed for laying in a single room, were utilized in three sequential experiments.

## Materials and Methods

### *Birds, Housing, and Management*

Hy-Line International provided two cases of hatching eggs from flocks at 56 wk-of-age for both White and Brown layer strains. The eggs were incubated (NMC2000, NatureForm, Jacksonville, FL) at the University of Georgia Poultry Research Center. After 21 d of incubation the hatched chicks were removed and female chicks were identified by fast feathering (White) or

down pattern (Brown). Hatchability for the White strain was 90.6% and resulted in 220 female chicks. Hatchability for the Brown strain was 78% resulting in 177 female chicks. White and Brown chicks were reared intermingled in an environmentally controlled facility from day-of-hatch through 15 wk-of-age. Pullets were reared on a concrete floor covered with new pine shavings in a single room (24 x 30 ft | 7.32 × 9.14 m) with access to a trough feeder line, nipple drinker lines, and perches (Hy-Line, 2006-2008). The photoperiod program followed the recommended Hy-Line management guide. At 15 wk-of-age, pullets were weighed and then selected within 1 standard deviation of mean body weight by strain, resulting in 162 White (1.12 kg) and 153 Brown (1.38 kg) selected pullets. Mortality was limited to a single bird and signs of cannibalism were not apparent.

***Experiment 1.*** At 15 wk-of-age, pullets were placed by strain in the three housing systems: conventional cages (1 x 2 in | 2.54 x 5.08 cm, 16 gauge galvanized wire that were newly constructed), elevated wire slats (0.75 x 3 in | 1.90 x 7.62 cm, 12.5 gauge white PVC coated, sanitized reused), and all new pine shavings covered concrete floors. A total of 6 pens were used in Experiment 1 with duplicate pens (one for White and one for Brown pullets) of each housing system. For the conventional cages, each pen contained 9 colony cages (24 wide x 18 in deep x 18 in high | 61 cm wide x 45.7 cm deep x 45.7 cm high). There were 6 White hens housed per cage (72 $in^2$/hen | 465 $cm^2$/hen) or 5 Brown hens housed per cage (86 $in^2$/hen | 555 $cm^2$/hen). There were 54 White or Brown hens housed in the all wire slat pens and the all shavings floor pens (1.8 $ft^2$/hen | 0.16 $m^2$/hen). The cage and cage-free housing densities were compliant to UEP recommendations (United Egg Producers, 2010). All hens in the 6 pens were housed in the same room (24 x 30 ft | 7.32 × 9.14 m), fed the same pelleted feed *ad libitum* from a central alley (6 x 24 ft | 1.83 x 7.32 m) accessing each pen, and were subjected to the same

environmental conditions (temperature and humidity ranges, ventilation, light intensity, and photoperiod program). Throughout the experiment, all birds were provided feed formulated to meet the nutritional requirements outlined in the Hy-Line Brown commercial layer management guide (8 diets; Hy-Line, 2006-2008). Trough feeders were used for hens in cages, while two tube/pan feeders (41.5 in | 105 cm circumference with 14 partitions) were used for hens housed in the wire slats and shavings pens. One-story front roll-out nest boxes with rubber finger nest pads were provided for hens housed on wire slats and shavings at a stocking density of 4.5 hens/nest (12 nests/54 hens). Perches providing 5.3 in | 13.5 cm/hen were also placed in the wire slats and shavings pens. Eggs were collected by hand twice daily (11:00 and 15:00) and recorded for each pen. Hens were initially beak trimmed at 34 wk-of-age and beaks re-blunted as needed at monthly intervals in an effort to control cannibalism. Egg production and any mortality were recorded daily. All experimental animal procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia prior to placement of the chicks. Starting at 22 wk-of-age, at monthly intervals for 8 mo consecutively, eggs from the 6 pens were collected and the eggshells sampled (n=80 eggs for Experiment 1).

***Experiment 2.*** At 52 wk-of-age, all remaining hens were moved into triple-deck battery cage units and placed 2 hens/cage (one White and one Brown hen/cage) that were 12 in | 30.5 cm wide, 18 in | 45.7 cm deep, and 18 in | 45.7 cm high in the room containing the remainder of the hatch mate hens that had been utilized for egg production in other research projects. Each hen's previous housing system (cages, slats, or shavings) designation was recorded during moving. After 5 wk in the triple-deck battery cages, eggs were collected and sampled weekly from 57 to 61 wk-of-age, (n=50 eggs for Experiment 2).

***Experiment 3.*** Both the remaining Hy-Line White and Hy-Line Brown hens used in Experiments 1 and 2 were used in four sequential trials with a total of 45 hens/trial from 56-72 wk-of-age. White and Brown hens were commingled in either conventional colony cages (86 $in^2$/hen | 555 $cm^2$/hen a total of 3 cages), on all wire slats (6.4 $ft^2$/hen | 0.6 $m^2$/hen), or on all shavings flooring systems (6.4 $ft^2$/hen | 0.6 $m^2$/hen). They were placed back into the room in which they were housed from 15 to 51 wk-of-age in Experiment 1, without clean-out. For each trial, a total of 15 hens were placed into each of the three housing systems. Commingled hens had access to the same feeding and watering systems and at 12 d after reintroduction, eggs were collected, processed, and sampled at 59, 63, 67 and 71 wk-of-age as described for Experiment 2 (n=36 eggs for Experiment 3).

***Egg Sampling and Washing***

***Experiment 1.*** On each of eight replicate days (at 22, 25, 29, 34, 38, 42, 46, and 51 wk-of-age), up to 30 eggs were collected from each pen for bacterial analysis. On the day of sampling, starting at 06:00 all eggs present in the pens were collected, recorded, and excluded from that day's sample to assure only freshly laid eggs (within 2 h) were sampled each replicate day. At 10:00 and again at 14:00 all eggs present were aseptically collected from each pen, marked, and placed into new cardboard egg flats (one egg flat/pen). Only visibly intact eggs that were laid in the nest boxes (for hens on slats or shavings) were selected for sampling. The collected eggs were then held uncovered overnight at 12°C and approximately 70% RH in an onsite egg cooler. The following morning (08:00) each flat of eggs was placed into a clean plastic bag and the 6 flats of eggs were transported from the farm to the laboratory. Twelve representative eggs (of the 30 eggs from each pen) that were not to be washed were aseptically placed onto a sanitized plastic egg flat and remained in the laboratory. Twelve representative

eggs from each pen remained on the egg flat (remaining 6 eggs for each pen were discarded), were placed back into the plastic bag, and transported to the egg processing facility (Jones et al., 2005). Groups of 6 eggs from each of the 6 pens were spray-washed together in a single batch using a commercial egg washing solution (80 mL/26.5 L of Liquid egg wash 101, BioSentry, Stone Mountain, GA). The solution at pH of 11 was heated to 50°C and sprayed onto the eggs for 1 min at 5 psi | 34.5 KPa from the heated recirculation washing solution tank while eggs were rotated on spindles identical to those used in commercial washing equipment. Eggs were aseptically removed from the rollers of the egg spraying machine, placed into new foam egg cartons by pen, and dried for approximately 15 sec with a hand held blower producing 124°C air. The second batch of 6 eggs from each of the 6 pens was then placed onto the rollers, sprayed together, removed, placed into the same foam egg carton with the first batch, and then dried. The cartons were then closed and placed into a cardboard egg box for transport back to the laboratory by 10:00.

Ten of the twelve eggs (washed and non-washed groups for each pen) were sampled which included the eggshell and shell membranes for aerobic bacteria (APC), *Escherichia coli*, and coliforms. If the remaining two eggs for each pen that were not selected for sampling were not needed as replacement eggs (for eggs found to have cracks in the eggshell or were inadvertently cracked during handling), they were discarded. Using a new latex glove each time, each egg was cracked open on a sterile surface and the internal contents were discarded. The eggshell and shell membranes were then crushed by hand and forced into a sterile 50 mL centrifuge tube. Twenty mL of 0.85% saline were then added to the sample. Sterile glass rods were used to further crush the eggshell and shell membranes for 1 min and mix the sample with the saline solution (Berrang et al., 1991; Musgrove et al., 2005).

***Experiments 2 and 3.*** Eggs sampled in Experiments 2 and 3 were collected and selected as described for Experiment 1, but were washed using a small scale egg washing unit (Model EEW-30-G-R, Modernmatic, Lancaster, PA) operating at 48°C, pH 11, at 10 psi | 68.9 KPa, for a wash time of 1 min. The egg washing compound used in Experiments 2 and 3 was 25 g/10 L of DBC-A egg wash powder with 1 mL/6 L Antifoam B (BioSentry) resulting in a 50 ppm free chlorine solution in the 172 L heated recirculation tank. The main differences between the egg spraying machines were as follows. For Experiment 1, the eggs rotated in place while receiving a constant spray pattern while for Experiments 2 and 3, the eggs rotated while proceeding down the conveyer and therefore received a varied sanitizing spray pattern. Also the tank reservoir capacity for Experiment 1 was 26.5 L and for Experiments 2 and 3 was 172 L, and the spray pressure in Experiment 1 was 5 psi | 34.5 KPa which was raised to 10 psi | 68.9 KPa in Experiments 2 and 3.

***Bacteriological Analysis***

In Experiments 1, 2, and 3, 1 mL of crushed eggshell rinsate was collected from each sample to prepare serial dilutions to $10^{-4}$. For APC enumeration of non-washed eggs, 1 mL was transferred directly from the rinsate and the $10^{-2}$ and the $10^{-4}$ dilutions to duplicate APC Petrifilm™ (3M Health Care, St. Paul, MN) plates. For APC enumeration of washed eggs, 1 mL was transferred directly from the rinsate and the $10^{-2}$ dilution to duplicate APC Petrifilm™ plates. In Experiment 1 only, to enumerate *E.coli*/coliforms from both the non-washed and washed eggs, 1 mL was transferred directly from the rinsate and the $10^{-2}$ dilution to duplicate *E. coli*/coliform Petrifilm™ (3M Health Care) plates. All plates were incubated at 37°C for 24-48 h. Colonies on the APC and *E. coli*/coliform plates were enumerated following the manufacturer's directions and counts were converted to $\log_{10}$cfu/mL of crushed eggshell rinsate.

### *Statistical Analysis*

Analysis of variance according to the general linear model (GLM) procedure (SAS, 2005) was used to test for differences in APC due to wash treatment and laying hen strain. Tukey's Honest Significant Difference (HSD) test was used to identify differences due to housing system. Only positive rinsate samples were averaged. All differences reported as significant were evaluated at $P<0.05$. The prevalence of *E. coli* and coliforms among white and brown non-washed and washed eggs was insufficient for statistical testing. Dixon's Q test was applied once to identify and reject an individual egg outlier data within each housing system for non-washed and washed eggs for each sampling day (Dean and Dixon, 1951).

## Results and Discussion

### *Experiment 1*

Hen-day egg production from 22 to 51 wk-of-age for White hens was 74, 74, and 77% for those housed on shavings, on slats, or in cages, respectively. Hen-day egg production for Brown hens during the same period was 77, 75, and 80% for those on shavings, on slats, or in cages, respectively. Mortality and hens removed due to injury through 51 wk-of-age for White hens on shavings, on slats, or in cages was 2, 4, and 9%, respectively. Mortality and hens removed due to injury for Brown hens on shavings, on slats, or in cages was 44, 66, and 27%, respectively. Elevated mortality and injury among Brown hens in this study was primarily associated with cannibalism and the requirement to promptly remove and/or euthanize severely injured hens. Cannibalism has been previously reported to be the primary cause of increased mortality (20-30%) among Brown laying hens housed in floor systems (Abrahamsson and Tauson, 1998; Tauson et al., 1999).

Non-washed white eggs produced in the shavings pen had significantly higher APC levels (3.8 $\log_{10}$cfu/mL of rinsate) than eggs produced on slats (3.2 $\log_{10}$cfu/mL), which had similar levels to eggs produced in cages (3.1 $\log_{10}$cfu/mL; Table 3.1). Washing significantly reduced APC levels of white eggs produced on shavings, on slats, or in cages to 2.2, 1.3, and 2.2 $\log_{10}$cfu/mL, respectively. White eggs produced on slats that were washed had significantly lower APC levels than the eggs produced in cages or on shavings (Table 3.1), resulting in a significant interaction between housing and treatment. This low APC level following washing eggs from hens on slats may be attributed to the restricted air flow over the eggs while they sat in the roll-out nest egg tray in contrast to the unrestricted air flow around the eggs from hens in cages located in front and below the feed troughs. The initial higher APC levels (Table 3.1) for non-washed eggs from the Brown hens on slats (4.1 $\log_{10}$cfu/mL) or White (3.8 $\log_{10}$cfu/mL) or Brown hens on shavings (4.2 $\log_{10}$cfu/mL) may have overshadowed this benefit following washing.

Aerobic bacterial levels of non-washed brown eggs produced on shavings (4.2 $\log_{10}$cfu/mL) or on slats (4.1 $\log_{10}$cfu/mL) were significantly higher than those produced by hens in cages (3.0 $\log_{10}$cfu/mL; Table 3.1). Washing significantly reduced APC levels for brown eggs produced on shavings, on slats, or in cages to 2.2, 2.5, and 1.3 $\log_{10}$cfu/mL, respectively. Washed brown eggs produced in cages had significantly lower APC levels than those eggs produced on slats and shavings (Table 3.1).

Non-washed brown eggs produced on shavings (4.2 $\log_{10}$cfu/mL) or on slats (4.1 $\log_{10}$cfu/mL) had significantly higher APC levels than non-washed white eggs produced on shavings (3.8 $\log_{10}$cfu/mL) or on slats (3.2 $\log_{10}$cfu/mL; Table 3.1). Washed brown eggs

produced in cages (1.3 $\log_{10}$cfu/mL) had significantly lower APC levels than washed white eggs produced in cages (2.2 $\log_{10}$cfu/mL).

The prevalence of *E. coli* and coliforms among white and brown non-washed and washed eggs was insufficient for statistical testing. *E. coli* prevalence among non-washed white eggs was reduced from 15, 11.3, and 11.3% (shavings, slats, and cages, respectively) to 3.8% following washing for eggs from all three housing systems. Similarly, washing reduced coliform prevalence among white eggs produced on shavings, on slats, or in cages from 16.3, 12.5, and 12.5% to 3.8, 8.8, and 3.8%, respectively. Overall, coliform prevalence was slightly higher among non-washed brown eggs produced on shavings (*E. coli* 25% and coliforms 28.8%) and on slats (*E. coli* 16.3% and coliforms 22.5%) than in cages (*E. coli* 6.3% and coliforms 12.5%). Once subjected to the spray wash treatment, only 3.8% of brown eggs (identical to the percentage for white eggs) produced in each housing type were positive for *E. coli*, while 6.3, 3.8, and 6.3% (shavings, slats, and cages, respectively) of the Brown eggs were positive for coliforms.

***Experiment 2***

After all hens were moved from the shavings, slats, and cages room to the 2-hen cages in a separate room, the non-washed eggs had low APC levels at 0.8 $\log_{10}$cfu/mL. Levels ranging from 0.6 to 1.0 $\log_{10}$cfu/mL (Table 3.1) did not differ between hen strains and were not influenced by previous housing system. The average APC level for non-washed eggs (0.8 $\log_{10}$cfu/mL) was more than 1.0 log less than the average APC value attained for spray washed eggs (1.9 $\log_{10}$cfu/mL) in Experiment 1. Furthermore, following washing, the APC levels for eggs in Experiment 2 were further reduced to an average of 0.2 $\log_{10}$cfu/mL, a 0.6 log reduction. APC prevalence in Experiment 2 for eggs from the triple-deck caged hens after washing was

53% compared to 74% when hens were previously housed on shavings, slats, or cages in Experiment 1. Housing hens in cages without shavings and with manure removal belts resulted in lower eggshell APC for both non-washed and washed eggs (compared to eggs from hens housed in a room with shavings, slats, and cages).

***Experiment 3***

Moving hens from the triple-deck cages into the same room used in Experiment 1 (remained empty without clean-out from 52-56 wk) into the same shaving, slats, or cage pens resulted in non-washed eggs having APC levels similar to those reported in Experiment 1. For caged hens APC levels were 2.8-3.0 $\log_{10}$cfu/mL (3.0-3.1 $\log_{10}$cfu/mL in Experiment 1), for hens on slats 3.0-3.1 $\log_{10}$cfu/mL (3.2-4.1 $\log_{10}$cfu/mL in Experiment 1), and for hens on shavings 3.6-3.8 $\log_{10}$cfu/mL (3.8-4.2 $\log_{10}$cfu/mL in Experiment 1). The APC levels differed by less than 0.2 $\log_{10}$cfu/mL between hen strains (Table 3.1). The levels of APC for non-washed eggs were within 0.4 log below the APC values attained for non-washed eggs in Experiment 1, although hens in Experiment 3 were at 28% of the hen density used in Experiment 1. In Experiment 3, washing eggs lowered APC levels to 0.4-0.7 $\log_{10}$cfu/mL, a 2.7 log reduction. APC prevalence in Experiment 3 after washing was 80-86% for eggs from hens in cages (White and Brown), 69-86% for hens on slats, and 82-88% for hens on shavings. These percentages were similar to the results from Experiment 1 for hens in cages (62-74%), hens on slats (55-81%), and hens on shavings (84-90%).

Although the hens' density per pen in Experiment 3 was less than one-third (28%) of that used in Experiment 1 (15 hens/pen compared to 54 hens/pen in Experiment 1) and the resulting total room density less than one-sixth (14%; 45 hens/room compare to 315 hens/room), eggs from hens in all three housing systems in Experiment 3 had high levels of APC (within 0.4

$log_{10}$cfu/mL) except for Brown hens on slats, which were 1 $log_{10}$cfu/mL lower. Washing eggs continued to significantly reduce APC to 0.4-0.7 $log_{10}$cfu/mL. The egg washing machine and washing solutions used in Experiments 2 and 3 continued to out-perform the equipment and chemicals used in Experiment 1 by 1.5-2.0 $log_{10}$cfu/mL. APC prevalence for eggs in Experiment 3 was 69-88%, comparable to the prevalence (55-90%) in Experiment 1.

The influence of housing systems on eggshell bacterial contamination has been demonstrated in previous studies and, in general, eggs produced in alternative systems such as furnished cages and aviaries have higher eggshell bacterial levels than eggs produced in conventional cages (Harry, 1963; Quarles et al., 1970; De Reu et al., 2005; Mallet et al., 2006; Wall et al., 2008). In the current study (Experiment 1), non-washed white eggs produced on shavings had significantly higher APC levels (3.8 $log_{10}$cfu/mL) and a higher *E. coli* and coliform prevalence than eggs produced on slats (3.2 $log_{10}$cfu/mL) or in cages (3.1 $log_{10}$cfu/mL). The higher APC levels and total coliform (*E. coli* and coliforms combined) prevalence on the shells of white eggs produced in the all-shavings pen was likely due to the presence of excreta in the shavings and contact between the hen's feet and the nest pad. Hens may transport fecal matter and other contaminants from the shavings area to the nest boxes on their feet and increase the potential for eggshell contamination within the nest during lay and while the egg rolls out of the nest into the covered area. Tauson et al. (1999) reported poorer foot hygiene among hens housed in systems with shavings/litter areas compared to those housed in conventional cages (no shavings area). Aerobic bacterial levels reported by Wall et al. (2008) for non-washed white eggs produced in conventional cages (2.7 $log_{10}$cfu/mL) or in furnished cages (3.0 $log_{10}$cfu/mL) were similar to the levels reported in this study in Experiment 1 (3.1 $log_{10}$cfu/mL for hens in cages and 3.2 $log_{10}$cfu/mL for hen on slats). These results could be influenced by the fact that

hens housed on wire floors (i.e. cages and slats) are for the most part separated from their manure.

When comparing bacterial levels of brown eggs, non-washed eggs produced on all wire slats or shavings had significantly higher APC levels than those produced in cages. Similarly, De Reu et al. (2005) reported higher aerobic eggshell bacteria on eggs from Brown hens housed in an aviary system compared to a conventional cage system. Total coliform prevalence was also higher among non-washed brown eggs produced on shavings. Increased bacterial levels of non-washed brown eggs produced on slats (4.1 $\log_{10}$cfu/mL) and on shavings (4.2 $\log_{10}$cfu/mL) may have been attributable to the presence of nest boxes in both housing systems. Hy-Line Brown laying hens were about 25% larger by body weight than the Hy-Line W-36 (White) laying hens. Hen size may influence eggshell bacterial levels as larger hens will consume more feed and water daily, eventually producing more manure daily, which can potentially contaminate the hens' feet and eggs. Eggshell APC levels of brown eggs (3.0 $\log_{10}$cfu/mL) produced in cages were similar to those of white eggs (3.1 $\log_{10}$cfu/mL) produced in cages. For the caged hens, hen size may not have been an important factor as the Brown hens were housed at a lower density than the White hens (5 vs 6 hens). *E. coli* and coliform prevalence was lower among White and Brown non-washed eggs produced in cages compared to those produced on slats and shavings. These results are similar to those of Singh et al. (2009) who reported lower *E. coli* and coliform contamination levels on eggs from hens in cages than on eggs from nest boxes for white and brown laying hens.

Washing eggs significantly reduced the number of aerobic bacteria recovered from the eggshells of white and brown eggs produced in all three housing systems. Washed white eggs produced on slats had significantly lower aerobic bacteria levels than those produced on shavings

and in cages. Since the APC levels of non-washed white eggs produced on slats and in cages were statistically similar and eggs from both housing systems were subjected to the same washing procedures, this suggests that greater numbers of APC were removed from the eggs from hens housed on slats than from eggs from hens housed in cages. Washing eggs significantly reduced the number of aerobic bacteria recovered from the surface of brown eggs produced on slats and on shavings to comparable levels, which remained significantly higher than those of eggs produced in cages. This trend was also observed among non-washed brown eggs. Overall, washing eggs reduced aerobic bacterial levels of white and brown eggs by 1.5 and 1.8 $\log_{10}$cfu/mL, respectively. Once subjected to the wash treatment, the *E. coli* and total coliform prevalence among white and brown eggs were reduced to 3.8 and 5.4%. When identifying *Enterobacteriaceae* from unwashed and washed shell eggs, Musgrove et al. (2004, 2005) reported significantly fewer numbers of *Enterobacteriaceae* recovered from washed eggs. From a food safety prospective, if eggs are not going to be washed it is important that eggs are produced in a housing system with as little contamination as possible and collected frequently, as eggs are susceptible to bacterial contamination at various stages of production. Our results indicate that the housing system allowing for the least amount of eggshell aerobic bacteria for non-washed eggs contamination would be the conventional cages, followed by the all wire slats, and then the all shavings floor pen.

In summary, the eggshells of eggs collected from hens housed in cages had lower levels of APC bacteria than eggs from hens housed on slats or shavings. In addition, washing eggs significantly lowered eggshell bacteria levels ($P< 0.01$) and after washing eggs from hens housed on shavings, on slats, or in cages, the level of bacteria recovered did not differ between housing environments in Experiment 3 (when the small scale egg processing equipment was used).

When all hens were moved to triple-deck cage units in a separate room (2 hens/cage), with manure removal and the absence of shavings, there were subsequently lower levels of aerobic bacteria recovered from both non-washed and washed eggs for both White and Brown hens. After moving hens back to the shavings, slats, and cages room, the level of APC on the eggshells rapidly increased to levels recovered in Experiment 1, although the hen density was two-thirds lower in Experiment 3. However, in Experiment 3, following washing, much lower levels of aerobic bacteria (<0.7 $\log_{10}$cfu/mL) were observed among eggs from all three housing systems. For unwashed eggs, APC levels are lowest in housing systems that separate hens from manure and shavings. Spray sanitizing eggs using commercial style egg processing equipment where eggs received varied sanitizing spray patterns lowered APC to 0.7 $\log_{10}$cfu/mL compared to 2.0 $\log_{10}$cfu/mL for spraying eggs in place as they rotate. The influence of housing systems in which the eggs were laid was no longer apparent. Following adequate washing of nest clean eggs, the resulting eggshell APC levels are comparable for eggs from White and Brown hens housed on shavings, on wire slat, or in cage housing systems.

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Table 3.1. Eggshell aerobic plate counts from non-washed and washed eggs produced by White and Brown hens housed in cages, on slats, or on shavings from Experiments 1, 2, and 3.

| Treatment | Hen housing system | | |
|---|---|---|---|
| | Cages | Slats | Shavings |
| | ----------$log_{10}$cfu/mL of eggshell rinsate--------- | | |
| Experiment 1, n=80 | | | |
| White laying hens | | | |
| Non-washed | $3.1^{b,Y} \pm 0.9$[1] | $3.2^{b,Z} \pm 1.0$ | $3.8^{a,Z} \pm 0.8$ |
| Washed | $2.2^{a,Y} \pm 1.6$ | $1.3^{b,Z} \pm 1.0$ | $2.2^{a,Y} \pm 1.1$ |
| Prevalence %[2] | 62 | 55 | 90 |
| Brown laying hens | | | |
| Non-washed | $3.0^{b,Y} \pm 0.9$ | $4.1^{a,Y} \pm 0.9$ | $4.2^{a,Y} \pm 0.9$ |
| Washed | $1.3^{b,Z} \pm 0.9$ | $2.5^{a,Y} \pm 1.4$ | $2.2^{a,Y} \pm 1.3$ |
| Prevalence % | 74 | 81 | 84 |
| Experiment 2, n=50 (all hens 2/cage) | | | |
| White laying hens | | | |
| Non-washed | $0.7^{ab,Y} \pm 0.4$ | $1.0^{a,Y} \pm 0.8$ | $0.6^{b,Y} \pm 0.6$ |
| Washed | $0.3^{a,Y} \pm 0.9$ | $0.0^{a,Y} \pm 0.3$ | $0.2^{a,Y} \pm 0.6$ |
| Prevalence % | 54 | 67 | 72 |
| Brown laying hens | | | |
| Non-washed | $0.8^{a,Y} \pm 0.6$ | $0.7^{a,Y} \pm 0.6$ | $0.8^{a,Y} \pm 0.6$ |
| Washed | $0.1^{a,Y} \pm 0.4$ | $0.1^{a,Y} \pm 0.4$ | $0.3^{a,Y} \pm 0.6$ |
| Prevalence % | 52 | 74 | 60 |
| Experiment 3, n=36 | | | |
| White laying hens | | | |
| Non-washed | $2.8^{b,Y} \pm 0.8$ | $3.1^{b,Y} \pm 1.2$ | $3.6^{a,Y} \pm 0.7$ |
| Washed | $0.5^{a,Y} \pm 0.8$ | $0.4^{a,Y} \pm 0.7$ | $0.7^{a,Y} \pm 0.7$ |
| Prevalence % | 80 | 69 | 88 |
| Brown laying hens | | | |
| Non-washed | $3.0^{b,Y} \pm 0.7$ | $3.0^{b,Y} \pm 1.0$ | $3.8^{a,Y} \pm 0.6$ |
| Washed | $0.4^{a,Y} \pm 0.6$ | $0.6^{a,Y} \pm 0.8$ | $0.7^{a,Y} \pm 0.7$ |
| Prevalence % | 86 | 86 | 82 |

[1] Means and standard deviation.

[2] Percent positive samples from total number of samples taken.

[a-b] Means within a row with different letters differ significantly $P<0.05$.

[Y-Z] Means for White and Brown laying hens within a housing system for non-washed or washed eggs with different letters differ significantly $P<0.05$.

Hens age during sampling for Experiment 1 = 22-51 wk, Experiment 2 = 57-61 wk, and Experiment 3 = 59-71 wk.

# CHAPTER 4

# HORIZONTAL TRANSMISSION OF *SALMONELLA* AND *CAMPYLOBACTER* AMONG CAGED AND CAGE-FREE LAYING HENS[1]

[1]J. F. Hannah, J.L. Wilson, N.A. Cox, L.J. Richardson, J.A. Cason, and R.J. Buhr. To be submitted to Avian Diseases.

**Summary**

In each of five trials, laying hens (56-72 wk-of-age) were challenged orally, intracolonally, and intravaginally with *Salmonella* and *Campylobacter*. One wk post inoculation, challenged hens (n=3) were commingled with non-challenged hens (n=12) in conventional wire cages, on all wire slats, or on all shavings floors. After 12 days, challenged and non-challenged hens were euthanized for sample collection. Ceca were aseptically collected from all hens, and the spleen, liver/gallbladder (LGB), lower (LRT) and upper (URT) reproductive tracts, and ovarian follicles (mature and immature) were aseptically collected from challenged hens only. Samples were equally divided and cultured separately for *Salmonella* and *Campylobacter*. There was no significant (P>0.05) difference in the horizontal transmission of *Salmonella* among non-challenged hens housed in cages (12%), on slats (15%), and on shavings (14%). Among challenged hens housed in cages, *Salmonella* was recovered only from the cecum (20%) and LRT (13%) samples. *Salmonella* was recovered from 25% of the cecum, 12% of the spleen, 19% of the LGB, 25% of the LRT, and 19% of the URT samples, collected from challenged hens housed on slats. Fifteen percent of the cecum, spleen, and LRT samples and 8% of the LGB and URT samples collected from challenged hens housed on shavings were positive for *Salmonella*. Horizontal transmission of *Campylobacter* among non-challenged hens was significantly lower in cages (28%) than on shavings (47%), with slats (36%) being intermediate. *Campylobacter* was recovered from 27% of the of the cecum, 13% of the LRT, 7% of the URT, and 17% of the follicle samples collected from challenged hens housed in cages. Among challenged hens housed on slats, *Campylobacter* was recovered from 44% of the cecum, 6% of the spleen, 19% of the LGB, 12% of the LRT, 6% of the URT, and 14% of the follicle samples. Among

challenged hens housed on shavings, *Campylobacter* was recovered from 46% of the cecum, 8% of the LRT, 8% of the URT, and 40% of the follicle samples.

**Introduction**

*Salmonella* and *Campylobacter* are common causes of food-borne bacterial gastroenteritis in the United States and worldwide and are considered to be the most important zoonotic pathogens with regards to poultry (28,32). *Salmonella* infection among laying hens is a primary food safety concern for the commercial table egg industry (21,34) as *Salmonella enterica* serovar Enteritidis is the only pathogen that intermittently contaminates eggs (26) and eggs are the main food source for transmission of *S.* Enteritidis to humans. Greig and Ravel (24) recently analyzed the international food-borne outbreak data reported between 1988 and 2007, and found that 43% of *S.* Enteritidis outbreaks were associated with eggs. Eggs can become contaminated prior to oviposition as a result of the reproductive tissues (ovary and oviduct) being infected, at oviposition when the eggshell passes through the cloaca, or after oviposition when the egg comes in contact with contaminated environmental surfaces (20), and these routes of contamination have been identified as trans-ovarian, oviductal, and trans-eggshell (3,4). The prevalence of *S.* Enteritidis among eggs produced by naturally infected hens is low. Humphrey et al. (27) and Poppe et al. (30) reported overall *S.* Enteritidis prevalence among egg content as 0.55% and less than 0.06%, respectively. Using available data on the occurrence of *S.* Enteritidis in U.S. laying hens and eggs, Ebel and Schlosser (18) estimated that one in every 20,000 (0.005%) eggs produced annually would be contaminated with *S.* Enteritidis.

Although *Campylobacter* has been recovered from the reproductive tissues of broiler breeder (6,9) and laying hens (8,12), eggs have not been identified as a significant food-borne source of *Campylobacter* infection in humans. Studies have shown that the prevalence of

*Campylobacter* among table eggs is low (1,30,44). *Campylobacter* spp. accounted for 0.6% of international egg associated food-borne outbreaks, while *Salmonella* spp. accounted for 97.4% (24). When sampling eggs produced by laying hens known to be positive for *Campylobacter jejuni*, Doyle (15) recovered *C. jejuni* from two eggshell surfaces and no egg contents. *C. jejuni* was also recovered from the interior eggshell and shell membranes of inoculated eggs subjected to refrigeration (15). Neill et al. (35) recovered *C. jejuni* from shell membranes, but not from the albumen or yolk.

In the national and international table egg industries, commercial laying hens are primarily housed in conventional battery cages (2,28). Although there are several advantages to cage management, including lower production costs, increased egg production, and increased hen livability, this housing system has been extensively criticized for providing a barren and confined environment that physically restricts laying hens from performing many of their natural behaviors (42,45). These concerns have lead to the development and proposal of legislation in the U.S. and other countries to ban conventional cages and implement alternative systems intended to improve hen welfare (7,17,33,42). Conventional cages will also be banned in the European Union by 2012 (**19**). To address growing hen welfare concerns associated with caged housing and meet consumer demand for cage-free products, a portion of table egg producers have transitioned to alternative, cage-free production systems (25). It has been estimated that 5% of U.S. table eggs are produced by hens housed in alternative production systems (46).

Several studies have focused on the effects of housing system on eggshell contamination of table eggs (13,31,40), but few studies have evaluated the effects of housing system on the prevalence and transmission of *Salmonella* and *Campylobacter* among laying hens housed in cage and cage-free systems. Pieskus et al. (37) showed no significant difference in the

prevalence of *Salmonella* between hens housed in cage and aviary systems. Green et al. (25) found no differences in *Salmonella* prevalence between caged and cage-free laying hens, and De Vylder et al (14) concluded that housing laying hens in alternative production systems would not increase *Salmonella* colonization or shedding. One of the main advantages associated with conventional cages is that, because hens are efficiently separated from their feces, the risk for fecally shed and transmitted diseases among hens is reduced. However, with flock size being typically larger in caged housing systems than in cage-free housing systems, Van Hoorebeke et al. (47) considered housing laying hens in conventional cages a significant risk factor for the spread of *S.* Enteritidis and *S.* Typhimurium. *Campylobacter* has been recovered from naturally infected caged (12) and cage-free (44) laying hens, but limited data is available on the influence of these production systems on *Campylobacter* transmission among laying hens. Housing environment has been reported to play an important role in the recovery of *C. jejuni* from broiler chickens with *C. jejuni* being recovered from floor raised (65%) broilers at a significantly higher level than cage raised (37%) broilers (48). As some table egg producers change to alternative and cage-free systems to comply with legislative requirements and meet consumer demand, it is important to determine what effect housing systems may have on the spread of *Salmonella* and *Campylobacter* among laying hens housed on the floor.

## Materials and Methods

### *Inoculation and Experimental Design*

In each of five sequential trials, nine laying hens (Hy-Line W-36 white and Hy-Line brown at 56, 61, 65, 70, and 72 wk-of-age) were challenged by three routes orally (1 mL), intravaginally (1 mL), and intracolonally (1 mL) with *Salmonella* (day 1) and *Campylobacter* (day 2). A nalidixic acid-resistant marker strain of *Salmonella* Typhimurium (5, 11) was used in

trials 1 and 2 (average titer, 1.1 x $10^9$ cfu/mL), while nalidixic acid-resistant *Salmonella* Enteritidis (provided by N.A. Cox, USDA/ARS Russell Research Center, Athens, GA) was used in trials 3, 4, and 5 (average titer, 3.3 x $10^8$ cfu/mL). A gentamicin-resistant marker strain of *Campylobacter coli* (10) was used in trials 1, 2, and 3 (average titer 5.9 x $10^8$ cfu/mL), and a field strain of *Campylobacter jejuni* (provided by N.A. Cox) was used in trials 4 and 5 (average titer, 1.2 x $10^8$ cfu/mL). Challenged hens were then housed in individual cages in isolation, and 5 days post-inoculation, challenged hens were commingled with non-challenged hens at a ratio of 1 challenged hen per 4 non-challenged hens in adjacent conventional colony cages (86 $in^2$/hen | 555 $cm^2$/hen), on all wire slats (6.4 $ft^2$/hen | 0.6 $m^2$/hen), or on shavings flooring systems (6.4 $ft^2$/hen | 0.6 $m^2$/hen). Prior to hen placement, the pens used in this study were sampled via stepped on drag swabs (5) and all pens tested negative for *Salmonella* and *Campylobacter*, although the pens had not been cleaned from previous flock use. The room used for this study contained duplicate pens for each housing system, with a pen for cages, slats, and shavings on each side of the room. Trials 1, 3, and 5 were conducted in the left set of pens approximately 5 wk apart, while trials 2 and 4 were conducted in the right set of pens approximately 6 wk apart. A trough feeder was used for hens in cages, while one tube/pan feeder (41.5 in | 105 cm circumference with 14 partitions) was used for hens housed in the wire slats and shavings pens. A one-story front roll-out nest box with rubber finger nest pads was provided for hens housed on wire slats and shavings at a stocking density of 2.5 hens/nest (15 hens/6 nests). Perches providing 19.1 in | 48.5 cm/hen were available in the wire slats and shavings pens. For each trial, a total of fifteen hens were placed in each housing system. Commingled hens had access to the same feeding and watering systems, and all hens were subjected to the same room environmental conditions (temperature and humidity ranges, ventilation, light intensity, and

photoperiod program). Twelve days after placement, challenged and non-challenged hens were euthanized by electrocution and then samples collected.

***Organ Samples***

Ceca were aseptically collected from both challenged and non-challenged hens. One cecum was designated for *Salmonella* analysis, while the other cecum was designated for *Campylobacter* analysis. From only the challenged hens, the spleen, liver/gallbladder (LGB), upper (URT: infundibulum, magnum, and isthmus) and lower (LRT: shell gland and vagina) reproductive tracts, and ovarian follicles (mature and immature; trials 4 and 5, only) were aseptically collected. After separating the LRT and URT, each segment was placed on a clean surface and aseptically (using sterile utensils when dividing both the LRT and the URT) divided lengthwise, providing one half for *Salmonella* analysis and one half for *Campylobacter* analysis. Each sample was transferred to a sterile sample bag, placed on ice, and transported to the laboratory for analysis. An average weight for each sample type was obtained. The samples within the plastic bags were then smashed with a rubber mallet to expose the internal contents. Physiological saline (0.85%) was added to the LGB, spleen, and follicle samples at a ratio of 1 times the weight of the sample (mL/g). All LGB, spleen, and follicle samples were equally divided for *Salmonella* and *Campylobacter* analysis. Buffered peptone water (BPW; 1%; Acumedia, Lansing, MI) and TECRA® enrichment broth (TECRA®, Frenchs Forest, NSW, Australia) with supplements (*Campylobacter* selective supplement containing trimethoprim, rifampicin, and polymyxin; TECRA®) were added at a ratio of 3mL/g of sample for *Salmonella* and *Campylobacter* analysis, respectively. All samples were then placed in a Stomacher 400 (Fisher Scientific, Hampton, NH) and stomached for 1 min. Samples for *Salmonella* analysis

were incubated at 37°C for 24 hr, and samples for *Campylobacter* analysis were incubated microaerophilicly at 42°C for 48 hr.

***Fecal Samples***

During each trial, approximately 5 g of fresh feces was aseptically collected from each challenged hen 4 days post-inoculation. Each sample was placed in a sterile 50 mL centrifuge tube and transferred to the laboratory for analysis. A standard volume of 30 mL of BPW was added to each fecal sample, and all samples were vortexed. Five mL of the suspension was transferred from each sample to 45 mL of prepared TECRA® broth for *Campylobacter* analysis. Samples in BPW for *Salmonella* analysis were then incubated at 37°C for 24 hr. Samples in TECRA® broth for *Campylobacter* analysis were incubated microaerophilicly at 42°C for 48 hr.

***Environmental Samples***

In each trial, pens were sampled via stepped on drag swabs (n=2/pen) for *Salmonella* and *Campylobacter*. Presoaked drag swabs (DS-001, Solar Biologicals, Inc., Ogdensburg, NY) were unwound and dragged across the litter beneath the cages, the slat floor, and the shavings floor in a figure 8 pattern around the pen (5). Swabs were stepped on 4 times during sampling with a clean, disposable boot cover that was put on when entering each pen. The nipples on each drinker line (n=1/pen) were also sampled during trials 4 and 5. Using a gloved hand, each nipple (n=2/cage; n=10/line in the shavings and slats pens) was swabbed with an open gauze swab (n=2/pen). Individual floor and nipple drinker swab samples were placed in a sterile sample bag and transported to the laboratory. One hundred mL of BPW were added to each sample. All samples were massaged by hand to loosen any attached debris. Five mL of BPW was transferred from each sample to 45 mL of prepared TECRA® for *Campylobacter* analysis. Drag swabs

samples in BPW were incubated for 24 hr at 37°C for *Salmonella* analysis. Samples for *Campylobacter* analysis were incubated microaerophilicly at 42°C for 48 hr.

***Egg Samples***

During trials 4 and 5, eggs produced by isolated and commingled challenged hens were sampled for *Salmonella* and *Campylobacter*. For identification purposes, only brown laying hens were challenged with *Salmonella* and *Campylobacter*. This was done to ensure that after challenged hens were commingled with non-challenged hens, only brown eggs produced by challenged hens were collected for sampling. Eggs were collected daily, placed on a clean flat, and held in an onsite egg cooler at 12°C and 70% RH until sampled. Eggs from trial 4 were taken to the laboratory for sampling 16 days after collection began and eggs from trial 5 were taken 10 days after collection began. Eggs produced by challenged caged hens held in isolation were pooled (trial 4, n=3-5 eggs/sample; trial 5, n=2-5 eggs/sample) by hen. Forty-two eggs were collected for sampling in trial 4, and 40 eggs were collected for sampling in trial 5. Eggs produced by commingled hens were pooled (trials 4 and 5, n=4-5 eggs/sample) by housing system. A total of 97 and 54 eggs were collected for sampling in trials 4 and 5, respectively. Eggs within each pooled sample were cracked on a sterile surface. The internal contents of eggs pooled by hen were released into a gloved hand to separate the yolk from the albumen and the internal contents of eggs pooled by housing system were discarded. The eggshell and shell membranes were crushed by hand and placed in a sterile sample bag. For eggs pooled by hen, the albumen was discarded and the yolk was transferred to a sterile petri dish. Five mL of yolk was collected with a sterile syringe and transferred to a sterile sample bag. The vitelline membrane was removed with sterile forceps, rinsed with distilled water, and transferred to a sterile 50 mL centrifuge tube. The eggshell, vitelline membrane, and 5 mL yolk samples from

eggs within each pooled sample were combined by sample type and placed in individual sampling containers. To maintain aseptic technique, new gloves and sterile forceps and syringes were used between pooled samples.

Buffered peptone was added to each sample bag at a ratio of 20 mL/eggshell or pooled vitelline membrane, and 10 mL/5 mL yolk material. The vitelline membrane samples were vortexed, and all samples were incubated for 24 hr at 37°C for *Salmonella* analysis. Following incubation, 20 mL of BPW from each eggshell, vitelline membrane, and yolk sample were transferred to 100 mL of TECRA®. Samples were incubated microaerophilicly for 48 hr at 42°C for *Campylobacter* analysis.

***Plating procedures***

Two loops (20 µL) from each sample for *Salmonella* analysis were streaked onto Brilliant Green Sulfa (BGS) Agar containing 200 ppm nalidixic acid. BGS plates were incubated at 37°C for 24 hr and colony forming units characteristic of *Salmonella* were selected and subjected to slide agglutination tests using *Salmonella* O antisera (Becton Dickinson, Sparks, MD) for serogroup (Trials 1 and 2, Group B; Trials 3-5, Group $D_1$) confirmation. Samples for *Campylobacter* analysis were streaked (20 µL) onto Campy-Cefex with gentamicin (Sigma Aldrich, St. Louis, MO) (Trials 1-3, *C. coli* used) or without gentamicin (Trials 4 and 5, *C. jejuni* used). Samples from trials 4 and 5 were also streaked onto Campy-Cefex plates with gentamicin to ensure that any *Campylobacter* recovered was *C. jejuni* and not residual *C. coli*. Campy-Cefex plates were incubated in a microaerophilic atmosphere at 42°C for 48 hr. Following incubation, characteristic colony forming units were confirmed by observation, through phase-contrast microscopy, of the distinctive spiral morphology and darting motility of *Campylobacter* on a wet mount.

### *Statistical Analysis*

Chi-square and Fisher's exact test (SAS, Cary, North Carolina) were used to identify differences in *Salmonella* (*S*. Typhimurium or *S*. Enteritidis) and *Campylobacter* (*C. coli* or *C. jejuni*) colonization due to housing system (cages, slats, or shavings). Differences were considered significant at $P<0.05$.

## Results

Prior to commingling of the hens, *S*. Typhimurium was recovered from 100% (17/17) of the fecal samples collected from challenged hens in trials 1 and 2, and *S*. Enteritidis was recovered from only 57% (16/28) of the fecal samples collected in trials 3, 4, and 5. Prior to commingling of the hens, *C. coli* was recovered from 65% (17/26) of the fecal samples collected from challenged hens in trials 1, 2, and 3, and *C. jejuni* was recovered from 100% (19/19) of the fecal samples collected in trials 4 and 5.

There was no significant ($P>0.05$) difference in the horizontal transmission of *Salmonella* (Table 4.1) among non-challenged hens housed in cages (12%), on slats (15%), and on shavings (14%). Of the samples collected from challenged hens housed in cages, *Salmonella* was recovered only from the cecum (20%) and LRT (13%). Among challenged hens housed on slats, *Salmonella* was recovered from 25% of the cecum, 12% of the spleen, 19% of the LGB, 25% of the LRT, and 19% of the URT samples. *Salmonella* was recovered from 15% of the cecum, spleen, and LRT samples and 8% of the LGB and URT samples collected from challenged hens housed on shavings. *Salmonella* was not recovered from any of the ovarian follicles sampled. Collectively, *Salmonella* was recovered from 53 tissue-organ samples, and approximately 98% of the *Salmonella* recovered was confirmed as *S*. Typhimurium. The only *Salmonella* positive sample confirmed as *S*. Enteritidis was a LRT collected from a challenged hen housed on slats.

Challenged hens were inoculated with *S*. Enteritidis in trials 3, 4, and 5, but *S*. Enteritidis was not recovered from any of the non-challenged hens used in these trials. However, residual *S*. Typhimurium from trials 1 and 2 was recovered from cecum samples from non-challenged hens in last three trials, and when these data (Table 4.2) were included the horizontal transmission of *Salmonella* was significantly lower in cages (15%) and on slats (20%) than on shavings (38%). Residual *S*. Typhimurium was also recovered from the cecum and LRT of challenged hens. Among challenged hens housed in cages, on slats, and on shavings, the percentage of cecum samples positive for *Salmonella* increased to 27, 38, and 31%, respectively, and the percentage of LRT samples positive for *Salmonella* increased to 27, 44, and 31%, respectively. The only LGB and URT samples positive for residual *S*. Typhimurium were collected from one hen housed on shavings. All spleen and ovarian follicle samples collected from challenged hens in the last three trials were negative for *S*. Typhimurium.

Horizontal transmission of *Campylobacter* (Table 4.3) was significantly lower in cages (28%) than on shavings (43%), and horizontal transmission on slats (36%) was intermediate to that of the caged and shavings housing systems. Among challenged hens housed in cages, *Campylobacter* was recovered from 27% of the cecum, 13% of the LRT, 7% of the URT, and 17% of the follicle samples. For challenged hens housed on slats, *Campylobacter* was recovered from 44% of the cecum, 6% of the spleen, 19% of the LGB, 12% of the LRT, 6% of the URT and 14% of the follicle samples. *Campylobacter* was recovered from 46% of the cecum, 8% of the LRT, 8% of the URT, and 40% of the follicle samples collected from challenged hens housed on shavings. *C. jejuni* (n=81) was recovered from more tissue-organ samples than *C. coli* (n=18), accounting for 82% of total *Campylobacter* isolated. The percentage of cecum samples positive for *C. coli* and *C. jejuni* is presented in Figure 4.1. For both the non-challenged and

challenged hens, only *C. jejuni* was recovered from the caged housing system, while *C. coli* was recovered from slats and shavings housing systems at lower levels than *C. jejuni*. Approximately 2% of the cecum samples collected from non-challenged hens housed on slats were positive for *C. coli* and 34% were positive for *C. jejuni*. Among cecum samples collected from non-challenged hens housed on shavings, 21 and 22% were positive for *C. coli* and *C. jejuni*, respectively. For challenged hens housed on slats, 13% of the ceca samples collected were positive for *C. coli* and 38% were positive for *C. jejuni*. Among cecum samples collected from challenged hens housed on shavings, 16 and 31% were positive for *C. coli* and *C. jejuni*, respectively. All *Campylobacter* isolated from the spleen, LGB, URT, and ovarian follicle samples was *C. jejuni*. The LRT samples from hens housed in cages and on slats were positive for *C. jejuni*, and one LRT from a hen housed on shavings was positive for *C. coli*.

During trial 1, the cage and shavings pens were positive for *S.* Typhimurium and the slat pen was positive for *C. coli* via stepped on drag swabs (Table 4.4). During trial 2, the cage, slat, and shavings pens were positive for *S.* Typhimurium and the shavings pen was positive for *C. coli*. All pens used in trial 3 were negative for *S.* Enteritidis and *C. coli*, but positive for *S.* Typhimurium. *S.* Enteritidis was not recovered from any of the environmental samples (floor and nipple drinker swab samples) taken during trials 4 and 5. *C. jejuni* was recovered from the floors of the cage, slat, and shavings pens and the nipple drinkers of the cage pen during trial 4. The floor of the shavings pen used in trial 4 was also positive for *S.* Typhimurium. During trial 5, all pen floors were positive for *C. jejuni*, and the floor and nipple drinkers in the shavings pen, as well as the nipple drinkers in the slats pen were positive for *S.* Typhimurium. Results indicate that the sampling methods used were sufficient for recovering *S.* Typhimurium from the environment.

All eggshell, vitelline membrane, and yolk samples (n=10) from eggs produced by challenged hens held in isolation (pooled by hen) during trial 4 were negative for *S.* Enteritidis and *C. jejuni*. All eggshell and vitelline membrane samples (n=21) from eggs produced by commingled challenged hens (pooled by housing system) were negative for *S.* Enteritidis and *C. jejuni*. During trial 4, *S.* Typhimurium was recovered from 1/6 and 1/8 eggshell samples from eggs produced on slats and shavings, respectively. From eggs produced by isolated challenged hens in trial five, 1/10 eggshell samples were positive for *S.* Enteritidis. All other samples were negative for *S.* Enteritidis and *C. jejuni*. The eggshell and vitelline membrane samples (n=11) from eggs produced by commingled hens were negative for *S.* Enteritidis and *C. jejuni*.

**Discussion**

The horizontal transmission of *Salmonella* among non-challenged hens housed in cages, on slats, and on shavings was similar, and *Salmonella* prevalence among all hens was relatively low, ranging from 0-25%. This is partially due to the seemingly poor colonization of the *S.* Enteritidis strain used to challenge hens in the last three trials. *S.* Enteritidis was recovered from only one of 151 tissue-organ samples collected from challenged hens inoculated with *S.* Enteritidis. If *S.* Enteritidis did colonize and persist within the cecum of challenged hens, it is likely that the levels of bacteria shed into the environment after commingling were minimal as *S.* Enteritidis was not recovered from any cecum samples collected from non-challenged hens or environmental samples collected from each housing system. *S.* Typhimurium was recovered only from cecum and LRT samples of challenged hens housed in cages, suggesting that the bacteria did not spread to and colonize within other abdominal organs. Colonization of the cecum resulted from oral and intracolonal inoculation, while colonization of the LRT likely resulted from intravaginal inoculation. However, the oviduct can become contaminated through

ascending infection from the cloaca (20,36). In addition to cecum, LRT, and URT samples, *S.* Typhimurium was recovered from spleen and LGB samples of challenged hens housed on slats and shavings, indicating that the bacterial infection became systemic in these hens. *S.* Typhimurium did colonize in the LRT and translocate to the URT, suggesting that the contents of eggs produced by challenged hens could be contaminated prior to oviposition. However, *Salmonella* was not recovered from any of the ovarian follicles sampled, implying that the egg yolks would not be contaminated. *S.* Typhimurium colonized within the intestinal tract and translocated to other organs of hens at higher rates than *S.* Enteritidis. Hen age may have contributed to the poorer colonization of *S.* Enteritidis. Laying hens challenged with *S.* Enteritidis were 65, 70, and 72 wk of age and in general, hens with more established intestinal microflora are less susceptible to *Salmonella* colonization. However, *S.* Enteritidis has been recovered from laying hens of a similar age (29). The strain of *S.* Enteritidis used in this study may have been lacking factors needed to proliferate within the intestinal tract of laying hens. The colonization rate of marker *S.* Enteritidis may be misleading as a field strain of *S.* Enteritidis is likely to colonize hens at a higher rate and therefore, have a greater potential for horizontal transmission.

With the inclusion of residual *S.* Typhimurium, the horizontal transmission of *Salmonella* among non-challenged penmate hens was significantly greater in the shavings system than in the caged and slats housing systems. The levels of *S.* Typhimurium excreted through the feces of hens in trials 1 and 2 were sufficient enough for the bacteria to persist in the environment of each housing system and infect non-challenged and challenged hens used in subsequent trials. *S.* Typhimurium was recovered from environmental samples through the duration of the study (16 wk). Although residual *S.* Typhimurium was recovered from the cecum of non-challenged and

challenged hens and LRT of challenged hens in each housing system, the largest increase in *Salmonella* prevalence was among hens housed on shavings. It is thought that the risk for disease and pathogen transmission increases among hens housed in cage-free floor systems because they are not separated from their feces (16), and the litter in the all-shavings pen played an important role in the persistence and transmission of *S.* Typhimurium in the current study. Among challenged hens in each housing system, the percentage of cecum and LRT samples positive for *Salmonella* were similar, and ascending infection from the cloaca likely resulted in contamination of the LRT.

There have been conflicting reports regarding the influence of housing system on *Salmonella* prevalence among laying hens. While some studies have indicated that housing system has no effect on *Salmonella* in laying hens (25,37), other studies have reported a higher prevalence of *Salmonella* in caged flocks than in cage-free flocks (29,43,44,47). *Salmonella* spread minimally among non-challenged hens housed in cages and our results suggest that housing laying hens in cages is not a significant risk factor for the transmission of *Salmonella*. However, the number of hens used in this study is considerably lower than number of hens in a commercial facility. Flock size has been reported to have an effect on *Salmonella* prevalence among caged layers (29,34,43) as number of hens housed in caged facilities is generally larger than the number of hens kept in cage-free facilities.

*S.* Enteritidis is the primary serovar associated with laying hens and table eggs, and studies have shown that even when laying hens are orally challenged with large doses ($10^9$ cfu/mL) of *S.* Enteritidis, the incidence of egg contamination is reasonably low (yolk 2.5-7%; albumen 0-2%) (22,23). From eggs produced by challenged hens held in isolation, *S.* Enteritidis was detected in only one pooled eggshell sample suggesting that at the time of lay, *Salmonella*

was present on the eggshell(s) or within eggshell membranes when the egg was laid. The eggshell samples collected from eggs produced in the slats and shavings pens that were positive for *S*. Typhimurium (trial 4) were likely contaminated in the housing system, prior to collection. Although residual *S*. Typhimurium was not recovered from the environment of the all slats pen in trial 4, it was likely present in the environment as the percentage of positive ceca collected from non-challenged hens increased from 15 to 20.

The horizontal transmission of *Campylobacter* among non-challenged penmate hens was significantly greater on shavings than in cages, and the horizontal transmission of *Campylobacter* among non-challenged hens housed on slats was similar to that of hens housed in cages and on shavings. The litter in the shavings pen contributed to the survival of the *Campylobacter* that was shed through the feces of challenged hens. Coprophagia, or the consumption of feces, contributes to the persistence of *C. jejuni* infections in poultry, and the survival of *Campylobacter* in damp litter prolongs the shedding period of broilers (41). Overall, more ceca were positive for *Campylobacter* than *Salmonella* indicating that *Campylobacter* may spread to pen or cage-mates and persist within a flock longer than *Salmonella*. This trend held for *C. jejuni* in all housing systems and *C. coli* in the shavings housing system only. In the current study, *C. jejuni* (n=81; 82%) was recovered from more tissue-organ samples than *C. coli* (n=18; 18%), but among naturally infected commercial laying hens, Cox et al. (12) found that *C. jejuni* and *C. coli* accounted for 50 and 49%, respectively, of *Campylobacter* isolated and that co-colonization occurred in <10% of the hens. In each housing system, *Campylobacter* was recovered from both segments of the reproductive tract and the ovarian follicles, while *Salmonella* was not. Cox et al. (9) also recovered *Campylobacter* (19%) from ovarian follicles of broiler breeder hens at a higher rate than *Salmonella* (1%). There are two primary

implications associated with the recovery of *Campylobacter* from reproductive tissues of hens; one being the possible production of contaminated eggs and the other being vertical transmission of *Campylobacter* from breeder hens to their progeny (8).

There was no significant difference in the horizontal transmission of *Salmonella* between caged and cage-free housing systems. However, when residual *S*. Typhimurium was taken into account, the shavings housing system provided the greatest horizontal transmission. The shavings housing system also provided the greatest horizontal transmission of *Campylobacter*. With regards to food safety, overall results indicate that the caged housing system provides the lowest horizontal transmission of *Salmonella* and *Campylobacter* among egg laying hens.

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Table 4.1. Percentage of tissue samples positive for *Salmonella* (*S. Typhimurium* and *S. Enteritidis*, combined) from non-challenged and challenged laying hens within each housing system.

| | Non-challenged hens | | Challenged hens | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Housing System | n | Cecum | n | Cecum | Spleen | LGB | LRT | URT | Follicles [A] |
| Cages | 60 | 12 | 15 | 20 | 0 | 0 | 13 | 0 | 0 |
| Slats [B] | 61 | 15 | 16 | 25 | 12 | 19 | 25[D] | 19 | 0 |
| Shavings [C] | 58 | 14 | 13 | 15 | 15 | 8 | 15 | 8 | 0 |

[A] Follicles (mature and immature) sampled only during trials 4 and 5; cages n= 6, slats n=7, and shavings n=5.

[B] n=61 non-challenged hens and n=16 challenged hens because one additional non-challenged and challenged hen placed during trial 4.

[C] n=58 non-challenged hens because two hens died during trial 5; n=13 challenged hens because one hen died during trials 2 and 5.

[D] One of the *Salmonella* positive LRT samples was the only sample from which *S.* Enteritidis was recovered.

LGB=Liver/gallbladder

LRT=Lower reproductive tract (shell gland and vagina)

URT=Upper reproductive tract (infundibulum, magnum, and isthmus)

Table 4.2. Percentage of tissue samples positive for *Salmonella* (trials 1-5), with the inclusion of residual *S*. Typhimurium (from trials 1 and 2) recovered in trials 3-5.

| | Non-challenged hens | | Challenged hens | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Housing System | n | Cecum | n | Cecum | Spleen | LGB | LRT | URT | Follicles [A] |
| Cages | 60 | 15[E] | 15 | 27 | 0[*] | 0[*] | 27 | 0[*] | 0[*] |
| Slats [B] | 61 | 20[E] | 16 | 38 | 12[*] | 19[*] | 44 | 19[*] | 0[*] |
| Shavings [C] | 58 | 38[D] | 13 | 31 | 15[*] | 15 | 31 | 15 | 0[*] |

[A] Follicles (mature and immature) sampled only during trials 4 and 5; cages n= 6, slats n=7, and shavings n=5.

[B] n=61 non-challenged hens and n=16 challenged hens because one additional non-challenged and challenged hen placed during trial 4.

[C] n=58 non-challenged hens because two hens died during trial 5; n=13 challenged hens because one hen died during trials 2 and 5.

[D,E] Percentages within columns with different letters differ significantly ($P<0.05$).

[*] No change. Results are the same as reported in Table 4.1.

LGB=Liver/gallbladder

LRT=Lower reproductive tract (shell gland and vagina)

URT=Upper reproductive tract (infundibulum, magnum, and isthmus)

Table 4.3. Percentage of tissue samples positive for *Campylobacter* (*C. coli* and *C. jejuni*, combined) from non-challenged and challenged laying hens within each housing system.

| | Non-challenged hens | | Challenged hens | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Housing System | n | Cecum | n | Cecum | Spleen | LGB | LRT | URT | Follicles [A] |
| Cages | 60 | 28[E] | 15 | 27 | 0 | 0 | 13 | 7 | 17 |
| Slats [B] | 61 | 36[DE] | 16 | 44 | 6 | 19 | 12 | 6 | 14 |
| Shavings [C] | 58 | 47 [D] | 13 | 46 | 0 | 0 | 8 | 8 | 40 |

[A] Follicles (mature and immature) sampled only during trials 4 and 5; cages n= 6, slats n=7, and shavings n=5.

[B] n=61 non-challenged hens and n=16 challenged hens because one additional non-challenged and challenged hen placed during trial 4.

[C] n=58 non-challenged hens because two hens died during trial 5; n=13 challenged hens because one hen died during trials 2 and 5.

[D,E] Percentages within columns with different letters differ significantly ($P<0.05$).

LGB=Liver/gallbladder

LRT=Lower reproductive tract (shell gland and vagina)

URT=Upper reproductive tract (infundibulum, magnum, and isthmus)

Table 4.4. *Salmonella* and *Campylobacter* recovered from environmental samples taken from each housing system during each trial. [A]

| | *S.* Typhimurium | | *S.* Enteritidis | | *C. coli* | | *C. jejuni* | |
|---|---|---|---|---|---|---|---|---|
| | Floor[B] | Nipple drinkers[C] | Floor | Nipple drinkers | Floor | Nipple drinkers | Floor | Nipple drinkers |
| Trial 1 | | | | | | | | |
| Cages | + | na[D] | na | na | - | na | na | na |
| Slats | - | na | na | na | + | na | na | na |
| Shavings | + | na | na | na | - | na | na | na |
| Trial 2 | | | | | | | | |
| Cages | + | na | na | na | - | na | na | na |
| Slats | + | na | na | na | - | na | na | na |
| Shavings | + | na | na | na | + | na | na | na |
| Trial 3 | | | | | | | | |
| Cages | +[E] | na | - | na | - | na | na | na |
| Slats | + | na | - | na | - | na | na | na |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Shavings | + | na | - | na | - | na | na | na |
| Trial 4 | | | | | | | | |
| Cages | - | - | - | - | na | na | + | + |
| Slats | - | - | - | - | na | na | + | - |
| Shavings | + | - | - | - | na | na | + | - |
| Trial 5 | | | | | | | | |
| Cages | - | - | - | - | na | na | + | - |
| Slats | - | + | - | - | na | na | + | - |
| Shavings | + | + | - | - | na | na | + | - |

[A] *S.* Typhimurium and *C. coli* used in trials 1 and 2, *S.* Enteritidis and *C. coli* used in trial 3, and *S.* Enteritidis and *C. jejuni* used in trials 4 and 5.

[B] Sampled via stepped on drag swab.

[C] Nipples of drinker line sampled with open gauze swab during trials 4 and 5 only.

[D] Not applicable.

[E] Any *S.* Typhimurium recovered from samples taken during trials 3-5 was residual *S.* Typhimurium from trials 1 and 2.

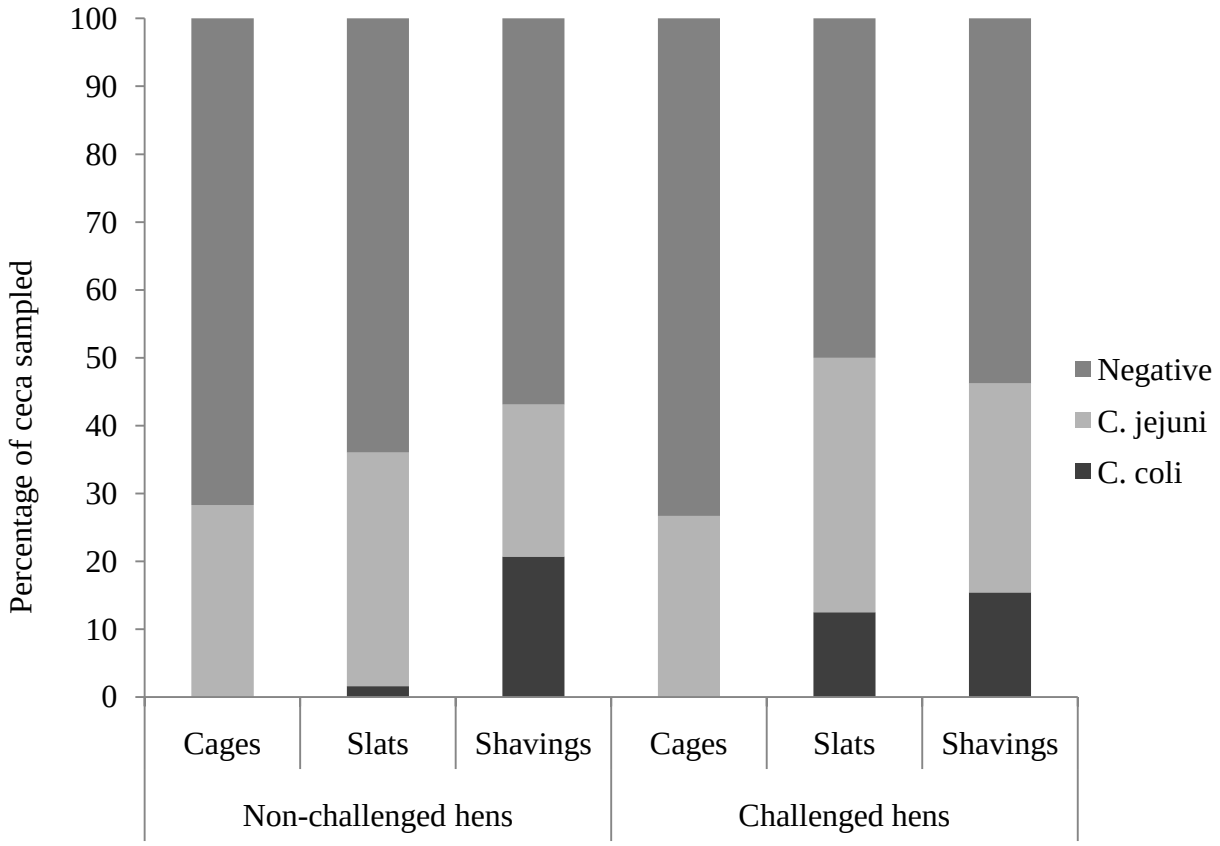


Figure 4.1. Percentage of ceca samples collected from non-challenged and challenged hens in each housing system positive for *C. coli* and *C. jejuni*.

# CHAPTER 5

# COLONIZATION OF A MARKER AND FIELD STRAIN OF *SALMONELLA* ENTERITIDIS AND A MARKER STRAIN OF *SALMOENLLA* TYPHIMURIUM IN VANCOMYCIN PRETREATED AND NON-PRETREATED LAYING HENS

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**Summary**

This study was conducted to evaluate the effects of a vancomycin pre-treatment on the ability of marker (nalidixic acid-resistant) *S.* Enteritidis ($SE^M$), field *S*. Enteritidis ($SE^F$), and marker *S.* Typhimurium ($ST^M$) strains to colonize within the intestinal and reproductive tracts and translocate to other organs of Leghorn laying hens. In each of three trials, caged hens (76, 26, and 33 wk-of-age) were divided into 6 groups designated to receive $SE^M$, $SE^F$, or $ST^M$, and half were pretreated with vancomycin (n=12). Vancomycin-treated hens received 10mg/kg body weight for 5 d to inhibit Gram-positive bacteria within the intestines. On day 6, all hens were challenged orally, intravaginally and intracolonally with *Salmonella* and placed into separate floor pens by *Salmonella* strain. Two wk post-inoculation, all hens were euthanized and the ceca, spleen, liver/gallbladder (LGB), upper (URT) and lower (LRT) reproductive tracts, and ovarian follicles were aseptically collected, and analyzed for *Salmonella*. Results for the three hen's ages were not different and therefore, were combined, and Chi-square and Fisher's exact test were used to identify significant differences ($P<0.05$) in colonization. The vancomycin pretreatment had no significant effect on the colonization ability of $SE^M$, $SE^F$, or $ST^M$. The marker strain of *S*. Enteritidis was recovered from 21% of ceca, 4% of LGB, and 9% of LRT samples. The field strain of *S*. Enteritidis was recovered from 88% of ceca, 96 % of spleen, 92% of LGB, 30% of LRT, 4% of URT and 13% of follicle samples. *S*. Typhimurium was recovered from 100% of ceca, 74 % of spleen, 91% of LGB, 30% of LRT, 9% of URT and 9% of follicle samples. Among ceca, spleen and LGB samples, $SE^F$ and $ST^M$ colonization was significantly greater than $SE^M$ colonization. Overall prevalence of *Salmonella* in the reproductive tracts of challenged hens was relatively low, ranging from 4-30%.

## Introduction

Animals are reservoirs for many zoonotic pathogens, including *Salmonella* (21, 28), and this is partially due to the ability of the microorganism to either persist in the animal's intestinal tract or translocate to and invade other abdominal organs (18). Invasive pathogens pose a greater threat to food safety as contamination can be spread on the surface and into the interior of a food. An important example of this is egg-associated salmonellosis (18). *Salmonella* infection among laying hens is a food safety concern for the commercial table egg industry and *Salmonella enterica* serovar Enteritidis is currently the primary cause of egg-associated salmonellosis (4, 7, 13). Greig and Ravel (13) recently analyzed the international food-borne outbreak data reported between 1988 and 2007, and found that egg associated outbreaks (n=584) were due to *S.* Enteritidis (73.7%), other *S.* enterica (15.3%), and *S. enterica* serovar Typhimurium (8.4%). The detection prevalence of *S*. Enteritidis among the contents of eggs produced by naturally infected hens has been reported to be relatively low (<1.0%) (19, 26).

Antimicrobial resistant or marker strains of *Salmonella* have been used in many scientific studies (1, 5, 15, 16) as they generally remain viable and are readily identifiable with simplified cultivation methods. However, that acquisition of mutations in antibiotic target genes, caused by inducing antibiotic resistance have been associated with fitness costs such as a slower growth rate and reduced virulence (24). The poor colonization of a *S*. Enteritidis marker strain in a previous study may have been influenced by hen age (56-72 wk-of-age) compared to young broilers (5-7 wk-of-age) (14). In general, chickens become more resistant to *Salmonella* colonization with age (from chicks to maturity) and the establishment of intestinal microflora (11, 23), but it is unclear how further increases in age beyond maturity may affect *Salmonella* colonization.

The objectives of this study were to 1) compare the colonization ability of *Salmonella* Enteritidis and *S.* Typhimurium marker strains to that of a *S.* Enteritidis field strain and 2) evaluate the effects of vancomycin antibiotic pretreatment on the ability of the *Salmonella* strains to colonize within and translocate within laying hens. Vancomycin pretreatment has been used to enhance *Salmonella* colonization in 4 wk-old broilers (29). Findings from this study would help determine if lowering intestinal microflora by vancomycin pretreatment improves *Salmonella* colonization in mature Leghorn laying hens.

## Materials and Methods

### *Experimental Design and Inoculation*

In each of three separate trials, Leghorn laying hens (Hy-Line W-36) were randomly allocated to 1 of 6 treatment groups: 1) nalidixic acid-resistant *S.* Enteritidis marker ($SE^M$; provided by N.A. Cox, USDA/ARS Russell Research Center, Athens, GA) strain-no vancomycin pretreatment, 2) nalidixic acid-resistant $SE^M$ strain-with vancomycin pretreatment, 3) *S.* Enteritidis field ($SE^F$; provided by K.W. Post, North Carolina Diagnostic Laboratory, Raleigh; of different origin than $SE^M$) strain-no vancomycin pretreatment 4) $SE^F$ strain-with vancomycin pretreatment, 5) nalidixic acid-resistant *S.* Typhimurium marker [$ST^M$; provided by N.A. Cox and previously used by Buhr et al. (2) and Cox et al. (3)] strain-no vancomycin pretreatment, and 6) nalidixic acid-resistant $ST^M$ strain-with vancomycin pretreatment. Hens were housed in separate isolated pens in individual wire cages according to their designated vancomycin pretreatment. Hens that were allocated to the vancomycin pretreatment groups were housed separately and received 0.5 mL of a vancomycin (Sigma Aldrich, St. Louis, MO) solution (prepared at 10 mg/kg body weight) orally for 5 days to reduce Gram-positive intestinal microflora (17). On the sixth day, all hens were challenged orally (1 mL), intravaginally (1 mL), and intracolonally (1 mL) with either $SE^M$ average $1.1 \times 10^8$ cfu/mL, $SE^F$ average $2.0 \times 10^9$

cfu/mL, or $ST^M$ average 2.4 x $10^8$ cfu/mL. After inoculation, hens were placed in separate isolated floor pens (7'x7' | 2.1m x 2.1m) on fresh pine shavings with access to a nest box (6 nests/box), water and feed *ad libitum*, and a 16 h photoperiod. A total of 3 pens ($SE^M$, $SE^F$, and $ST^M$) were used as non-pretreated and vancomycin pretreated hens inoculated with the same strain of *Salmonella* were placed in the same pen. The hens used in trials 1, 2, and 3 were 76, 26, and 33 wk-of-age, respectively. In the first trial (n=18 hens), 3 hens were used in each pretreatment group (6 hens/pen; 8.2 $ft^2$/hen | 0.8 $m^2$/hen). In the second trial (n=30 hens), 5 hens were used in each pretreatment group (10 hens/pen; 4.9 $ft^2$/hen | 0.4 $m^2$/hen), and in the third trial (n=24 hens), 4 hens were used in each pretreatment group (8 hens/pen; 6.1 $ft^2$/pen | 0.6 $m^2$/hen). Two weeks post-inoculation, all hens were euthanized by electrocution for sample collection.

***Organ Samples***

The ceca, spleen, liver/gallbladder (LGB), upper (URT: infundibulum, magnum, and isthmus) and lower (LRT: shell gland and vagina) reproductive tracts, and ovarian follicles were aseptically collected from all hens for *Salmonella* analysis. Each sample was transferred to a sterile sample bag, placed on ice, and transported to the laboratory for analysis. An average weight for each sample type was obtained. The samples within the plastic bags were smashed with a rubber mallet to expose the internal contents of the samples. Sterile buffered peptone water (BPW; 1%; Acumedia, Lansing, MI) was added at a ratio of 3 times the weight of the sample (mL/g). All samples were then placed in a Stomacher 400 (Fisher Scientific, Hampton, NH) and stomached for 1 min. Samples were incubated at 37°C for 24 hr.

***Fecal Samples***

To collect fecal samples, hens from each pen were placed into individual units of a 3 unit portable wire cage system lined with clean brown craft paper. Approximately 3 g of fresh fecal

material was aseptically collected from each hen. After sample collection, hens were returned to their designated floor pen. To maintain aseptic technique, new gloves were used for each fecal sample and clean craft paper was used for each group of hens placed in the portable cage system. Fecal samples were collected 1 wk post-inoculation during each trial. Each fecal sample was placed in a sterile 50 mL centrifuge tube and transferred to the laboratory for analysis. A standard volume of 30 mL of BPW was added to each fecal sample, and all samples were vortexed. Samples in BPW for *Salmonella* analysis were incubated at 37°C for 24 hr.

***Environmental Samples***

During each trial, the floors of each pen were sampled by stepped on drag swabs (n=1/pen). Presoaked drag swabs (DS-001, Solar Biologicals, Inc., Ogdensburg, NY) were unwound and dragged across the shavings floor in a figure 8 around the pen (2). Swabs were stepped on 4 times during sampling with a clean, disposable boot cover that was put on upon entering each pen. The nipples on each drinker line were also sampled for *Salmonella*. Using a gloved hand, each nipple (8/line) was swabbed with an open gauze swab (n=1/pen). Individual floor and nipple drinker swab sample were placed in a sterile sample bag and transported to the laboratory. One hundred mL of BPW was added to each sample. All samples were massaged by hand to loosen any attached debris. Swab samples were incubated at 37°C for 24 hr for *Salmonella* analysis.

***Egg Samples***

Eggs were collected from each pen daily, placed on a clean flat, and held in an onsite cooler at 5°C until sampled. Eggs were pooled (trials 1 and 3, n=5 eggs/sample; trial 2, n=8 eggs/sample) by pen. A total of 129 eggs (23 samples), 152 eggs (25 samples), and 167 eggs (28 samples) were collected from hens challenged with $SE^M$, $ST^M$, and $SE^F$, respectively. Eggs

within each pooled sample were cracked on a sterile surface and the internal contents were discarded due to the low incidence of *Salmonella* contamination among contents (10, 11). The eggshell and shell membranes were crushed by hand and placed in a sterile sample bag. Shell samples from eggs within each pooled sample were combined and placed in the same sample bag. To maintain aseptic technique, new gloves were used between pooled samples. Sterile BPW was added to each sample bag at a ratio of 20 mL/eggshell and all samples were incubated at 37°C for 24 hr for *Salmonella* analysis.

***Plating procedures***

Following incubation, 2 loops (20 µL) from each sample for $SE^M$ and $ST^M$ analyses were streaked onto Brilliant Green Sulfa (BGS) Agar (Acumedia, Lansing, MI) containing 200 ppm nalidixic acid (Sigma Aldrich, St. Louis, MO). BGS plates were incubated for 24 h at 37°C. Colonies characteristic of *Salmonella* were selected and subjected to the slide agglutination tests using *Salmonella* O Antisera (Becton Dickinson, Sparks, MD) for serogroup (A-I followed by Group $D_1$ for $SE^M$ and Group B for $ST^M$) confirmation. For $SE^F$ samples, 0.1 mL of incubated BPW was transferred to 9.9 mL of Rappaport-Vassiliadis (RV; Becton Dickinson, Sparks, MD) broth and 0.5 mL of incubated BPW was transferred to 9.5 mL of Tetrathionate (TT; Becton Dickinson, Sparks, MD) broth. The RV and TT broths were incubated at 42°C for 48 h. Two loops from the incubated RV and TT broths were streaked onto BGS and Xylose-Lysine-Tergitol 4 (XLT4; Acumedia, Lansing, MI) plates, and all plates were incubated at 37°C for 24 h. Suspect colonies were picked and transferred to triple sugar iron (TSI; Becton Dickinson, Sparks, MD) and lysine iron agar (LIA; Acumedia, Lansing, MI) slants. Slants were incubated at 37°C for 24 h. Presumptive colonies were then subjected to slide agglutination tests using *Salmonella* O Antisera for serogroup (A-I followed by Group $D_1$ for $SE^F$) confirmation.

***Statistical Analysis***

Chi-square and Fisher's exact test were used to identify differences in *Salmonella* colonization due to bacterial strain (3; $SE^M$, $SE^F$, and $ST^M$) and vancomycin pretreatment (2; non-pretreated and pretreated hens). Since it was determined that Vancomycin pretreatment had no impact on colonization for any of the three *Salmonella* strains, therefore non-pretreatment and pretreatment data were combined and reanalyzed by strain only. Differences were considered significant at $P<0.05$.

**Results**

There was no significant difference ($P<0.05$) in $SE^M$, $SE^F$, and $ST^M$ colonization (Table 1) between non-pretreated and vancomycin pretreated hens for all samples collected. $SE^M$ was recovered from 17% (4/23) of the fecal samples collected. $SE^F$ was recovered from 42% (5/12) of the fecal samples, and $ST^M$ was recovered from 100% (23/23) of the fecal samples.

For $SE^F$ and $ST^M$ challenged hens colonization was significantly greater among cecum, spleen, and LGB samples than $SE^M$ challenged hens. $SE^M$ was recovered from 21% of cecum, 4% of the LGB, and 9% of the LRT samples, and all positive samples were collected from different hens. $SE^F$ was recovered from 88% of the cecum, 96% of the spleen, 92% of the LGB, 30% of the LRT, 4% of the URT, and 13% of the follicle samples. Six out of the seven LRT samples that were positive for $SE^F$ were collected from hens that also had positive ceca. The URT (n=1) and ovarian follicle (n=3) samples that were positive for $SE^F$ were collected from four different hens. *S.* Typhimurium was recovered from 100% of the cecum, 74 % of the spleen, 91% of the LGB, 30% of the LRT, 9% of the URT and 9% of the follicle samples. The two URT samples that were positive for $ST^M$ were collected from hens that had positive LRT samples. Of the two ovarian follicle samples that were positive for $ST^M$, one was collected from

a hen with positive LRT and URT samples, while the other was collected from a hen with negative LRT and URT samples.

$SE^M$ was not recovered from any of the floor or nipple drinker swab samples taken throughout the study (Table 1; n=3). $SE^F$ was recovered from the floor and nipple drinker swab samples taken during trials 1 and 3, but samples taken during trial 2 were negative for $SE^F$. $ST^M$ was recovered from drag swab samples taken during trials 2 and 3 and nipple drinker swab samples taken during all three trials. Results indicate that the sampling methods used were sufficient for recovering $ST^M$ and $SE^F$ but not $SE^M$ from the pen environment.

All eggshell samples (n=23) from eggs produced by hens challenged with $SE^M$ were negative (Table 1). From eggs produced by hens challenged with $SE^F$, 21/28 (75%) eggshell samples were positive. From eggs produced by hens challenged with $ST^M$, 18/25 (72%) eggshell samples were positive.

**Discussion**

Vancomycin is effective against Gram-positive bacteria, and studies have shown that Gram-positive bacteria are abundant in and predominately cultured from the avian intestinal tract (12, 22, 27). Reducing established intestinal microflora should make the hens more susceptible to *Salmonella* infection. However, in this study, vancomycin pretreatment had no significant effect on the ability of the *Salmonella* strains to colonize within the ceca and reproductive tract and to translocate to other organs of the laying hens. These results suggest that hen's age (26, 33, and 76 wk-of-age) and the presence of established intestinal microflora were not an influential factors affecting *Salmonella* colonization by these three strains. It is likely that the $SE^M$ strain used in this study lacked the factors needed to colonize and proliferate within the intestinal tract of the laying hens.

$SE^F$ and $ST^M$ colonization was significantly greater among cecum, spleen, and LGB samples than $SE^M$ colonization. There was no significant difference in $SE^M$, $SE^F$ and $ST^M$ colonization of the reproductive tract. The marker strain of *S.* Enteritidis was recovered from the feces of 4 hens and the organs of 7 separate hens, suggesting that intestinal colonization did occur in approximately 50% (11/23) of the hens. However, it is likely that among the hens fecally excreting $SE^M$, the bacterial infection did not spread to other organs as the bacteria were not recovered from any organ samples collected. $SE^M$ was primarily recovered from ceca (21%) and LRT (9%) samples. Colonization of the cecum can be attributed to the oral and/or intracolonal routes of inoculation, while colonization of the reproductive tract most likely resulted from intravaginal inoculation, although the oviduct can become contaminated through ascending infection from the cloaca (6, 25). The $SE^M$ strain was recovered from the LGB of 1 hen (4%) suggesting that the bacterial infection became systemic. $SE^F$ was recovered from cecum, spleen, LGB, LRT, URT, and follicle samples, indicating that this strain of *S.* Enteritidis was more invasive than the $SE^M$ strain. $SE^F$ was recovered from 21/24 cecum samples, 22/24 spleen samples, and 23/24 LGB samples. Similar results were reported by Gast and Beard (8), who after orally challenging laying hens (ranging from 20 to 88 wk-of-age), recovered *S.* Enteritidis from 21/25 cecum samples, 20/24 spleen samples, and 19/24 liver samples two weeks post-inoculation. In the current study, all positive URT and ovarian follicle samples were collected from hens with positive spleen and LGB samples. These results suggest that the $SE^F$ translocated to the URT and can disseminate to the ovarian follicles via the vascular system, and that the yolks and contents of eggs produced by the infected hens could become contaminated prior to oviposition. $ST^M$ was also recovered from cecum, spleen, LGB, LRT, URT, and follicle samples. The $SE^F$ and $ST^M$ strains used in this study seemed to be equal in their ability to

colonize the ceca and reproductive tracts and to translocate to other organs of laying hens. Keller et al. (20) also found that *S.* Enteritidis had no selective advantage over *S.* Typhimurium (field strain) in its capacity to invade reproductive tissues.

The levels of $SE^F$ excreted through the feces of hens in trials 1 and 3 were sufficient enough for the bacteria to be detected in the environment and $ST^M$ was recovered from environmental samples in all 3 trials. Environmental contamination likely contributed to the increased rate of $SE^F$ (75%) and $ST^M$ (72%) recovery from eggshell samples. Hens used in this study were housed in cages prior to placement into floor pens and without being acclimated to or familiar with nesting behavior, they laid the majority of their eggs on the shavings covered floor in a communal nest area. Therefore, eggs were susceptible to environmental surface contamination and the hens feet.

Hen age (26, 33, and 76 wk-of-age) and vancomycin pretreatment to reduce established intestinal microflora had no affect *Salmonella* colonization and desemination. Both $SE^F$ and $ST^M$ colonized the ceca, spleen, and LGB at significantly higher rates than $SE^M$, and were recovered from environmental and eggshell samples, while $SE^M$ was not. The $SE^M$ strain did not colonize well within the laying hen and was not recovered from the environmental or eggshell samples, suggesting that when in competition for nutrients during culture, this $SE^M$ is difficult to recover. Since the poor colonization of $SE^M$ cannot be attributed to the presence of established intestinal microflora, it may be associated with the induction of antibiotic resistance and repeated lab culture.

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Table 5.1. Percentage of samples positive for *Salmonella* from vancomycin (VNC) pretreated and non-pretreated laying hens.

| | n[A] | Cecum | Spleen | LGB | LRT | URT | Follicles | Floor [B] | Nipple drinkers [C] | Eggshells |
|---|---|---|---|---|---|---|---|---|---|---|
| SE[M] no VNC | 12 | 8 | 0 | 8 | 8 | 0 | 0 | | | |
| SE[M] with VNC | 11 | 36 | 0 | 0 | 9 | 0 | 0 | | | |
| Total for SE[M] | 23 | 21[E] | 0[E] | 4[E] | 9 | 0 | 0 | - | - | 0[E] |
| SE[F] no VNC | 12 | 83 | 100 | 100 | 17 | 0 | 17 | | | |
| SE[F] with VNC | 12 | 92 | 92 | 83 | 42 | 8 | 8 | | | |
| Total for SE[F] | 24 | 88[D] | 96[D] | 92[D] | 30 | 4 | 13 | + | + | 75[D] |
| ST[M] no VNC | 11 | 100 | 73 | 91 | 18 | 18 | 18 | | | |
| ST[M] with VNC | 12 | 100 | 75 | 83 | 42 | 0 | 0 | | | |
| Total for ST[M] | 23 | 100[D] | 74[D] | 91[D] | 30 | 9 | 9 | + | + | 72[D] |

[A] n=11 hens because one hen from the respective pretreatment group died during trial 2.

[B] Sampled via stepped on drag swab.

[C] Nipples of drinker line sampled with open gauze swab.

[D, E] Percentages within columns with different letters are significantly different ($P<0.05$).

$SE^M$=*Salmonella* Enteritidis marker strain

$SE^F$=*Salmonella* Enteritidis field strain

$ST^M$=*Salmonella* Typhimurium marker strain

LGB=Liver/gallbladder

LRT=Lower reproductive tract (shell gland and vagina)

URT=Upper reproductive tract (infundibulum, magnum, and isthmus)

# CHAPTER 6

# SUMMARY AND CONCLUSIONS

Laying hens used in the commercial table egg industry are primarily housed in conventional battery cages, and one of the most controversial issues facing the table egg industry today is hen housing and welfare. With alternative, cage-free production becoming an international trend, it is important to determine what effect housing system has on eggshell contamination and pathogen transmission among laying hens. This study and majority of literature indicate that the eggshells of eggs collected from hens housed in conventional cages had lower levels of APC than eggs from cage-free hens housed on slats or shavings. Washing eggs significantly lowered eggshell bacteria levels. When hens were moved from the shavings, slats, and cages room to triple-deck cage units in a separate room, lower levels of aerobic bacteria were recovered from both non-washed and washed eggs. After moving hens back to the shavings, slats, and cages room at one-third hen density, the level of APC on the eggshells rapidly increased to levels recovered in the first experiment, indicating the significance of housing system. However, following washing of nest clean eggs, the resulting eggshell APC levels are comparable for eggs from hens housed in cages, on slats, or on shavings. These data demonstrate that from a food safety prospective, if eggs are not going to be washed, it is important that they are produced in a housing system with as little contamination as possible and collected frequently (within two hours of lay), as eggs are susceptible to bacterial contamination at various stages of production (from the time of lay to the time of processing).

When residual *S*. Typhimurium was taken into account, horizontal transmission was greatest in the shavings housing system. There was no significant difference in the horizontal transmission of *Salmonella* between caged and cage-free housing systems. These results were partially due to the poor colonization of the *S*. Enteritidis marker strain used in the study. It did not colonize well within challenged laying hens and was not recoverable from the environment. However, the marker strain of *S*. Typhimurium colonized within challenged and non-challenged pen mate hens and persisted in the environment throughout the duration of the study.

Horizontal transmission of *Campylobacter* was also greatest in the shavings housing system. Overall results indicate that the caged housing system provides the lowest horizontal transmission of *Salmonella* and *Campylobacter* among laying hens. This was one of the first known studies conducted to determine the horizontal transmission of *Campylobacter* among caged and cage-free laying hens. With regards to *Salmonella* these data conflict with previously published commercial reports that caged production increases the prevalence of *Salmonella* infection in laying hens.

Hen age and the presence of an established intestinal microflora were thought to be possible factors in the poorer colonization of the *S*. Enteritidis marker strain. However, data from the third study indicate the vancomycin pretreatment intended to reduce Gram-positive intestinal microflora had no significant effect on *Salmonella* colonization. The *S*. Enteritidis field strain and the *S*. Typhimurium marker strain colonized the tissues of laying hens at significantly higher rates than the *S*. Enteritidis marker strain. Since the poor colonization of *S*. Enteritidis marker strain cannot be attributed to established intestinal microflora, it may be due to the acquisition of antibiotic resistance and lab passage. This is supported by the fact that the field strain of *S*. Enteritidis, absent of antibiotic resistance, was more invasive than the marker

strain. However, it is difficult to attribute the poor colonization of the *S*. Enteritidis marker strain solely to the acquisition of antibiotic (nalidixic acid) resistance considering the marker strain of *S*. Typhimurium colonized within and translocated to internal tissues of laying hens at reasonably high rates. These data demonstrate that there are serovar, strain, and host specific factors (i.e. age, broiler, breeder, or layer) that can affect *Salmonella* colonization in laying hens.

# APPENDICES

The appendices are included to provide data from two supplemental studies that were conducted without an extensive literature review, statistical analysis, or discussion of the results. Appendix A describes methods used to obtain (in vitro) the growth curves of four different strains of *Salmonella*. Appendix B describes methods used to evaluate the colonization potential of a second marker strain of *S.* Enteritidis in broilers.

# APPENDIX A

# GROWTH PROFILES OF FOUR DIFFERENT STRAINS OF *SALMONELLA* IN BUFFERED PEPTONE

## Materials and Methods

Fifty mL of buffered peptone (BP; 1%; Acumedia, Lansing, MI) with 25 ppm nalidixic acid (Sigma Aldrich, St. Louis, MO) was inoculated with a field strain of *S*. Enteritidis. The BP was incubated at 37°C for 24 hr. Following incubation, two loops (20 µL) from the incubated *Salmonella* sample were streaked onto Brilliant Green Sulfa (BGS; Acumedia, Lansing, MI) Agar containing 200 ppm nalidixic acid. The BGS plates were incubated at 37°C for 24 hr. Colonies were selected and streaked onto fresh BGS plates and refrigerated.

Four separate suspensions containing approximately $10^2$ cfu/mL of a nalidixic acid-resistant *S*. Enteritidis marker strain ($SE^M$), the *S*. Enteritidis field strain ($SE^F$), the newly induced nalidixic acid-resistant *S*. Enteritidis marker strain ($SE^{FM}$), and a nalidixic acid-resistant *S*. Typhimurium marker strain ($ST^M$) were prepared. For each strain of *Salmonella,* 0.4 mL was transferred to 5 wells (for 5 replications) of a Honeycomb 2 cuvette plate (Labsystems, Inc., Franklin, MA). Plates were placed in the incubator tray of a computer-operated Bioscreen C microbiology reader (Thermo Electron Corp., West Palm Beach, FL). Bacterial cultures were incubated at 37°C for 24 hr to measure optical densities.

## Results

See Figure A.1.

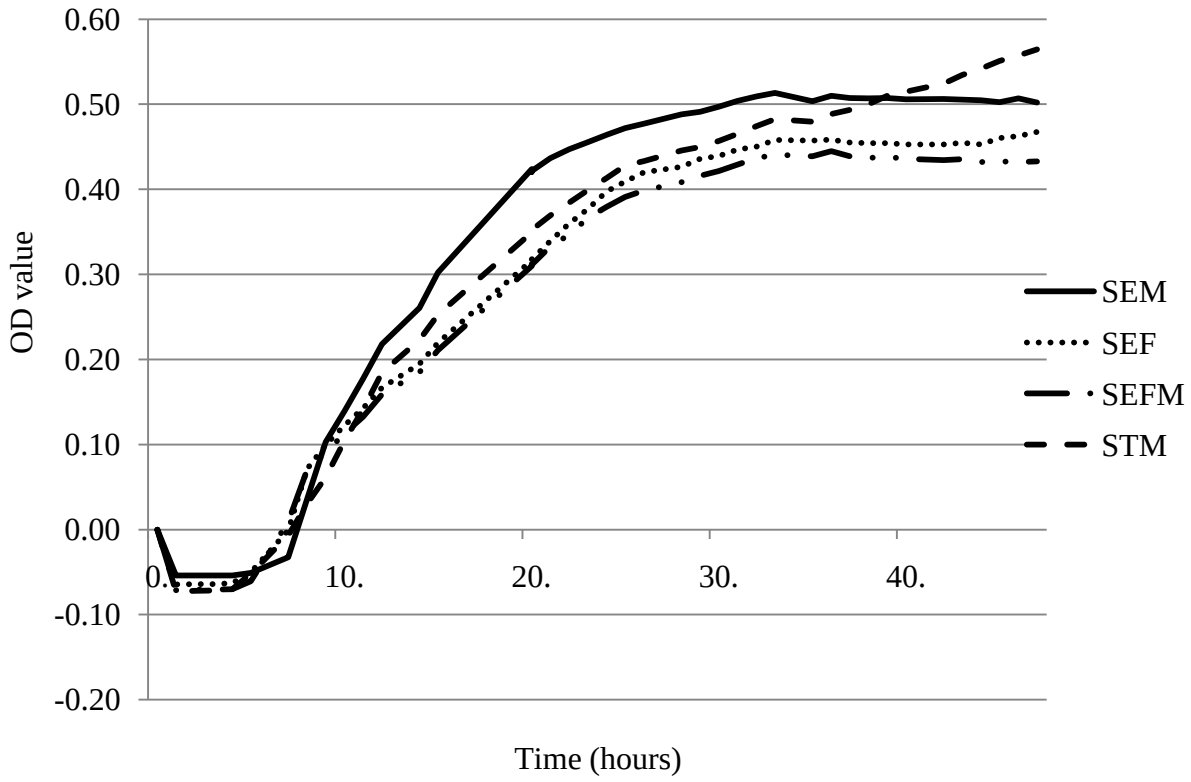


Figure A.1. Growth curves of the *S*. Enteritidis marker strain ($SE^{M}$), the *S*. Enteritidis field strain ($SE^{F}$), the marker strain made from *S*. Enteritidis field strain ($SE^{FM}$), and the *S*. Typhimurium marker strain ($ST^{M}$).

APPENDIX B

THE COLONIZATION POTENTIAL OF A SECOND MARKER STRAIN OF *S.* ENTERITIDIS IN BROILERS

## Materials and Methods

### *Inoculation and experimental design*

In each two replications, 12 broilers were challenged orally (1 mL) with either moderate ($2.8x10^4$ cfu/mL) or high ($2.8x10^8$ cfu/mL) levels of the second nalidixic acid-resistant *S.* Enteritidis marker strain ($SE^{FM}$). Broilers receiving moderate or high levels of *Salmonella* were housed in separate pens. One wk post-inoculation, all birds were euthanized by electrocution for sample collection.

### *Ceca samples*

Ceca were aseptically collected from all broilers. Each sample was transferred to a sterile sample bag and transported to the laboratory for analysis. An average sample weight was obtained. The samples within the plastic bags were then smashed with a rubber mallet to expose the internal contents. Buffered peptone water (BPW; 1%; Acumedia, Lansing, MI) was added at a ratio of 3mL/g of sample. All samples were then placed in a Stomacher 400 (Fisher Scientific, Hampton, NH) and stomached for 1 min. Ceca samples in BPW were incubated at 37°C for 24 hr.

### *Plating procedures*

Two loops (20 µL) from each incubated ceca sample were streaked onto Brilliant Green Sulfa (BGS) Agar containing 200 ppm nalidixic acid. BGS plates were incubated at 37°C for 24

hr and colony forming units characteristic of *Salmonella* were selected and subjected to slide agglutination tests using *Salmonella* O antiserum (Group $D_{1;}$ Becton Dickinson, Sparks, MD) for serogroup confirmation.

**Results**

*S.* Enteritidis was recovered from 17% (4/24) of the ceca samples collected from broilers challenged with a moderate ($10^4$) level of bacteria. *S.* Enteritidis was recovered from 96% (23/24) of the ceca samples collected from broilers challenged with a high ($10^8$) level of bacteria.