INVESTIGATION OF THE IMPACTS OF HATCHERY PRACTICES ON INTESTINAL MICROFLORA OF LATE-STAGE EMBRYOS AND EARLY POST-HATCH CHICKS

A Thesis

by

BRETT D. MEISINGER

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Chair of Committee,	John B. Carey
Co-chair of Committee,	Craig D. Coufal
Committee Members,	Audrey P. McElroy
	James A. Byrd
Head of Department	Audrey P. McElroy

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ABSTRACT

Commercial hatcheries are a key part of the poultry industry, and the quality of stock produced is influential to the chick's life throughout production. Previous studies with broiler hatching eggs have determined that despite the sanitation and vaccination plans currently in place at the commercial level, chicks are leaving the hatchery with a high intestinal tract microbial load. The proper handling and sanitation of eggs at breeder facilities and throughout the duration of the hatching process is critical in lessening the exposure risk of pathogenic bacteria that can penetrate viable eggs and replicate internally. The objectives of this research were to examine the impact of hatchery sanitation methods, such as formaldehyde application, in addition to the use of *in ovo* injection for vaccine administration on intestinal tract microflora of late incubation embryos and neonatal chicks. Additionally, a study on the membrane microbial distribution of late-stage hatching eggs was performed to evaluate the relationship of internal and external pipping, and the relationship to intestinal tract microbiota.

Microbiological evaluations determined that embryos and newly hatched chicks subjected to formaldehyde application resulted in a reduction of the microbial load while formaldehyde was in use, yet when formaldehyde use ceased, the intestinal tract counts began to approach the counts of the no formaldehyde treatment group. Similar to the intestinal tract microbiota results, the air plate enumeration dropped during treatment for the formaldehyde application group, and approached the non-treated group on 21 d after formaldehyde application ceased. Based on the results, formaldehyde application in the hatchers did not significantly impact the colonization of the chick's intestinal tract in the late stages of broiler hatching egg incubation.

Microbial evaluations were also performed to determine the effects of *in ovo* injection on chick intestinal tract microbiota from 19 to 21 d of incubation. The results of the experiment

concluded that significant differences existed at 20 d of incubation in Trial 1 with the no *in ovo* injected treatment group on the TSA aerobic (P= < 0.0001) and TSA anaerobic (P=0.0012) plate counts when compared to the *in ovo* injected treatment group. Similarly, TSA aerobic plate counts were significantly higher (P= 0.0409) in the *in ovo* injected treated group. The 21 d results differed between trials. On 21 d of incubation, microbial enumeration of TSA aerobic and anaerobic were not significantly affected by the use of *in ovo* injection. Trial 1 concluded that *in ovo* injected eggs on 21 d had a greater enumeration value, while in Trial 2 the no *in ovo* injected eggs had a greater enumeration value. Results of this experiment conclude that *in ovo* injection is not a primary source of hatchery contamination due to the elevated microbial counts present in the non-*in ovo* injection groups for both trials.

A third study performed to evaluate the presence of microorganisms in the eggshell membranes of hatching eggs. Eggshell membranes were extracted from White Leghorn fertile eggs collected from nest and floor locations. Enumeration of the microorganisms in the membranes concluded that there were no differences between the eggs from the nest and floor. Eggshell membranes at day of lay were contaminated with both aerobic and anaerobic bacteria, and fungi regardless of the location the eggs originated. A comparison of eggshell membrane microbial counts and gastrointestinal microbiota was performed in late-stage Ross 708 broiler hatching eggs. The objective was to determine if there was a link between contaminated membranes and the pipping process. Results concluded that the same bacteria were found in the membrane and intestinal tract on most samples; however, additional research is warranted.

DEDICATION

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NOMENCLATURE

APC	Aerobic plate count
BEA	Bile Esculin Agar
cfu	Colony forming unit
d	day
EMB	EMB Agar
EYA	Egg Yolk Agar
Form	Formaldehyde application treatment
h	hour
LOD	limit of detection
log	logarithmic
min	minute(s)
mL	milliliter
MSA	Mannitol Salt Agar
No Form	No formaldehyde application
PBS	phosphate buffer solution
s	seconds
SE	Standard Error
TSA	Tryptic Soy Agar for aerobic plate count
TSA-AN	Tryptic Soy Agar for anaerobic plate count
µm	Micrometer
UV	ultraviolet light

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

The microbial load found in commercial hatching eggs varies greatly depending on location of egg deposition and other external factors. Broiler breeder house designs are equipped with designated nest boxes and a built-in conveyer belt to deliver fertile eggs to the storage and sorting area (McElroy, et al., 1989). At the time of collection, eggs are sorted based on size, shell quality, and visual cleanliness. Eggs are then stored prior to transport to the hatchery in a hypothermic environment. Eggs that are visually soiled often have lower hatchability, and soiled eggs are left unwashed and separated from good quality hatching egg stock (Van den Brand, et al., 2016). Good egg handling and sanitation procedures are imperative to limiting the potential sources of contamination of hatching eggs because eggshell contamination can have negative effects far beyond the hatchery environment. If contamination of an egg occurs, bacteria found within the developing chick can survive through the live poultry production chain and lead to a food safety concern in poultry products (Coufal, et al., 2003).

Formaldehyde is a known chemical disinfectant, that when used at balanced levels, can reduce eggshell surface microbial counts (Acklund, et al., 1980; Braswell et al.,1970). Previous studies have indicated that successful administration of formaldehyde application in commercial hatchers can reduce eggshell surface and air plate enumeration. This has been achieved by testing a multitude of drop intervals, formaldehyde volume and withdrawal periods in late-stage incubation (Cadirci, 2009). The effect of formaldehyde application on chick intestinal microbiota is unknown. Limited research has evaluated this sanitation method's effect on intestinal microbiota and warrants further review at the commercial level.

As poultry production has become an industrial scale business to meet the demand for more poultry meat production, *in ovo* vaccine injections have become the alternative to posthatch, hand-vaccinated methodologies (Saeed, et al., 2019). The ideal timeframe to inject eggs from a developmental standpoint is when the yolk sac begins to absorb into the abdomen and internal pipping occurs for the most direct vaccine administration (Williams, 2007). *In ovo* technology uses the direct administration of vaccines to enhance the overall immune response and health of the chicken embryo (Saeed, et, al., 2019). Due to penetration of the injection needle breaking an egg's natural line of defense, the potential for transfer of bacteria through the shell membrane is possible (Meijerhof, et al., 1997). This exposure of the internal contents of hatching eggs and hatcher environments is of interest as a potential egg contamination source in late-stage incubation.

Limited research has been published on the relationship between the microbiota of the eggshell membrane and the chick intestinal tract during late-stage incubation. Past research found that low microbial levels were present in chick embryo intestinal samples prior to 18 d of incubation (Cantu, 2018; Cantu 2021). However, an exponential increase in embryo intestinal tract microbiota was observed during 19 to 21 d of incubation when pipping occurs. In addition, it was of interest to compare White Leghorn hatching eggs laid on the floor and eggs laid in nesting boxes to establish a baseline for eggshell membrane microbiota and a method for sample extraction. The primary objectives of this study were to evaluate the effects of formaldehyde application and *in ovo* injection on late-stage commercial broiler hatching eggs. Additionally, the relationship between eggshell membrane microbiota and intestinal microbiota at internal egg pipping, external egg pipping, and hatch in broiler hatching eggs is of interest.

CHAPTER II

LITERATURE REVIEW

Innate egg defenses

Eggs of avian species possess effective and efficient methods to defend the embryo from continuous external exposure to potential microbial contamination (Kulshreshtha, et al., 2022). These defenses include the cuticle (bloom), the shell, the inner and outer shell membranes, and the albumen (Berrang, 1999). There are two types of defense mechanisms; nonspecific and adaptive (Hincke, et al., 2019). Nonspecific mechanisms act against pathogens in a non-targeted way. These methods include cellular and antimicrobial molecules, in addition to macrophages and heterophils. Adaptive mechanisms on the other hand are pathogen-specific and will only act upon and directly manipulate replication of a pathogen by the induction of antimicrobial agents or by phagocytosis (Hincke, et al., 2019).

The cuticle is a thin defensive feature that forms a barrier against microbial contamination, plugging the pores on the shell surface, and ultimately decreasing the egg's porosity (Hincke, et al., 2019). This proteinaceous substance, varying in thickness from 0.5 to 12.8 μm (Board and Fuller, 1974), is placed on the shell surface as it leaves the hen's vaginal cavity, dries, and partially fills the shell's pores (Berrang, et al., 1999). This feature protects embryos throughout the incubation, hatching, and production process.

The eggshell, compromised almost entirely from calcium carbonate, provides physical protection against microbial invasion. It is predominantly composed of the inner mammillary cone layer, central palisades, and the outer vertical crystal layers. (Wellman-Labadie, et al., 2008). The eggshell mineral is associated with an organic matrix composed of proteins glycoproteins and proteoglycans, which ultimately influences the base of this biomaterial during

its deposition (Nys et al, 2004). Shell quality, thickness, and porosity have been linked to the contamination rate of hatching eggs and vary depending on breed, species, and nutrition of the hen. Additionally, in studies where eggs are placed in direct contact with known pathogens, the rate at which microbial invasion occurs is greatly influenced by shell characteristics (Board and Fuller, 1974; Yamamoto, et al., 1997). Eggshell thickness is not directly linked to its protective capabilities. Thicker shelled eggs may have larger pores; thus, it has been argued that the coverage of the cuticle is of more importance to protective capabilities of eggs (Berrang, 1999).

In addition to the exterior egg's defenses, the eggshell membranes, both interior and exterior, have been reported to be the most challenging part of the egg's physical defenses. It is known that bacteria are able to penetrate through the cuticle into the interior of eggs (Berrang, et al., 1999 (2)). The two membranes act as a filtration barrier that can aid in the transmission blocking of microorganisms. Eggshell membranes consist of keratin layers that are encapsulated in glycoprotein. The inner and outer eggshell membranes have been found to not be effective in the control of yeasts and molds, and more to the defense of bacterial contamination (Board and Fuller, 1999). Earlier research suggests that between the two membranes, the inner shell membrane is more effective in reducing microbial contamination when directly compared to the outer shell membrane, partially due to the difference in thickness and protein makeup (Berrang, 1999).

The albumen also plays a key role in an eggs' natural defense mechanisms. The egg albumen consists of a thick and thin albumen, in addition to a chalaziferous layer, totaling 60% of an egg's weight. Its viscous consistency delays the inward movement of microorganisms toward the egg yolk. The viscosity of the thick albumen is much higher than of the thin due to its ovomucin concentration being four times that of thin albumen (Yamamoto, et al., 1997). This trait, along with an alkaline pH and inhibitory protein makeup creates a less than ideal

environment for microbial survivability and replication in the albumen (Board and Fuller, 1994; Wellman-Labadie, et al., 2008). In chicken eggs, the albumen averages 10% protein and 90% water. The albumen includes more than 40 different proteins, with the highest concentrations being ovomucinoid, lysozyme, ovalbumin, conalbumin, ovomucin, ovoglobulin, ovomacroglobulin, ovoglycoprotein, cystatin, flavoprotein, avidin, and ovoinhibitor (Yamamoto, et al., 1997). Ovalbumin is the major protein of the albumen, and constitutes about 54% of the total albumen protein. The conalbumin (ovotransferrin) and ovomucoid occupy about 12% and 11%, respectfully, with the latter portion of proteins each making up 4% or less of the total albumen protein (Cantu, 2021; Yamamoto, et al., 1997).

Chicken breed and type: differences in intestinal microbiota

Considerable physiological differences exist between chicken breeds selected for meat and egg production. These distinctions have affected the immune function and intestinal physiology of the birds (Simon, et al., 2016). Morphological differences are easily distinguishable between broiler chickens and White Leghorn layers, including: villus height, villus width, and crypt depth. These factors influence the absorptive ability of the bird's digestive tract. Larger features are associated with meat type breeds (Uni et al., 2014). Broiler strains (Ross vs Cobb) show variability in gut microbiota (Kim et al., 2015). Intestinal physiological differences are likely to influence the microbiota of each bird type and strain.

There are many other factors that could be responsible for microbiota differences between production breed birds. The growing environment and the variety of rearing facilities can greatly manipulate the types of microorganisms entering a bird's system (Gong, et al., 2008).
Most broilers are produced on the floor of chicken houses, while most layers are caged. Layer chickens can be kept in a wider array of housing condition including free range, battery-caged,

and cage-free. The microbiota difference between poultry flocks housed on recycled litter and caged birds being raised on wire can change the course of and speed at which preexisting microorganisms inhabit hosts in these settings (Pedroso, et al., 2013).

Microbial contamination of eggs

Regardless of their defensive properties, microbial contamination still occurs in poultry eggs. There are two types of microbial transmission; vertical and horizontal transmission. Vertical transmission occurs in poultry when microbes or potential pathogens are passed from the hen (transovarian) to the egg and potential embryo from a pre-existing infection (Callicott, et al., 2006). Horizontal transmission is the transmission of organisms between biotic or abiotic members in the same environment that is not directly from a parent to offspring relationship. It is the most common pathway for spread of microbial contamination in the early stages of broiler breeder fertile egg production. With horizontal transmission, the most vulnerable time for an egg is the first minute after it is laid. Providing clean nest boxes and flooring in breeding facilities is a critical intervention for contamination in the early stages of production (Coufal, et al., 2003; Cantu, 2021; Berrang et al., 1999). Horizontal transmission routes can include, direct fecal material contact, nest boxes, storage rooms, hatchery and breeder equipment, and other potentially infected eggs (Coufal, et al., 2003; Callicott, et al., 2006).

At the time of lay, a rapid temperature change occurs between the reproductive tract of the hen and the breeder facility environment. With this temperature change comes a pressure change, that in turn creates a negative pressure environment, causing the contents of the freshly laid eggs to contract (Coufal et al., 2003). This pressure change occurs throughout the transport and lifetime of the egg as it is moved and processed in the hatchery environment (Berrang et al., 1999). Eggs are moved from coolers to setters, setters to hallways and then back into hatchers.

Each temperature gradient has the potential to cause the egg contents to contract and creates an entrance portal for microbial transfer (Ratkowsky, et al., 1982; Cantu, 2021). Along with this temperature and pressure change comes moisture build up and condensation. Water is a known vector of microbes, and when presented into a drier environment, can aid in the growth and development of microbes and their transmission (Berrang, et al., 1999; Cantu, 2021). Microbial transmission can also occur during the development of eggs in both hatchers and setters. The incubator temperature and humidity requirements of chicken eggs are ideal for many species of bacteria and fungi. When introduced to this type of environment, sporulation and replication can occur, causing the spread to other eggs in the same environment (Ratkowsky, et al., 1982; Berrang, et al., 1999). Poultry hatchery environments have been found to be highly contaminated with a number of bacterial and fungal microorganisms, each of which is readily transmitted via air ventilation systems, hatchery workers, buggies and other hatchery equipment (Berrang et al., 1997). Many hatchery sanitation practices are in place, however, with the use of multi-stage incubation to increase efficiency, the potential for contamination amongst hatch groups is higher (Hulet, 2007).

Microbial contamination, pathogenic contamination in particular, is of a large concern for the poultry industry. Both gram-positive bacteria such as *Staphylococcus* spp. and *Enterococcus* spp., and gram-negative bacteria such as *E. coli* and can be found in hatchery production and are of concern (Ratkowsky, et al., 1982; Board and Fuller, 1994). Such contamination can greatly affect the hatchability and survivability of chicks (Berrang, et al., 1999). Previous research suggests that contamination in the embryotic stage can lead to colonization of the pathogen in the developing chick's intestinal tract (Cantu, 2018; Cantu, 2021). Ultimately, this early contamination can follow a broiler throughout production and into human food production (Berrang et al., 1999).

Pathogens of concern

Bacteria are one of the top contributors to microbial contamination in poultry hatching eggs. Bacteria are microscopic or ultramicroscopic single-celled organisms that are widely distributed in nature (Weiner., 1989). Bacteria are broken down into two main categories based on their anatomical variation, into gram-positive and gram-negative bacteria. This difference broadly segregates these organisms' cell wall structure, and their reaction to gram-staining. Furthermore, they can also be categorized based on their ability to form spores and whether they need oxygen to survive, or the lack thereof. Gram-positive bacteria have a distinctive purple appearance after a gram staining while gram-positive bacteria stain a pale reddish color (Steward, 2019). Gram-positive bacteria have a thick peptidoglycan layer in their cell wall. It is made of a mesh-like layer of polymer, made of sugars and amino acids unique to bacteria on the external surface of their cell wall with no outer lipid membrane (Beaveridge, 1999). They do not have any O-specific side chains present. Moreover, gram-positive bacteria have teichoic and lipoteichoic acids present. As for gram-negative bacteria, they have an outer lipid membrane present and a thin peptidoglycan layer. They have present O-specific side chains, but do not have teichoic and lipoteichoic acids (Steward, 2019). The differences between the structure of these two bacteria classifications can help in understanding the ability of certain bacteria to withstand certain sanitation and mitigation factors in poultry production.

Salmonella is a top concern when discussing pathogens in poultry production and is one of the bacteria most frequently linked with foodborne diseases (D'Aoust, et al., 2007; Montgomery, et al., 1999; Quist, 1963). Salmonella are gram-negative bacteria that live in the intestinal tract of poultry. Chickens that are infected with salmonella usually are asymptomatic and their overall production is not affected. The bacterium serotype and phage type are both

factors in the severity of milder symptoms including: weakness, loss of appetite and poorer growth rates. (Dar, et al., 2017). *S. typhimurium* is the main strain associated with salmonellosis in humans. This serovar has the ability to infect birds and contaminate eggs which makes it a potent infectious agent to humans and is of great concern to the poultry industry (Dar, et al., 2017).

Like Salmonella, *Escherichia coli* is a gram-negative bacterium. *E. coli* is rod-shaped and is naturally occurring in the lower digestive tract of poultry. This bacterium is also of great concern to poultry production due to its antibiotic resistance and ability to mutate. Some potential symptoms of an *E. coli* infection in chickens include arthritis, airsacculitis, cellulitis, and enteritis, in conjunction with potential secondary infection from previously listed symptoms (Montgomery, et al., 1999). *E. coli* infection has been noted in late embryonic stages (Cantu, 2018), and can lead to rapid dispersal of this bacterium at the time of chick placement. During the grow-out phase of poultry production, *E. coli* and other gram negatives can be quickly spread via the fecal oral route, further demonstrating the importance of early preventative hatchery sanitation procedures to lessen the microbial load of broiler chicks at placement (Dar, et al., 2017).

Staphylococcus aureus is a gram-positive, coccoid bacteria that that is typically found on skin, feathers, in the intestinal tract, and in the respiratory tract of poultry. It is a pathogen mainly related to food poisoning and is in the top three largest causes for food borne illnesses worldwide (Ali, et al., 2017). It can cause a number of different symptoms and infections including: pneumonia, meningitis, osteomyelitis, dermatitis, mild skin infections, toxic shock syndrome, and bumblefoot in poultry (Ali, et al., 2017; Shareef, et al., 2009). The methicillin resistance properties of this bacterium are high, and have been shown to be potentially zoonotic (Ali, et al., 2017). Since animal agriculture production is moving away from antibiotic use in recent years,

this is a problem for human health and should not be disregarded. Potential points of contact between poultry and humans include: contact with contaminated meat and egg products, occupational contact (farmers, butchers, meat packers), and in healthcare facilities as a secondary infection (Smith, 2015).

Enterococcus spp. are facultatively anaerobic, gram-positive, catalase-negative cocci. Members of this genus are commonly found in parts of avian digestive systems and are a normal cecal flora inhabitant (Stalker, et al., 2010). In poultry, *Enterococcus spp*. have been implicated in various clinical conditions such as femoral head necrosis, osteomyelitis, spondylitis, cellulitis, endocarditis, and secondary infections (Morishita, 2018; Stalker, et al., 2017). With the decrease in antibiotic usage in poultry production, the frequency of *Enterococcus spp.* infection will likely increase. These bacteria are transmitted via skin injuries, oral ingestion and inhalation of aerosols, further expressing the importance of hatchery sanitation and early preventative measures of microbial contamination.

Clostridium spp., like many bacteria, are normal inhabitants of the intestinal tract of avian species, with the potential to cause necrotic enteritis. *Clostridium spp*. are an anaerobic sporeforming bacterium that consists of over 200 known variants, including highly pathogenic strains associated with human health concerns (Pahalagedara, et al., 2020). All strains of *Clostridium perfrengens* have shown high susceptibility to penicillin, avilamycin, monensin, and narasin (Silva, et al., 2009). Silva, et al. (2009) demonstrated that 7.3% of *Clostridium spp*. strains showed intermediate sensitivity to linomycin, and 89.1% were considered susceptible. For tetracycline and bacitracin, 41% and 47.3% of strains, respectively, were considered resistant. Similar to other pathogens, yeasts and molds are also of interest when it comes to poultry and egg contamination. Fungi such as yeasts and molds, are classified as any member of the eukaryotic group of organisms. They are spore- producing, and feed on organic matter (Richards,

et al., 2017). Spore forming microorganisms are capable of surviving in less-than-ideal environments until favorable conditions are met, including temperature and humidity extremes (Board and Fuller, 1994). Some fungi are capable of producing mycotoxins, which can affect the quality and livability of avian species at the embryonic stage. In eggs meant for table consumption, this also poses a human health hazard (Haque, et al., 2020). Despite hatchery sanitation practices, some yeasts and molds are present in the hatchery system, in addition to being brought in on unwashed eggs from breeder facilities.

Hatcher sanitation

Egg disinfection methods vary depending on the egg's final destination point, that be for hatching purposes, table eggs, or for further processing. Common disinfectants used in the poultry industry include formaldehyde fumigation, hydrogen peroxide (H_2O_2), and ultraviolet (UV) radiation. Formaldehyde fumigation, besides being an excellent antimicrobial agent, is a toxic chemical. Formaldehyde (H_2CO, formalin, formol) is a gas at room temperature and is readily soluble in water. It is a cheap, non-corrosive disinfectant that kills most bacteria and fungi (including their spores) (Cadirci, 2009). Formaldehyde is able to act on the proteins and nucleic acids of microorganisms by attaching itself to primary amide and amino groups of proteins. Formaldehyde also forms stable methylene bridges and intermolecular cross-linkages, further enhancing its disinfectant abilities (Cadirci, 2009). The first published report of formaldehyde application in a commercial hatchery was in 1908 (Pernot, 1908). Formaldehyde can be applied as a liquid, but is more effective when used as a gas. In poultry production, formaldehyde is most commonly paired with potassium permanganate ($KMnO_4$) in a 2 to 1 ratio (Asuquo, and Okon, 1993; Cadirci, 2009), also referred to as formaldehyde fumigation. For

maximum germicidal activity from formaldehyde, a temperature range of 21 to 25 °C and humidity of 60 to 80% is necessary, coupled with an exposure time of 20 min (Chapple, 2020).

The addition of formaldehyde to hatchers can be useful in decreasing the microbial presence both aerosol and on surfaces. It was first utilized as a preincubation fumigant for chicken hatching eggs at a high level, with a goal in mind to reduce the number or pathogenic bacteria if present (Williams, 1970). However, as the usage of formaldehyde in production evolved, formaldehyde was later introduced as a mitigation step in the hatching process, referred to as formaldehyde application. Liquid 37% formalin 45-60 mL per cubic meter is used in the vapor administration (formaldehyde application system) to rooms or hatchers with late-stage embryos (Chapple, 2020). This vapor is circulated in the hatcher using installed fans to evenly distribute the formalin treatment. Adnan et al (2019) studied fumigation with formaldehyde gas at double and single strength of fertile broiler hatching eggs to distinguish differences in hatchability and embryonic growth characteristics. The study concluded that the hatchability and candling of eggs in the double strength formaldehyde treatment group were significantly < 0.05 better compared to the single strength formaldehyde group (Adnan, J et al., 2019).

Due to the carcinogenic nature of formaldehyde, some research suggests the use of other disinfectant methods, such as chloride dioxide (ClO_2) as a safer alternative (IARC, 2006). ClO_2 is a water soluble, yellow tinted gas that has a strong oxidative activity (Chung, et al., 2018; Patterson, et al., 1990). It is commonly used as a final egg washing step in the commercial egg industry. A study was conducted by Chung et al (2018) to determine if ClO_2 gas at two intervals (40 ppm and 20 ppm) caused deterioration of hatching eggs, compared to formaldehyde fumigation exposure. The results concluded that formaldehyde had similar efficacies ($P>0.05$) to CLO_2 at a concentration of 40 ppm, superior to the 20 ppm ClO_2 gas group ($p<0.001$).

Kim and Kim (2010) demonstrated that during the late-stage hatching process, aerobic bacterial contamination was over 300 cfu/hatcher in operating hatchers without formaldehyde treatment. In their study, four treatments including: a negative control group without formaldehyde application, 37% formaldehyde application rate administered once during the hatching process into a basin, 37% formaldehyde administered every 12 h throughout from transfer up until 12 h before chick removal, and 37% formaldehyde administered every 4 hours from the time of transfer up until 4 h before chick removal. Air plate samples were taken from 24 locations within the commercial hatchery including: the egg receiving room, storage room, hatcher corridor, setters, hatchers and the chick processing area. Results from the study showed that bacterial enumeration across treatments rose from 18 d to 21 d of incubation in a bacterial bloom. The study concluded that administering 37% formaldehyde into a basin at the time of transfer had a superior inhibitory effect on aerosol bacterial counts when compared to the other three treatments.

UV light and hydrogen peroxide are another method of hatchery sanitation used to control aerobic bacterial contamination in hatchers. UV light, combined with the use of hydrogen peroxide is known to be an effective hatching egg sanitation method for commercial use (Gottselig, et al., 2016; Coufal, et al., 2003). UV light has been used widely to sanitize hatcher air spaces, and when properly applied, has not affected the development of developing embryos (Bailey, et al., 1996). Bailey et al (1996) demonstrated that the use of UV light and hydrogen peroxide in hatchers during the last 3 d of incubation was an effective method for hatcher sanitation. UV lights were placed on the ceiling and on the floor of hatchers and measured with a UV meter to generate the ozone concentrations. Additionally, a second treatment group of hydrogen peroxide (2.5% in water from a stock solution of 50%) was applied during the last 3 d of incubation, every five minutes. Sampling of aerobic microbial contamination was performed

by placing duplicate agar plates over the air exhaust for 2 minutes (Bailey, et al., 1996). Results concluded that hydrogen peroxide was highly effective at sanitizing all locations within the hatcher where bacterial contamination was previously found. UV light was effective at reducing microbial loads; however, due to the inability of the UV light to penetrate all visible points in the hatcher, bacterial growth was still observed. The hydrogen peroxide resulted in significant kill of bacteria located in the treatment hatcher.

In ovo injection

In ovo injections machines were first introduced to broiler hatchery production systems to expedite vaccine administration. This removes the step of vaccinating day-old chicks via manual subcutaneous injection in the back of the neck (Ricks et al., 1999). These *in ovo* injection machines are capable of treating a range of 25,000 to 70,000 eggs per h (Peebles, 2018). The vaccine is designed to be given to embryos between incubation days 18 and 19, delivered through the shell to the hatch-positioned embryo. Eggs are placed in trays from the setter on a belt and the system gently lowers injection needles onto the top of the egg, puncturing through to deliver the vaccine. The needle is then drawn back up, where it is cleansed in a sterile wash (Johnson, et al., 1997). More advanced technology has established a candler attachment for the *in ovo* injection machine that removes clear eggs. Wakenell et al., (2002) showed that the vaccine was effective when given to the amnion, the optimal site of administration, and into the embryo's body. Injection into the allantoic sac or the air cell was ineffective.

The machine is capable of delivering a wide variety of vaccines and biologics to embryos. With a goal to produce larger chicks that are capable of optimizing daily rate of gain, prebiotics aid in reducing enteric diseases and enhance productivity (Berrocoso, et al., 2017). The idea of vaccine administration pre-hatch is that producers are able to facilitate an early

establishment of a healthy gut microbiome, before the potential of pathogenic exposure posthatch (Roto, et al., 2016). As mentioned previously, the risk of contamination pre-hatch for eggs is present, therefore the sooner intestinal tracts can be infiltrated with nonpathogenic microbes, the more productive birds can be in a grow-out setting.

Conclusions

Hatchery sanitation is a key aspect to the overall productivity and sustainability of commercial poultry operations. The key target for controlling bacterial contamination once chicks leave the hatchery is the last three days of incubation. Across the studies discussed in this literature review, bacteria increase in numbers rapidly towards the end of the hatching process in negative control groups. This bacterial bloom if not effectively treated can lead to disease and foodborne illness further down the production chain. It is known that this bacterial bloom occurs, but not what causes it. Further research is warranted to observe commercial hatchery late-stage incubation mitigation practices in attempt to pinpoint potential sources of this bacterial introduction.

Formaldehyde application and *in ovo* injection are both introduced at the time of transfer, and are of great interest to this study. It is known that formaldehyde application is effective at decreasing aerosol microbial contamination during the last three days of incubation; however, the effect of formaldehyde application on chick intestinal tract contamination is unknown. Additionally, *in ovo* injection, due to the introduction of foreign objects past an eggs' physical defense mechanisms has the potential for contamination. The ultimate goal of hatchery sanitation research is to narrow down the potential hatchery contamination sources at the commercial level. Late-stage incubation mitigation strategies affecting embryo and chick intestinal tract microbiota could answer these questions.

CHAPTER III

EVALUATION OF FORMALDEHYDE APPLICATION IN COMMERCIAL HATCHERS ON CHICK INTESTINE MICROBIOTA

Introduction

Limited research has evaluated the effects of formaldehyde application on chick intestine microbiota in broiler hatching eggs. Historically, air plate enumeration has been used to quantify the benefits of formaldehyde application during the final stages of incubation in commercial hatchers. Formaldehyde is commonly used as a disinfectant for poultry hatching eggs as a cheap method to kill most bacteria and fungi, including their spores (Acklund, et al., 1980; Braswell et al.,1970; Williams, 1980). Bacteria and fungi are present throughout each stage of egg handling, from the breeder flock to egg storage (Berrang, 1999; Wells, et al., 2010). The cuticle, a natural defense mechanism, which aids in blocking bacterial penetration, is the eggs' first line of defense. In addition, the antimicrobial and high pH properties of the albumen limit the number of bacteria that are able to populate near the developing embryo of fertile eggs (Berrang, 1999; Cadirci, 2009). As a result, formaldehyde application during the last 3 d of incubation is a common practice to lower the amount of airborne bacteria circulating in commercial hatchers (Yildirim et al., 2003).

Previous studies with formaldehyde application in commercial hatchers has shown to greatly reduce the shell surface bacterial load by up to 99.85% with high-level formaldehyde fumigation techniques (Williams, 1970). While formaldehyde is a proven anti-microbial agent, it is a toxic chemical, and if over applied can cause damage to the developing embryo (Cadirci, 2009). Application of formaldehyde has two main objectives: First, to reduce the microbial load present. Second, to minimize the amount of damage caused to the developing embryo.

Ultimately, the balance between reducing microbial count without causing embryo damage must be achieved or the results will be ineffective (Cadirci, 2009).

The objectives of the current experiment were: 1) to evaluate the impact of formaldehyde application in hatchers compared to hatchers without formaldehyde application on embryo and chick intestinal tract microbiota during the late stages of incubation and hatching process, and 2) monitor the impact of formaldehyde application in hatchers compared to hatchers without formaldehyde application on airborne microbial loads.

Materials and methods

Treatments

Experiment 1. Two trials were conducted in this experiment using commercial Ross 708 broiler hatching eggs. All eggs used in Trial 1 were obtained from the same commercial broiler flock (A) and all eggs used in Trial 2 were obtained from the same commercial broiler flock (B). On 19 d of incubation, all eggs were *in ovo* injected and moved into two hatchers. One hatcher was subjected to formaldehyde application, and the other hatcher was not. The formaldehyde treatment group was applied with formaldehyde in two-hour intervals beginning at the time eggs were transferred (on 19 d of incubation) with 4 oz of 37% formalin for the first application. After the initial formaldehyde application, 2 oz of 37% formalin was applied every two hours thereafter, with a withdrawal period (the end of 20 d) 12 hours before chicks were removed from the hatcher on 21 d of incubation. (Steinlage, et al., 2002). Sample collection intervals included 18 d of incubation (24 h pre-transfer), 19 d of incubation (at transfer), 20 d of incubation (21 h after transfer), and 21 d of incubation (at the time of chick removal from hatchers). Air plates of the 6 media specified in Table 2 were placed in each treatment hatcher for 2 minutes on 19 d, 20 d, and 21 d.

Table 1. Broiler breeder flock, equipment and hatch results Experiment 1.

Trial	Treatment Hatcher	Flock name	Age (weeks)	Hatcher	Setter	Number of eggs set	Hatch Percentage
1	Formaldehyde	A	40	9	17, 18	51,840	87.0 %
	No Formaldehyde			8	15, 16		
2	Formaldehyde	B	51	13	25, 26	51,840	81.0 %
	No Formaldehyde			14	27, 28		

Microbial analysis

Intestinal microbial enumeration. Two trials were conducted. Trial 1 had a sample size of 5 eggs or chicks per sample day, and Trial 2 had a sample size of 7 eggs or chicks per sample day. On 18 d and 19 d of incubation, eggs with viable embryos were collected and transported to the laboratory in a cooler with ice for 3 h. For 20 d and 21 d, hatched chicks were transported to the laboratory and euthanized with carbon dioxide gas. Due to a later hatch in Trial 2, 20 d embryos were collected instead of hatched chicks. Demonstrated in Figure 1, eggs or chicks were placed into a sanitizing solution (chlorhexidine diacetate) for 15 seconds prior to necropsy and intestinal tract removal. Eggs or chicks were set into the solution and embryos were broken out post-sanitizing. The euthanized embryos or chicks were necropsied using forceps and surgical scissors sterilized by dipping them in 100% ethanol followed by flaming. The intestinal tract of the embryo or chick was aseptically removed and placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI). Each sample was weighed and sterile PBS (pH 7.4 HiMedia Laboratories, West Chester, PA) was added to the bag at 4 times the weight of the intestinal tract sample. The sample was crushed and rolled to homogenize the contents of the Whirl-Pak bag. Ten-fold serial

dilutions were performed with the homogenized sample. A volume of 0.1 mL homogenized sample and each dilution was pipetted and spread onto media using sterile plastic spreaders (VWR International LLC, Radnor, PA). Table 2 demonstrated the media used in Experiment 1. After plating, media were incubated for 48 h at 37°C. Colonies were enumerated and calculated as log 10cfu/g of intestinal tract. Therefore, the limit of detection (LOD) was 40 cfu/g.

Air plate enumeration. Airborne bacteria in each hatcher were evaluated by placing six Petri dishes of agar media (air plates), 1 per media described in Table 2, were left in each treatment's hatcher for 2 minutes per sample day (19 d, 20 d, and 21 d). Plates were left on the top of stacked hatcher trays near the ceiling of the hatcher (Bailey, et al., 1996). Air plates were kept in a cooler with ice for 3 h and transported back to the laboratory for the duration of travel back to Texas A&M University before being incubated for 48 h at 37°C, and enumerated. Results are presented as colony forming units (cfu). Plates with more than 300 cfu were recorded as too numerous to count (TNTC) due to overgrowth from chick dust.

Figure 1. Intestinal tract enumeration for Experiment 1.

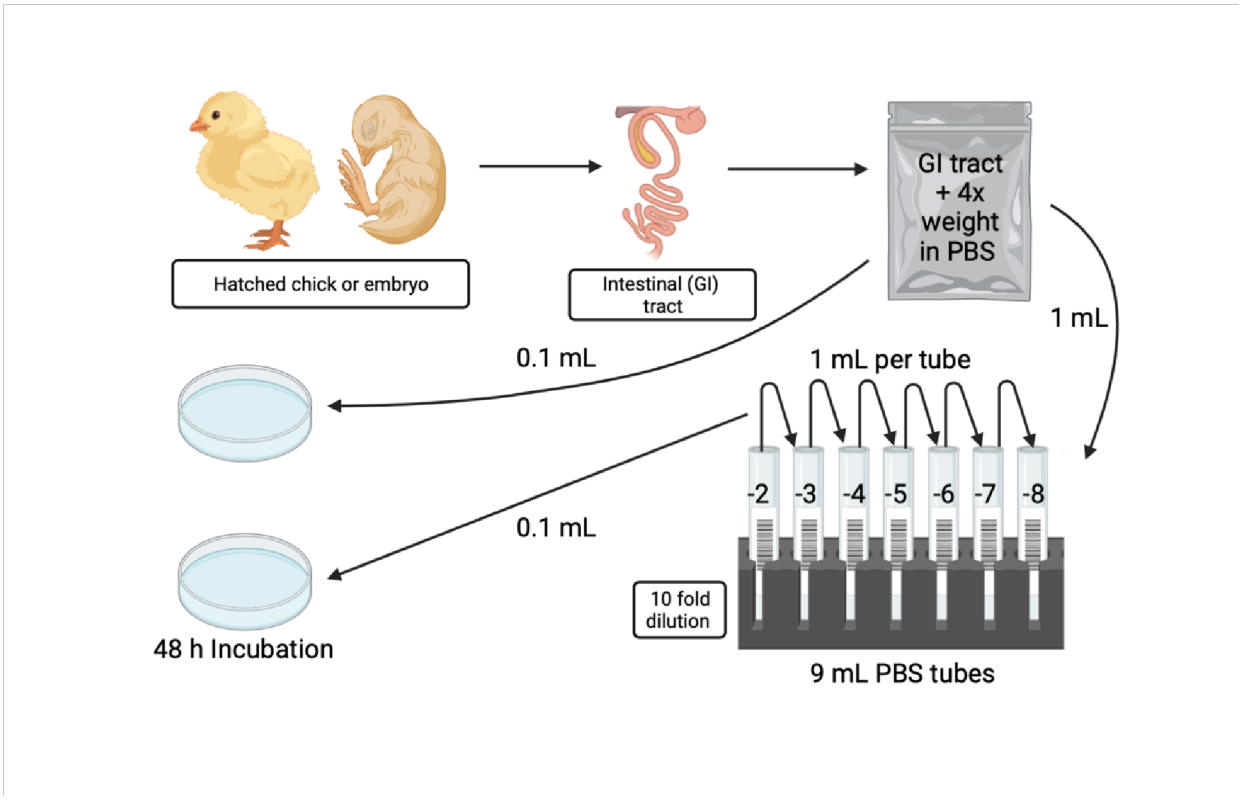

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Table 2. All media and corresponding microorganisms enumerated for intestinal samples and air plates in Experiment 1.

Media	Supplier	Organism
Tryptic Soy Agar (TSA)	BD Difco BBL Microbiology Distributor, Houston, TX	Total Aerobes and Total Anaerobes
Mannitol Salt Agar (MSA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Staphylococcus aureus*
Bile Esculin Agar (BEA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Enterococci* and Group D S*treptococci*
EMB Agar	Criterion, Hardy Diagnostics, Santa Maria, CA	*E. coli* and other gram-negative bacilli
Egg Yolk Agar (EYA) + 50% Egg Yolk Enrichment	HiMedia Laboratories Pvt. Ltd., Maharashtra, India	Clostridium spp

Statistical analysis Eggs that yielded zero colony counts for intestinal enumeration were assigned a value of 1.30 $\log_{10}$ cfu/g for statistical analysis because a value of log 0 /cfu is not attainable. Data collected was analyzed as a Generalized Linear Model (GLM) using JMP Pro 2016 software to compute student T-test P-values. Microsoft Excel 2021 was used to calculate the linear model means and standard error. If significance was detected, linear model means were separated and analyzed between the two treatment groups, with significance of $P < 0.05$. A statistical analysis was not performed for air plate enumeration because there were no replicates within the treatments.

Results and discussion

Intestinal microbial enumeration

Experiment 1, Trial 1 & 2. Results for Experiment 1 intestinal enumeration are presented in Tables 3 and 4. Results indicated that from 18 d to 21 d of incubation, formaldehyde application did not significantly decrease the intestinal microbiota in Trial 1 or Trial 2 on any of the media used for enumeration. On 18 d of incubation, little to no microbial growth was enumerated from the intestinal tract samples. On 19 d after eggs were transferred from the setters to the hatchers, an increase in the microbial growth was observed. On 20 d of incubation, 21 h after formaldehyde application began, formaldehyde application decreased the amount of microbial growth on all media used for intestinal tract enumeration. In between 20 d and 21 d of incubation as the chicks began to hatch, formaldehyde application ended 12 h before chicks were pulled from the hatchers. On 21 d of incubation in Trial 1 for hatched chick intestinal microbiota, the formaldehyde applied group of chicks showed an increase in the intestinal microbiota for total anaerobes, *Staphylococcus aureus*, and total anaerobes. The intestinal enumeration for *Enterococcus*, *E. coli* and other gram-negatives and *Clostridium spp* was higher in the no

formaldehyde application hatcher. In Trial 2, 21 d microbial growth enumeration was higher in the no formaldehyde treatment group, with no significant differences.

It should be noted that while all of the eggs for Experiment 1 were collected from the same breeder flock, the eggs were split up for the first 18 d of incubation into different setters. Table 1 depicts where the eggs for each treatment were incubated and hatched. Differences in the results for the two groups could be due to outside flock presence within the multistage setters used at this hatchery. It is possible that eggs could have been contaminated while in one setter and changed the microbiota of the eggs prior to formaldehyde application, contributing to some of the differences seen in Trial 1.

Based on this data, formaldehyde application was not effective at significantly decreasing the amount of intestinal tract microbiota during late-stage incubation of broiler hatching eggs. Formaldehyde application decreased the amount of microbial presence enumerated while formaldehyde application was occurring; however, the values of the formaldehyde application group approached that of the no formaldehyde application group after the application ended. Due to the smaller sample size collected and the differences in standard error, more trials are needed to solidify these findings. No additional differences were noted between treatments for subsurface microbial loads on any evaluated media.

Table 3. Intestinal enumeration plate count results (Log10 cfu/g ± SE) for Trial 1.

Media	Treatment	18 d	19 d	20 d	21 d
TSA-aerobic	Form	1.58 ± 0.14	4.64 ± 0.04	6.01 ± 1.34	11.17 ± 0.44
	No Form	1.60 ± 0.17	4.68 ± 0.04	6.76 ± 0.02	10.36 ± 0.78
	P-value	0.9117	0.4537	0.6031	0.3968
MSA	Form	1.42 ± 0.07	2.01 ± 0.71	3.11 ± 1.12	7.26 ± 1.22
	No Form	1.30 ± 0.00	1.30 ± 0.00	1.30 ± 0.00	5.29 ± 1.05
	P-value	0.1778	0.3739	0.1806	0.2559
BEA	Form	1.30 ± 0.00	4.60 ± 0.04	4.43 ± 1.35	7.39 ± 1.18
	No Form	1.30 ± 0.00	4.65 ± 0.04	6.63 ± 0.04	8.71 ± 0.10
	P-value	1.0000	0.4100	0.1775	0.3299
EMB	Form	1.46 ± 0.16	4.52 ± 0.02	3.55 ± 1.41	5.69 ± 1.79
	No Form	1.36 ± 0.06	4.56 ± 0.02	6.55 ± 0.05	8.64 ± 0.09
	P-value	0.5919	0.1918	0.1002	0.1742
TSA-anaerobic	Form	1.36 ± 0.06	4.69 ± 0.03	5.08 ± 1.42	10.05 ± 0.28
	No Form	1.30 ± 0.00	4.68 ± 0.07	7.69 ± 0.09	9.70 ± 0.26
	P-value	0.3739	0.9075	0.1746	0.3946
EYA	Form	1.36 ± 0.06	4.55 ± 0.04	4.69 ± 1.43	8.75 ± 0.66
	No Form	1.30 ± 0.00	4.67 ± 0.07	7.24 ± 0.21	9.17 ± 0.35
	P-value	0.3739	0.1853	0.1492	0.5975

Table 4. Intestinal enumeration plate count results (Log10 cfu/g ± SE) for Trial 2.

Media	Treatment	18 d	19 d	20 d	21 d
TSA-aerobic	Form	1.30 ± 0.00	1.76 ± 0.26	3.69 ± 0.16	6.47 ± 0.87
	No Form	1.30 ± 0.00	1.92 ± 0.38	3.85 ± 0.16	7.37 ± 0.74
	P-value	1.0000	0.7280	0.4954	0.4453
MSA	Form	1.30 ± 0.00	1.30 ± 0.00	1.34 ± 0.04	3.29 ± 0.68
	No Form	1.30 ± 0.00	1.34 ± 0.04	1.30 ± 0.00	4.24 ± 0.27
	P-value	1.0000	0.3559	0.3559	0.2341
BEA	Form	1.30 ± 0.00	1.30 ± 0.00	2.28 ± 0.20	6.38 ± 0.89
	No Form	1.30 ± 0.00	1.30 ± 0.00	2.60 ± 0.23	6.40 ± 0.68
	P-value	1.0000	1.0000	0.3284	0.9835
EMB	Form	1.30 ± 0.00	1.30 ± 0.00	2.29 ± 0.38	5.36 ± 0.65
	No Form	1.30 ± 0.00	1.30 ± 0.00	2.77 ± 0.41	6.91 ± 0.59
	P-value	1.0000	1.0000	0.4086	0.1028
TSA-anaerobic	Form	1.30 ± 0.00	1.34 ± 0.04	2.22 ± 0.41	6.13 ± 1.01
	No Form	1.34 ± 0.04	1.43 ± 0.09	2.68 ± 0.41	7.23 ± 0.71
	P-value	1.0000	0.4101	0.4458	0.3933
EYA	Form	1.30 ± 0.00	1.34 ± 0.04	1.73 ± 0.29	6.02 ± 1.00
	No Form	1.30 ± 0.00	1.34 ± 0.04	2.10 ± 0.39	6.82 ± 0.86
	P-value	1.0000	1.0000	0.4650	0.5530

Air plate microbial enumeration

Experiment 1, Trial 1 & 2. Results for air plate enumeration are presented in Tables 5 and 6. Similar to the results presented for intestinal enumeration, air plate enumeration on 18 d, 19 d, and 20 d displayed a decrease in the airborne bacteria found within the hatcher containing the formaldehyde treatment group, compared to the no formaldehyde treatment group. On 21 d, the formaldehyde treated group approached the level of airborne bacteria enumerated in the no formaldehyde treatment group. These findings mimic the effect formaldehyde application had on chick intestinal microbiota, and can be interpreted similarly. These results are similar to the results found in a study by Kim and Kim (2010). Formaldehyde application decreased the number of airborne bacteria found within the treatment hatchers when compared to a negative control. No additional differences were noted between treatments for subsurface microbial loads on any evaluated media.

Overall, data from Experiment 1 indicated that the use of formaldehyde application in late embryonic stage broiler hatchery production was able to reduce the intestinal and air plate enumeration of microorganisms while formaldehyde application was in progress. However, no significant differences were observed due to formaldehyde application. The lack of significance noted may be attributed to the application rate and volume used at the commercial hatchery. Formaldehyde application rate, withdrawal time and volume were not treatments for this study because the method of application at its current state was of interest.

Table 5. Air plate enumeration results (cfu/hatcher) for Trial 1.

Media	Treatment	19 d	20 d	21 d
TSA-aerobic	Form	3	90	[1]TNTC
	No Form	41	[1]TNTC	[1]TNTC
MSA	Form	2	21	4
	No Form	0	85	85
BEA	Form	1	65	120
	No Form	0	[1]TNTC	[1]TNTC
EMB	Form	0	85	8
	No Form	0	110	65
TSA-anaerobic	Form	0	110	240
	No Form	1	[1]TNTC	[1]TNTC
EYA	Form	3	58	265
	No Form	3	290	[1]TNTC

[1]TNTC= too numerous to count.

Table 6. Air plate enumeration results (cfu/hatcher) for Trial 2.

Media	Treatment	19 d	20 d	21 d
TSA-aerobic	Form	1	70	[1]TNTC
	No Form	0	103	[1]TNTC
MSA	Form	0	0	35
	No Form	0	1	51
BEA	Form	0	65	[1]TNTC
	No Form	0	78	[1]TNTC
EMB	Form	0	19	53
	No Form	0	15	118
TSA-anaerobic	Form	2	55	[1]TNTC
	No Form	1	81	[1]TNTC
EYA	Form	0	34	[1]TNTC
	No Form	0	63	[1]TNTC

[1]TNTC= too numerous to count.

Conclusions

Sanitation of broiler hatching eggs is an important preventative step in hatchery management to decrease the risk of microbial contamination. At this time, it is standard to use formaldehyde application in commercial hatchers during the late stages of incubation and hatching. This study indicated that the formaldehyde application protocol used at this commercial hatchery lowered airborne bacteria enumeration while formaldehyde application was in progress from 19 d to 20 d. On 21 d of incubation, the airborne bacteria enumeration results

for the formaldehyde treatment group approached the values of the no formaldehyde treatment group. Based on the results for air plate enumeration, formaldehyde at the given application rate was not effective at reducing air-borne bacteria in the commercial hatchery.

Intestine enumeration was not significantly affected by the application of formaldehyde during the late staged of incubation and the hatching process. Formaldehyde application lowered the amount of intestinal microflora on 20 d of incubation after formaldehyde application had been applied for 21 h post-transfer. On 21 d of incubation after formaldehyde application ended, the intestinal microbiota approached the values of the no formaldehyde application group. The results could be attributed to the inability of formaldehyde gas to penetrate beyond the shell membrane into the body cavity, ultimately not affecting the digestive tract. The 21 d intestinal tract enumeration was high for total aerobic and anaerobic bacteria in both trials, disproving the idea that formaldehyde application is an effective hatchery sanitation method at this application rate.

Overall, this study concluded that formaldehyde gas at the given application rate was not adequate in sanitizing hatching eggs in late-stage incubated broiler hatching eggs. There was a high standard error across media and days of incubation, partially due to the small sample size of this experiment. Limited research has been published on formaldehyde application rates and its ability to penetrate the internal contents of hatching eggs, affecting the intestinal microbiota. Further research is warranted to investigate the impact of formaldehyde at a higher application rate to solidify the findings from this chapter.

CHAPTER IV

EVALUATION OF *IN OVO* INJECTION IN COMMERCIAL HATCHERIES AND ITS EFFECT ON CHICK INTESTINE MICROBIOTA

Introduction

Poultry receive a variety of vaccines throughout the stages of production. *In ovo* injection machines were first introduced to broiler hatchery production systems to alleviate the step of vaccinating day-old chicks via manual subcutaneous injection in the back of the neck (Ricks et al., 1999). These injection machines are capable of administering vaccine at a rate of 25,000 to 70,000 eggs/h (Peebles, 2018). Marek's disease vaccine, infectious bursal disease vaccine, fowl pox vaccine, Newcastle disease vaccine, and coccidiostats are all approved vaccinations for *in ovo* injection use. Vaccinations are designed to be given to embryos between incubation days 18 and 19, delivered through the shell to the hatch-positioned embryo (Berrocoso, et al., 2017). Prebiotics are also approved to be administered pre-hatch by *in ovo* injection equipment, allowing producers to facilitate an early establishment of intestinal tract bacteria, before the potential of pathogenic exposure post-hatch (Roto, et al., 2016).

One key difference between eggs that are injected and those that are not is the creation of an entry hole into the egg (Williams and Hopkins, 2011). Avian eggs are naturally designed with a strict set of antimicrobial mechanisms to protect the growing embryo from foreign microorganisms. These barriers include the cuticle, eggshell, inner shell membrane and the outer shell membrane (Berrang, 1999), all of which are pierced with the *in ovo* injection needle. This creates a portal for microbes to enter and exit the eggs, increasing the potential for contamination to occur. *In ovo* injection machinery can be equipped with an egg candler and remover system, leaving the darker, developing eggs on racks. In addition to viable eggs, are those mid-dead,

latedead, and rotten eggs. This is undesirable due to the microbiome typically associated with these unwanted eggs.

The objectives of the experiment described in this chapter were: 1) to evaluate the impact of *in ovo* injection the intestinal microbiota of chicks during the late stages of incubation at hatch and 2) evaluate the impact of *in ovo* injection vaccines on airborne bacteria in hatchers during the late stages of incubation at hatch.

Materials and methods

Treatments

Experiment 2. Two trials were conducted in this experiment using commercial Ross 708 broiler hatching eggs. All eggs used in trial 1 were obtained from the same commercial broiler flock (A) and all eggs used in Trial 2 were obtained from the same commercial broiler flock (B). On 19 d of incubation, all of the eggs in one hatcher were *in ovo* injected after removal from the setters and all of the eggs in the no *in ovo* injected treatment were directly transferred from the setters to the hatcher. Sample collection intervals included 18 d of incubation (24 h pre-transfer), 19 d of incubation (at transfer), 20 d of incubation (21 h after transfer), and 21 d (at the time of chick removal from hatchers). Air plates of the 6 media specified in Table 8 were placed in each treatment hatcher for 2 minutes on 19 d, 20 d, and 21 d. Both hatchers were subject for 37% formaldehyde application post-transfer from 19 d of incubation to 20 d of incubation until 12 h prior to chick removal from hatchers.

Table 7. Broiler breeder flock, equipment and hatch results Experiment 2.

Trial	Treatment Hatcher	Flock name	Age (weeks)	Hatcher	Setter	Number of eggs set	Hatch Percentage
1	*In ovo* injection	A	41	14	27, 28	51,840	86.0 %
	No *in ovo* injection			15	29, 30		
2	*In ovo* injection	B	41	14	27, 28	51,840	89.0 %
	No *in ovo* injection			15	29, 30		

Microbial analysis

Intestinal microbial enumeration. Two trials were conducted. Trial 1 had a sample size of 5 eggs or chicks per sample day, and Trial 2 had a sample size of 7 eggs or chicks per sample day. On 18 d and 19 d of incubation, eggs with viable embryos were collected and transported to the laboratory in a cooler with ice for 3 h. For 20 d and 21 d, hatched chicks were transported to the laboratory and euthanized with Carbon Dioxide gas. Demonstrated in Figure 2, eggs or chicks were placed into a sanitizing solution (Chlorhexidine Diacetate) for 15 seconds prior to necropsy and intestinal tract removal. Eggs or chicks were set into the solution and embryos were broken out post-sanitizing. The euthanized embryos or chicks were necropsied using forceps and surgical scissors sterilized by dipping them in 100% ethanol followed by flaming. The intestinal tract of the embryo or chick was aseptically removed and placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI). Each sample was weighed and PBS (pH 7.4 HiMedia Laboratories, West Chester, PA) was added to the bag at 4 times the weight of the intestinal tract sample. The sample was crushed and rolled to homogenize the contents of the Whirl-Pak bag. Ten-fold serial dilutions were performed with the homogenized sample. A volume of 0.1 mL homogenized sample and each dilution was pipetted and spread onto media using sterile plastic spreaders (VWR International LLC, Radnor, PA). Table 8 demonstrated the media used in Experiment 1.

After plating, media were incubated for 48 h at 37°C. Colonies were enumerated and calculated as log 10cfu/g of intestinal tract. Therefore, the limit of detection (LOD) was 40 cfu/g.

Air plate enumeration. Airborne bacteria in each hatcher were evaluated by placing six Petri dishes of agar media (air plates), 1 per media described in Table 2, were left in each treatment's hatcher for 2 min per sample d (19 d, 20 d, and 21 d). Plates were left on the top of stacked hatcher trays near the ceiling of the hatcher (Bailey, et al., 1996). Air plates were kept in a cooler with ice for 3 h and transported back to the laboratory for the duration of travel back to Texas A&M University before being incubated for 48 h at 37°C, and enumerated. Plates with more than 300 colonies were recorded as too numerous to count (TNTC).

Figure 2. Intestinal tract enumeration for Experiment 2.

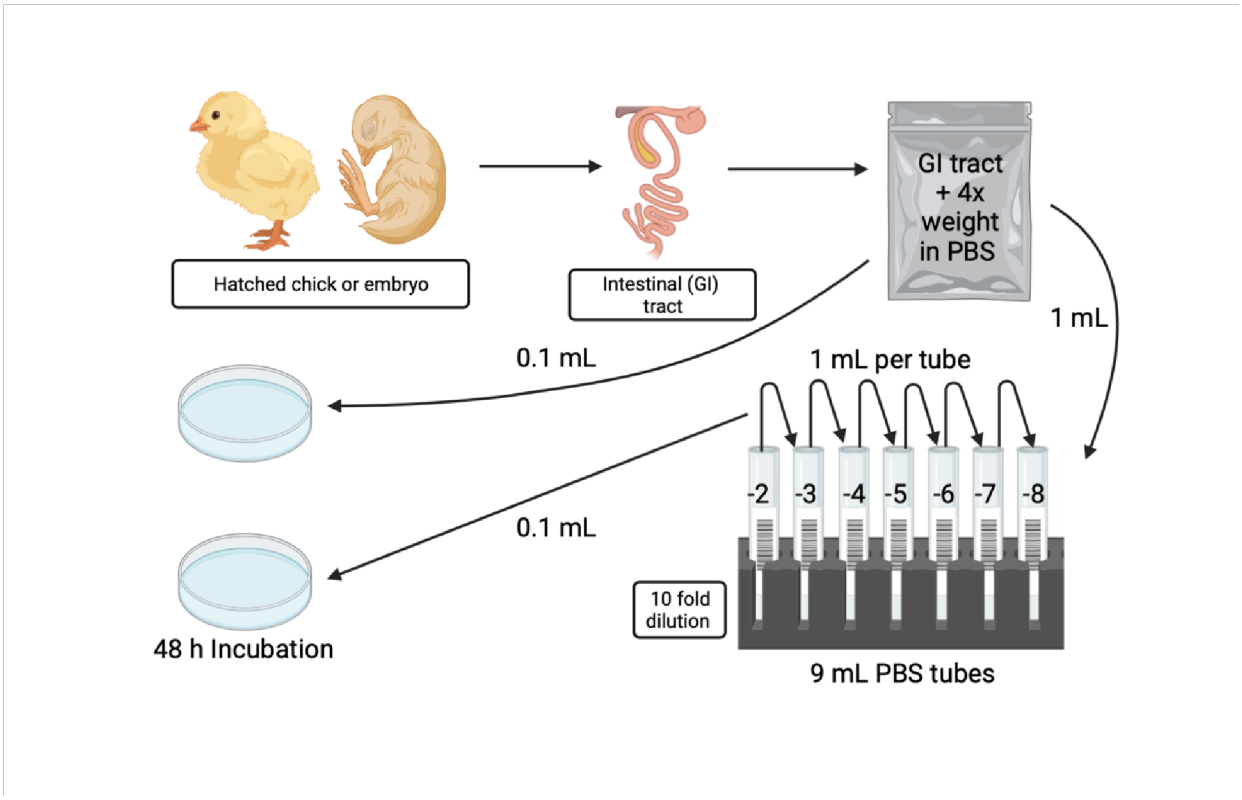

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Table 8. All media and corresponding microorganisms enumerated for intestinal samples and air plates in Experiment 2.

Media	Supplier	Organism
Tryptic Soy Agar (TSA)	BD Difco BBL Microbiology Distributor, Houston, TX	Total Aerobes and Total Anaerobes
Mannitol Salt Agar (MSA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Staphylococcus aureus*
Bile Esculin Agar (BEA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Enterococci* and Group D S*treptococci*
EMB Agar	Criterion, Hardy Diagnostics, Santa Maria, CA	*E. coli* and other gram-negative bacilli
Egg Yolk Agar (EYA) + 50% Egg Yolk Enrichment	HiMedia Laboratories Pvt. Ltd., Maharashtra, India	Clostridium spp

Statistical analysis Eggs that yielded zero colony counts for intestinal enumeration were assigned a value of 1.30 $\log_{10}$ cfu/g for statistical analysis because a value of log 0 /cfu is not attainable. Data collected was analyzed as a Generalized Linear Model (GLM) using JMP Pro 2016 software to compute student T-test P-values. Microsoft Excel 2021 was used to calculate the linear model means and standard error. If significance was detected, linear model means were separated and analyzed between the two treatment groups, with significance of $P < 0.05$. A statistical analysis was not performed for air plate enumeration because there were no replicates within the treatments.

Results and discussion

Microbial enumeration

Experiment 2, Trial 1 and 2. Results for Experiment 2 are presented in Tables 9 and 10. Results for intestinal enumeration on TSA- aerobic, MSA, BEA, EMB, TSA- anaerobic, and EYA agars on 18 d and 19 d displayed no significant differences with the use of *in ovo* injections for Trial 1 and Trial 2. Minimal microbial growth was observed on 18 d of incubation, with a slight increase in intestinal microbiota on 19 d. Intestinal microbiota increased at a faster rate from 19 d to 20 d of incubation, compared to the increase between 18 d to 19 d of incubation. On 20 d of incubation in Trial 1, results for *in ovo* injected (5.46 $\log_{10}$ cfu/g) hatching eggs and no *in ovo* (2.76 $\log_{10}$ cfu/g) hatching eggs concluded that *in ovo* injection significantly increased the intestinal tract microbiota for total aerobic bacteria enumeration ($P = < 0.0001$). Additionally in Trial 1, 20 d of incubation results for *in ovo* injected (5.03 $\log_{10}$ cfu/g) hatching eggs and no *in ovo* injected (3.63 $\log_{10}$ cfu/g) hatching eggs concluded that *in ovo* injection significantly increased the intestinal tract microbiota for total anaerobic bacteria enumeration

(P=0.0012). In Trial 2, results for *in ovo* injected (3.67 $\log_{10}$ cfu/g) hatching eggs and no *in ovo* (2.02 $\log_{10}$ cfu/g) hatching eggs concluded that *in ovo* injection significantly increased the intestinal tract microbiota for total aerobic bacteria enumeration (P= 0.0409). The 20 d total anaerobic bacteria growth observed in Trial 2 was higher in the *in ovo* injection sample group, but was not significantly different from the no *in ovo* injection group. This increase in microbial counts could be accredited to the earlier introductory period of eggshell membrane and eggshell puncture from the *in ovo* injection needle. Bacteria residing in the air cell of developing hatching eggs and in the eggshell membranes had more potential to expel into the 20 d hatcher environment and transfer to a larger number of neighboring eggs. Eggs in the treatment group not subjected to *in ovo* injection did not have the addition of an *in ovo* injection needle, and had a later time of exposure. These eggs were not exposed until the natural hatching process occurred, when the developing embryo internally and externally piped.

On d 21 of incubation in Trial 1, the microorganisms in the no *in ovo* injection treated hatcher approached that of the *in ovo* injected treatment group for TSA- aerobic and TSA- anaerobic intestinal enumeration. TSA- aerobic and TSA- anaerobic enumeration values were not significantly different (P= 0.3182 and P= 0.4532, respectively). Results from intestinal tract enumeration indicated that *in ovo* injected eggs had a higher number of colony forming units across all media used in Experiment 2, Trial 1. Elevated enumeration levels in the *in ovo* injected eggs of *Staphylococcus aureus*, *Enterococcus* spp, and *E. coli* and other gram-negative bacteria all approached levels of significance (P= 0.0707, P= 0.0707, and P= 0.0946 respectively) in favor of the hypothesis.

Results in Trial 2 differed for 21 d incubation results. Between 20 d of incubation and 21 d of incubation, results for intestinal tract enumeration shifted. The no *in ovo* injection treatment group had a higher number of colony forming units detected for total aerobic bacteria,

Enterococcus spp, *E. coli* and other gram-negative bacteria, total anaerobic bacteria, and *Clostridium* spp. Results for Trial 2, 21 d of incubation were not significantly different between treatments; however, *Enterococcus* spp (P=0.0811) and *Clostridium* spp (P= 0.0547) approached significance in favor of the *in ovo* injection group reducing the intestinal tract microbial presence. *Staphylococcus aureus* enumeration was higher in the in ovo injected treatment group, but not at a significant level. This shift in microbial enumeration on 21 d of incubation may be attributed to differences in the location at which eggs were incubated. Table 7 displays the setters and hatchers used for Experiment 2. The same breeder flock was used for both treatments; however, eggs from the breeder flock were split up for the first 18 d of incubation in setters. Eggs had exposure to different outside flocks in the multistage setters and could have been contaminated prior to transfer. This separation could be attributed to the differences seen between trials. No additional differences were observed between treatments for intestinal tract microbial loads on any evaluated media.

Table 9. Intestinal enumeration plate count results (Log10 cfu/g ± SE) for Trial 1.

Media	Treatment	18 d	19 d	20 d	21 d
TSA-aerobic	*In ovo*	1.30 ± 0.00	3.56 ± 0.06	5.46 ± 0.18	7.16 ± 0.43
	No *in ovo*	1.34 ± 0.04	3.52 ± 0.09	3.53 ± 0.08	6.41 ± 0.56
	P- value	0.3506	0.7400	< 0.0001*	0.3182
MSA	*In ovo*	1.34 ± 0.04	2.62 ± 0.35	3.05 ± 0.71	3.56 ± 0.92
	No *in ovo*	1.34 ± 0.04	2.43 ± 0.46	2.45 ± 0.47	1.30 ± 0.00
	P- value	0.9962	0.7532	0.5085	0.0707
BEA	*In ovo*	1.30 ± 0.00	1.82 ± 0.32	2.68 ± 0.85	6.59 ± 0.76
	No *in ovo*	1.30 ± 0.00	1.56 ± 0.26	3.00 ± 0.18	3.29 ± 1.32
	P- value	1.0000	0.5454	0.7321	0.0707
EMB	*In ovo*	1.30 ± 0.00	1.30 ± 0.00	1.30 ± 0.00	6.26 ± 0.70
	No *in ovo*	1.30 ± 0.00	1.36 ± 0.06	1.79 ± 0.49	3.36 ± 1.29
	P- value	1.0000	0.3739	0.3739	0.0946
TSA-anaerobic	*In ovo*	1.30 ± 0.00	2.94 ± 0.44	5.03 ± 0.20	6.70 ± 0.75
	No *in ovo*	1.30 ± 0.00	3.47 ± 0.15	3.63 ± 0.07	5.85 ± 0.77
	P- value	1.0000	0.3102	0.0012*	0.4532
EYA	*In ovo*	1.30 ± 0.00	1.98 ± 0.42	1.30 ± 0.00	6.68 ± 0.63
	No *in ovo*	1.30 ± 0.00	1.88 ± 0.36	1.30 ± 0.00	4.74 ± 1.21
	P- value	1.0000	0.8664	1.000	0.2052

*Indicates significance when P= < 0.05.

Table 10. Intestinal enumeration plate count results (Log10 cfu/g ± SE) for Trial 2.

Media	Treatment	18 d	19 d	20 d	21 d
TSA-aerobic	*In ovo*	1.30 ± 0.00	2.14 ± 0.35	3.67 ± 0.60	6.48 ± 0.50
	No *in ovo*	1.43 ± 0.09	1.86 ± 0.30	2.02 ± 0.35	7.33 ± 0.62
	P- value	0.1995	0.5519	0.0409*	0.3070
MSA	*In ovo*	1.30 ± 0.00	1.30 ± 0.00	1.50 ± 0.11	2.63 ± 0.47
	No *in ovo*	1.39 ± 0.09	1.44 ± 0.14	1.30 ± 0.00	1.92 ± 0.45
	P- value	0.3559	0.3554	0.1250	0.2937
BEA	*In ovo*	1.30 ± 0.00	1.49 ± 0.179	3.34 ± 0.69	4.93 ± 1.05
	No *in ovo*	1.30 ± 0.00	1.30 ± 0.00	2.35 ± 0.30	7.29 ± 0.61
	P- value	1.0000	0.3559	0.2210	0.0811
EMB	*In ovo*	1.30 ± 0.00	1.30 ± 0.00	2.30 ± 0.81	4.62 ± 1.15
	No *in ovo*	1.30 ± 0.00	1.30 ± 0.00	1.53 ± 0.23	7.29 ± 0.46
	P- value	1.0000	1.0000	0.3870	0.1241
TSA-anaerobic	*In ovo*	1.39 ± 0.09	2.50 ± 0.34	3.25 ± 0.67	5.40 ± 0.87
	No *in ovo*	1.30 ± 0.00	2.11 ± 0.36	2.48 ± 0.24	7.25 ± 0.58
	P- value	0.3559	0.4519	0.3155	0.1047
EYA	*In ovo*	1.30 ± 0.00	1.72 ± 0.29	3.07 ± 0.72	3.58 ± 0.99
	No *in ovo*	1.30 ± 0.00	1.77 ± 0.27	2.06 ± 0.17	6.15 ± 0.66
	P- value	1.0000	0.8876	0.2162	0.0547

*Indicates significance when P= < 0.05.

Air plate microbial enumeration

Experiment 2, Trial 1 & 2. Results for air plate enumeration are presented in Tables 11 and 12. Results between trials differed. Trial 1 results indicated that eggs in the *in ovo* injection treatment after transfer had an increase in aerosol colony counts present in the hatcher containing the *in ovo* injected treatment group. This trend continued into 20 d and 21 d of incubation, where the no *in ovo* injection treatment approached the *in ovo* injection treatment hatcher. As for Trial 2, air plate colony counts were similar between the *in ovo* injected and the no *in ovo* injected treatments, with a slight increase in some values on the no *in ovo* injected hatcher as sample collection time approached 21 d of incubation. There were no additional differences detected between treatments for the *in ovo* injected and no *in ovo* injected air plated counts for any media evaluated.

Overall, data from Experiment 2 indicated that the use of *in ovo* injection did not greatly affect aerosol bacteria within the hatchers during the late stages of incubation. Differences amongst the media used were observed, however differences could be attributed to variation between the incubation location and prior contamination before 18 d of incubation.

Table 11. Air plate enumeration results (cfu/hatcher) for Trial 1.

Media	Treatment	19 d	20 d	21 d
TSA-aerobic	*In ovo*	0	189	[1]TNTC
	No *in ovo*	0	173	268
MSA	*In ovo*	0	1	2
	No *in ovo*	0	38	3
BEA	*In ovo*	0	87	248
	No *in ovo*	0	93	190
EMB	*In ovo*	0	11	15
	No *in ovo*	0	16	11
TSA-anaerobic	*In ovo*	1	225	[1]TNTC
	No *in ovo*	1	185	280
EYA	*In ovo*	0	170	[1]TNTC
	No *in ovo*	0	165	200

[1]TNTC= too numerous to count.

Table 12. Air plate enumeration results (cfu/hatcher) for Trial 2.

Media	Treatment	19 d	20 d	21 d
TSA-aerobic	*In ovo*	1	160	[1]TNTC
	No *in ovo*	1	200	[1]TNTC
MSA	*In ovo*	0	16	9
	No *in ovo*	0	43	47
BEA	*In ovo*	1	145	[1]TNTC
	No *in ovo*	1	180	[1]TNTC
EMB	*In ovo*	0	7	55
	No *in ovo*	0	22	61
TSA-anaerobic	*In ovo*	1	160	[1]TNTC
	No *in ovo*	0	TNTC	[1]TNTC
EYA	*In ovo*	1	42	39
	No *in ovo*	0	40	55

[1]TNTC= too numerous to count.

Conclusions

Vaccination protocols and the sanitation procedures associated with commercial broiler production are critical in preventing cross contamination of microorganisms across a hatchery's production inventory. Currently, commercial hatcheries have shifted away from handvaccinating day-old poultry and moved to *in ovo* vaccination of 18 d to 19 d embryonic chicks to increase efficiency and cut back on hatch day labor costs. However, this new vaccination method breaks through an eggs' natural defense layers, protecting itself against pathogens and foreign material,

creating the potential for airborne bacteria to enter the injection hole on an egg postvaccination. Significant differences were observed in total aerobic and anaerobic bacteria in Trial 1 due to *in ovo* injection and total aerobic bacteria in Trial 2, but the difference did not continue into 21 d. It can be concluded from Experiment 2 that the *in ovo* injection treatment did not significantly increase the chick intestinal microbiota of 21 d incubation (day-old chicks). The results for Trial 1 and Trial 2 demonstrated that 21 d intestinal tract microbiota for the no *in ovo* injected eggs were not close to 0 log cfu/g, indicating that *in ovo* injection is not the source of contamination occurring during the incubation of broiler hatching eggs.

Differences in the air plate enumeration observed between the two treatments could be due to variation between eggs within the same breeder flock used for this experiment, in addition to the separation of the breeder flock's eggs during the first 18 d of incubation in setters prior to transfer. Hatchers are sanitized the same prior to the experiment, and both hatchers were subject to formaldehyde application. Additionally, the high standard error seen in Experiment 2 can be attributed to the low sample size used for the study. Limited research has evaluated vaccination methods and its ability to affect chick intestinal microbiota. Further research is warranted to observe the effect of *in ovo* injection on chick intestinal microbiota during the late stages of incubation; however, in the experiment *in ovo* injection did not conclusively result in an increase a of hatchery contamination at the commercial level.

CHAPTER V

EVALUATION OF EGGSHELL MEMBRANE AND CHICK INTESTINE MICROBIOTA INTERACTION IN LATE-STAGE INCUBATION OF WHITE LEGHORN AND BROILER HATCHING EGGS

Introduction

In order to protect the embryo, eggs are equipped with antimicrobial attributes to help defend eggs against pathogenic microorganisms. These antimicrobial components include the cuticle, inner and outer eggshell membrane, and the albumen. The cuticle is a thin defensive feature that forms a barrier against microbial contamination, plugging the pores on the shell surface, and ultimately decreasing the egg's porosity (Hincke, et al., 2019). The two eggshell membranes act as a filtration barrier against microorganisms that enter through the eggshell (Board and Fuller, 1999). The albumen is composed of antimicrobial proteins and an alkaline pH, which is able to defend the embryo against unwanted microorganisms (Yamamoto, et al., 1997; Board and Fuller, 1999; and Wellman-Labadie, et al., 2008).

Limited research has evaluated sampling techniques for eggshell membrane extraction. In order to evaluate the microorganism transmission between exterior sources and the internal contents of the egg, two separate sampling points will need to be observed; the eggshell membranes and the embryo or hatched chicks' intestinal tract. In addition to sampling techniques, the correlation between the late-stage hatching process is of interest. Near the time of hatch, avian species position their head under their wing, with their beak pointed towards the air cell. Around 19 d of incubation, the embryo's egg tooth punctures the membrane (internal pipping) (Tong et al., 2013). The chick stays in this position for around 24 h as the yolk sac absorbs into the abdominal cavity. Next, the embryo breaks through the eggshell (external

pipping). Once the yolk is fully absorbed the embryo will rotate its body and push its feet against the shell to fully break out of the egg (Tong, et al., 2013). Results from chapters III and IV indicated an increase in chick intestinal microbiota at the time internal and external pipping occurs, warranting further investigation. The microbial bloom is not a new discovery during this timeframe during the hatching process; however, it is of interest. If the microbial bloom can be decreased to a significant level, the likelihood of pathogenic bacteria entering the broiler production chain is less likely to occur. Past research has not indicated the source of this contamination. It is hypothesized that the increase of intestinal microbiota at the time of hatch could be attributed to bacteria residing in the eggshell membrane. Cantu (2021) demonstrated that UV light and peroxide of freshly laid hatching eggs prior to incubation can reduce the surface and subsurface microbiota of eggs to a significant level. If the hypothesis supports these findings, further research is warranted.

The objectives of the experiments described in this chapter were: 1) to successfully remove eggshell membranes for future experimental design use, 2) to evaluate the differences between floor eggs and nest eggs in White Leghorn laying breeder flock pens as a preliminary experiment, 3) to investigate the relationship between the bacteria found in the eggshell membranes to the intestinal microbiota found within the embryo, and 4) to determine if the steps of the late-stage hatching process line up with the bacterial bloom.

Materials and methods

Preliminary Experiment 3.1. One preliminary experiment was conducted using White Leghorn hatching eggs from the Texas A&M University's fertile egg production flock. Nest eggs and floor eggs were evaluated to distinguish if the membrane extraction method was successful

in removing the eggshell membranes. Nest eggs were randomly selected from the rubber lined nesting locations provided in the breeder house. Floor eggs were randomly selected off of the floor of the breeder house, laid on a mixture of pine shavings and organic material. External surfaces of the eggs were visibly clean, and not scrubbed or washed prior to membrane extraction.

Commercial Hatchery Experiment 3.2 Two trials were conducted in this experiment using commercial Ross 708 broiler hatching eggs from a post-peak producing flock. There was no treatment for the experiment. Membrane samples from the egg and intestinal tract samples from the embryo were taken from the same egg to observe the microbial relationship between the internal and external pipping stages of hatching. Table 13 depicts the breeder flock, equipment and hatchers used for this experiment.

Table 13. Broiler breeder flock, equipment and hatch results for Experiment 3.2.

Trial	Flock name	Age (weeks)	Hatcher	Setter	Number of eggs set	Hatch Percentage
1	Flock A	53	2, 3	3, 4, 5	41,472	73 %
2	Flock A	56	2, 3, 4	4, 5, 6, 7	51,840	67 %

The same breeder flock's eggs were used for both trials in Experiment 3.2.

Microbial Analysis

Preliminary Experiment 3.1 Five nest eggs and 5 floor eggs were randomly selected on the day of lay. No prior incubation of the fertile eggs occurred. All laboratory procedures were conducted at the Texas A&M University Poultry Science Research and Teaching Center shown in Table 16. Prior to membrane removal, eggs were dipped in a sanitizing solution (chlorhexidine diacetate) for 15 seconds prior to opening each egg from the air cell end and discarding the internal contents. The eggshells, with 90% or more of the inner and outer shell membrane still

intact were rinsed with sterile PBS (pH 7.4; HiMedia Laboratories, West Chester, PA) and placed on a sterile pad.

Commercial Hatchery Experiment 3.2. Ten eggs were randomly selected for 18 d and 19 d of incubation. Five eggs and five hatched chicks were randomly selected for 20 d of incubation. Prior to membrane removal, unhatched eggs were placed in a cooler with ice for 3 h and chicks were euthanized with carbon dioxide gas prior to sampling. Eggs and chicks were dipped in a sanitizing solution (Chlorhexidine Diacetate) for 15 s prior to embryo removal. Eggs were placed into the solution and embryos broke out post-sanitizing. Eggs were cracked and embryos removed from the air cell end of the egg, keeping approximately 90% of the inner and outer membranes still intact for sampling. Day 20 chicks were dipped into the solution for 15 s prior to necropsy. Next, broken out embryos were placed onto a sterile pad and labeled alongside their corresponding eggshell (with membrane still intact).

Intestinal microbial enumeration

Commercial hatchery experiment 3.2 The euthanized embryos or chicks were necropsied using tongs and surgical scissors that were sterilized by dipping them in 100% ethanol followed by flaming. The intestinal tracts were aseptically removed and placed in WhirlPak bags (Nasco, Fort Atkinson, WI). Each sample was weighed, and sterile PBS (pH 7.4; HiMedia Laboratories, West Chester, PA) was added to the bag at 4 times the weight of the intestinal sample. The samples were crushed and rolled to homogenize the contents of the WhirlPak bag. Ten-fold serial dilutions were performed. Agar media used for intestinal enumeration are presented in Table 15. For agar plates, a volume of 0.l mL of each homogenized sample and each dilution was pipetted and spread onto each agar media using sterile plastic spreaders (VWR International LLC, Radnor, PA). After plating, media was incubated for 48 h at

37°C. Colonies were enumerated and calculated as $\log_{10}$ cfu/g of intestinal tract. Therefore, the limit of detection (LOD) was 40 cfu/g.

Eggshell membrane microbial enumeration

Preliminary Experiment 3.1 Once the embryos were removed and the interior of the 0 d eggs, the interior of the eggs was rinsed out with sterile PBS (pH 7.4; HiMedia Laboratories, West Chester, PA). The inner and outer eggshell membranes were removed using 2 sets of sterile tweezers. Tweezers were sterilized in between contact with the exterior surface of the eggshell and the interior contents of the membrane. Membrane samples were placed in tubes with 20 mL of sterile PBS (pH 7.4; HiMedia Laboratories, West Chester, PA). Once membranes were removed, they were homogenized via vortex. Ten-fold serial dilutions were performed. For agar plates, a volume of 0.l mL of each membrane rinse solution and dilution was pipetted and spread onto each agar media using sterile plastic spreaders (VWR International LLC, Radnor, PA). For Petri films, a volume of 1.0 mL of each membrane rinse solution and dilution was used. Agar media used for membrane enumeration are presented in Table 14. After plating, media was incubated for 48 h at 37°C. Colonies were enumerated and calculated as $\log_{10}$ cfu/ membrane. Therefore, the limit of detection was 20 cfu/membrane.

Commercial hatchery Experiment 3.2 Ten membrane samples from 18 d and 19 d of incubation, along with 5 membrane samples from 20 d were extracted using 2 sets of sterile tweezers. Tweezers were sterilized with ethanol in between contact with the exterior surface of the eggshell and the interior contents of the membrane. Membrane samples were placed in tubes with 20 mL of sterile PBS (pH 7.4; HiMedia Laboratories, West Chester, PA). Once membranes were removed, the samples were homogenized via vortex. Ten-fold serial dilutions are

performed. For agar plates, a volume of 0.l mL of each membrane rinse solution and dilution were spread onto each agar media using sterile plastic spreaders (VWR International LLC, Radnor, PA). Agar media used for membrane enumeration are presented in Table 15. After plating, media were incubated for 48 h at 37°C. Colonies were enumerated and calculated as $\log_{10}$ cfu/ membrane. Therefore, the limit of detection was 20 cfu/membrane.

Table 14. All media and corresponding microorganisms evaluated in Experiment 3.1.

Media	Supplier	Organism
Tryptic Soy Agar (TSA)	BD Difco BBL Microbiology Distributor, Houston, TX	Total Anaerobes
APC Petrifilm	3M United States, Maplewood, MN	Total Aerobes
Sabourad Dextrose Agar	BD Difco BBL Microbiology Distributor, Houston, TX	Fungi and Aciduric organisms

Table 15. All media and corresponding microorganisms evaluated in Experiment 3.2.

Media	Supplier	Organism
Tryptic Soy Agar (TSA)	BD Difco BBL Microbiology Distributor, Houston, TX	Total Aerobes and Total Anaerobes
Mannitol Salt Agar (MSA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Staphylococcus aureus*
Bile Esculin Agar (BEA)	Criterion, Hardy Diagnostics, Santa Maria, CA	*Enterococci* and Group D S*treptococci*
EMB Agar	Criterion, Hardy Diagnostics, Santa Maria, CA	*E. coli* and other gram-negative bacilli
Egg Yolk Agar (EYA) + 50% Egg Yolk Enrichment	HiMedia Laboratories Pvt. Ltd., Maharashtra, India	Clostridium spp

Figure 3. Modified eggshell membrane removal technique performed for membrane microbial load enumeration in Experiment 3.1 and Experiment 3.2

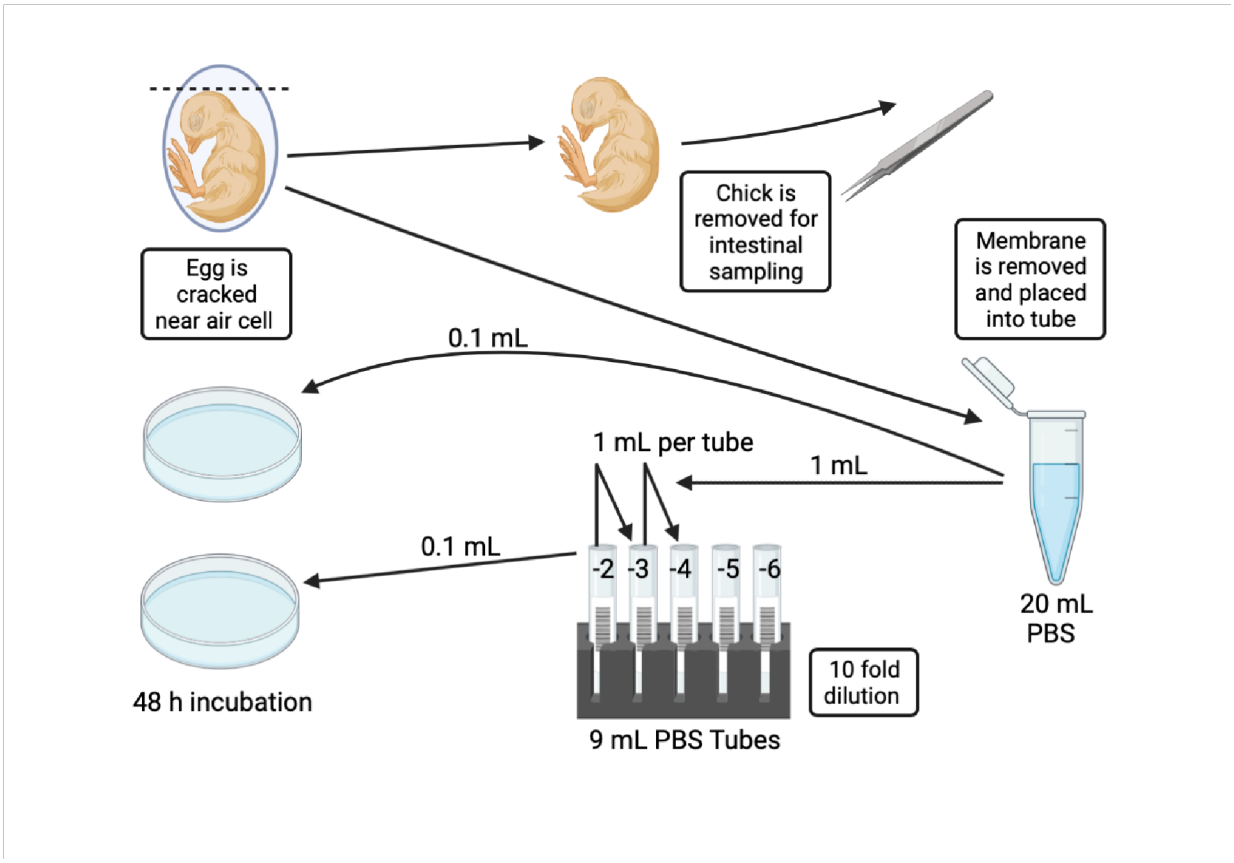

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Table 16. Breed, treatment, site of treatment and media evaluated per trial of Experiment 1.

Experiment	Trial	Breed	Sample type	Site of treatment
3.1	1	White Leghorn Layer	Membrane	Texas A&M University Poultry Science Center
3.2	1	Ross 708 broiler	Membrane and intestinal tract	Commercial hatchery
	2	Ross 708 broiler	Membrane and intestinal tract	Commercial hatchery

Statistical analysis Membrane samples that yield zero colony counts were assigned 1.0 $\log_{10}$ cfu/membrane for the statistical analysis. Intestinal samples that yield zero colony counts were assigned 1.3 $\log_{10}$ cfu/g of intestine for the statistical analysis. Data collected for Experiment 3.1 from the eggshell membrane was analyzed as a Generalized Linear Model (GLM) using JMP Pro 2016 software to compute student T-test P-values. Microsoft Excel 2021 was used to calculate the linear model means and standard error. If significance was detected, linear model means were separated and analyzed between the two treatment groups, with significance of $P < 0.05$.

A statistical analysis was not used for Experiment 3.2 because there was not a treatment applied to the commercial hatchers. Means and standard error were calculated for the eggshell membrane and intestinal tract comparison using Microsoft Excel 2021.

Results and discussion

Microbial enumeration

Experiment 3.1. Results for Experiment 3.1 are presented in Table 17. The results for membrane microbial enumeration on nest eggs and floor eggs for aerobic APC petri films, TSA-anaerobic, and Fungi SDA had no significant differences between the two sampling locations. The aerobic APC results show a slight increase in the microbial load on the floor eggs. The standard error (SE= 0.25) indicates differences amongst the sampled groups, with an insignificant p-value of P= 0.5374. The TSA- anaerobic plate count results from the next eggs displayed an increase in the number of colony forming units (3.75 $\log_{10}$ cfu/egg) compared to the floor eggs (3.58 $\log_{10}$ cfu/egg) with a p-value of P= 0.5928. The Fungi SDA results indicated that there was zero presence of these colony forming units detected from the nest egg samples, given a value of 1.3 $\log_{10}$ cfu/egg. Floor collected eggs displayed growth (1.70 $\log_{10}$ cfu/egg). The

results were not significant (P= 0.2025) when compared to the floor eggs. Due to the smaller sample size in this experiment, the standard error across each media is relatively high. Future experiments warrant a higher sample size to decrease the standard error. It can be concluded that there is no difference in the membrane enumeration of freshly collected White Leghorn hatching eggs based on the location of collection. No additional differences were observed between treatments for membrane microbial loads on any evaluated media.

Table 17. White Leghorn eggshell membrane results for Experiment 3.1, Trial 1. (Log_{10} cfu/membrane).

Medium	Nest Collection Average	Floor Collection Average	P-value
APC Petri film	2.94 ± 0.24	3.17 ± 0.25	0.5374
TSA-AN Agar	3.75 ± 0.26	3.58 ± 0.13	0.5928
SDA Agar	1.30 ± 0.00	1.70 ± 0.26	0.2025

Experiment 3.2. Results for Experiment 3.2 are presented in Tables 18 and 19, as averages of the samples for each media evaluated for each day of sampling. Ten samples were evaluated for each day of incubation, and a direct membrane and intestinal microbiota comparison was performed. The results of the membrane and intestinal tract microbiota comparison concluded that intestinal microbiota increased through each stage of the hatching process; internal pipping on 19 d, external pipping on 20 d and post-hatch on 20 d and 21 d of incubation. In addition to the intestinal microbiota increase, membrane microbiota increased. In the majority of eggs evaluated, the bacteria found in the membrane were also found to be in the intestinal tract. On a few occasions, the membrane colony forming units observed were below the limit of detection, while bacteria were found in the intestinal tract. Bacteria do not need to be enumerated in a large quantity to colonize in an uninhabited host in order to replicate. Cantu (2021) demonstrated low enumeration of pre-transfer intestinal tract. Experiment 3.2 results support these findings. When the pipping process begins on 19 d, intestinal microbiota increases. Five samples were taken on

20 d of incubation from embryos and five from hatched chicks. These embryos and chicks were removed from the hatchers at the same point of incubation. Post-hatch chicks displayed a higher intestinal tract enumeration, partially due to the longer exposure to the hatcher atmosphere. A membrane and intestinal tract analysis was not evaluated because there was not an eggshell to match up to post-hatched chicks on 20 d. Further research is warranted to explore the relationship between eggshell membrane and embryo and chick intestinal microbiota.

Table 18. Ross 708 broiler eggshell membrane (Log_{10} cfu/membrane) and intestinal tract (Log_{10} cfu/g) enumeration for Experiment 3.2, Trial 1.

Sample type	Medium	18 d embryos	19 d embryos	20 d embryos	20 d hatched chicks
Eggshell membrane	TSA	1.49 ± 0.27	1.47 ± 0.33	2.29 ± 0.41	N/A
	MSA	1.00 ± 0.00	1.86 ± 0.39	1.00 ± 0.00	N/A
	BEA	1.03 ± 0.26	1.33 ± 0.08	2.23 ± 0.54	N/A
	EMB	1.00 ± 0.23	1.10 ± 0.09	1.74 ± 0.49	N/A
	TSA-AN	1.00 ± 0.27	1.58 ± 0.31	2.22 ± 0.45	N/A
	EYA	1.00 ± 0.15	1.20 ± 0.25	1.87 ± 0.40	N/A
Intestinal tract	TSA	1.39 ± 0.04	1.94 ± 0.25	1.48 ± 0.19	2.26 ± 0.21
	MSA	1.30 ± 0.00	1.30 ± 0.00	1.30 ± 0.00	1.36 ± 0.22
	BEA	1.33 ± 0.03	1.42 ± 0.22	1.36 ± 0.12	2.72 ± 0.22
	EMB	1.30 ± 0.00	1.30 ± 0.00	1.33 ± 0.06	1.42 ± 0.55
	TSA-AN	1.38 ± 0.04	1.59 ± 0.30	1.81 ± 0.32	2.41 ± 0.55
	EYA	1.30 ± 0.00	1.38 ± 0.31	1.36 ± 0.28	1.30 ± 0.50

N/A indicates the lack of available eggshell membranes to match to the hatched chick intestinal tract.

Table 19. Ross 708 broiler eggshell membrane (Log_{10} cfu/membrane) and intestinal tract (Log_{10} cfu/g) enumeration for Experiment 3.2, Trial 2.

Sample type	Medium	18 d embryos	19 d embryos	20 d embryos	20 d hatched chicks
Eggshell membrane	TSA	1.49 ± 0.15	1.47 ± 0.19	2.29 ± 0.54	N/A
	MSA	1.09 ± 0.05	1.00 ± 0.00	1.12 ± 0.12	N/A
	BEA	1.03 ± 0.03	1.33 ± 0.15	2.23 ± 0.51	N/A
	EMB	1.00 ± 0.00	1.10 ± 0.10	1.74 ± 0.60	N/A
	TSA-AN	1.00 ± 0.00	1.58 ± 0.22	2.22 ± 0.55	N/A
	EYA	1.00 ± 0.00	1.20 ± 0.12	1.87 ± 0.54	N/A
Intestinal tract	TSA	1.39 ± 0.05	1.94 ± 0.28	1.48 ± 0.12	2.26 ± 0.71
	MSA	1.30 ± 0.00	1.30 ± 0.00	1.30 ± 0.00	1.36 ± 0.06
	BEA	1.30± 0.00	1.30 ± 0.00	1.30 ± 0.00	2.72 ± 0.87
	EMB	1.33 ± 0.03	1.30 ± 0.00	1.30 ± 0.00	2.72 ± 0.12
	TSA-AN	1.38 ± 0.08	1.59 ± 0.19	1.81 ± 0.44	2.41 ± 1.04
	EYA	1.30 ± 0.00	1.38 ± 0.08	1.36 ± 0.06	1.30 ± 0.00

N/A indicates the lack of available eggshell membranes to match to the hatched chick intestinal tract.

Conclusions

Hatchery sanitation procedures become increasingly important when it comes to eggshell membrane trends. Setting visually clean fertile eggs that are free of organic adhering matter are important for lessening the potential for horizontal contamination in a commercial hatchery setting. The chemical and physical defenses of eggs are naturally equipped with aid in the protection of developing embryos from external contamination; however, based on the results from this chapter, contamination is seen as early as the day eggs are laid. The White Leghorn fertile egg experiment demonstrated that contamination of eggshell membranes occurs the day eggs are laid. No significant differences were observed between eggs laid in nest boxes or eggs laid on the floor across the media used in this evaluation. The technique used for membrane removal was successful in removing 90% or more of the eggshell membranes for microbial enumeration. Between the two experiments conducted in this chapter, it became evident that the eggshell membrane degrades from the time eggs are laid and throughout the duration of

incubation. Eggshell membranes in the late stages of the broiler trials were much easier to remove.

One objective of the second experiment for this chapter was to determine if there was a link between the hatching process of chicks, and the microbiota of the membranes and the gastrointestinal tract. In most cases, the bacteria found in the membranes of eggs were also found in the intestinal tract of the corresponding embryo. In some cases, bacteria were present in the intestinal tract, but not in the membrane. Bacteria do not need to be present in large numbers in order to replicate and inhabit a host. It can be inferred that membrane enumeration in these cases fell below the limit of detection but were present, leading to undetected inoculation of the developing embryo's intestinal tract. During the last three days of incubation leading into chick hatching, embryos internally pip on 19 d, externally pip on 20 d and begin hatching from their eggshells between 20 d and 21 d. Samples collected from 20 d were split between embryos and post-hatch chicks. At the same incubation time, there is an increase in colony forming units on the post-hatch chicks compared to the embryos. Bacteria, depending on the strain, can have relatively short generation times. With the increased exposure to oxygen outside of the shell and airborne bacteria present in hatchers, chick microbiota enumeration increases in a short time period. Further research is needed to support the findings of this preliminary experiment. Evidence suggests a link between the pipping process through eggshell membranes and the increase of chick intestinal microbiota.

CHAPTER VI

CONCLUSIONS

Commercial broiler hatching eggs vary in the degree to which microorganisms inhabit the membrane and intestinal contents of developing and newly hatched chicks. It is important to reduce potential sources of contamination at the hatchery throughout the duration of an egg's incubation. Eggs that are visually soiled or washed have a lower hatchability, thus soiled eggs are left unwashed and separated from good quality hatching egg stock (Van den Brand, et al., 2016). If contamination of the eggs occurs, bacteria found within developing embryos and newly hatched chicks can make its way through the live poultry production chain and lead to a food safety concern in poultry products (Berrang, et al., 1999; Coufal, et al., 2003). In order to reduce bacteria in a hatchery setting, sanitation practices from the time eggs are laid at a breeder facility until the time of hatch are critical. Many egg defenses are in place to aid in the protection against invasive microorganisms. These defenses vary in their biological makeup and properties of resistance; however, the eggshell membrane is of interest. Limited research has been published on the microbiota of the eggshell membrane and its correlation to the intestinal tract microbiota of developing embryos.

One mitigation step historically used, formaldehyde application, is presented in latestage incubation to decrease airborne bacteria in hatchers. Formaldehyde is a known chemical disinfectant that when used at balanced levels, can improve the eggshell surface microbial counts (Acklund, et al., 1980; Braswell et al.,1970). At the time of transfer when formaldehyde application begins, eggs are transferred from the setters to the hatchers between 18 d and 19 d of incubation. During this transfer stage, eggs are *in ovo* vaccinated, a process designed to reduce labor and increase the efficiency of vaccinating neonatal avian species. The ideal timeframe to

inject eggs from a developmental standpoint is when the yolk sac begins to descend into the abdomen and internal pipping occurs for the most direct vaccine administration (Williams, 2007). This vaccination machine is capable of administering pre-hatch vaccines to enhance the overall immune response at an earlier stage than the traditional hand vaccinations at 21 d of incubation, hatch day (Saeed, et al., 2019). With this in mind, the primary objectives of this thesis were to evaluate the effects of formaldehyde application and *in ovo* injection on the chick's intestinal tract microbiota in late-stage incubation, in addition to comparing steps in the membrane pipping process and how they relate to chick intestinal tract microbiota.

Data from laboratory experiments demonstrated that eggs applied with formaldehyde had a lower intestinal tract microbiota count while formaldehyde was turned on. Yet, when formaldehyde was shut off 12 hours before samples were collected at the time of chick processing, values of the formaldehyde application group approached those of the no formaldehyde application group. Prior to this study, the effect of formaldehyde application and its effect on chick intestinal tract microbiota was minimally studied. The present study demonstrated that the current formaldehyde application rate was ineffective in significantly reducing bacteria in hatchers, potentially allowing pathogenic bacteria to enter the chick's digestive system and following it throughout production. Similarly, to the data recorded for intestinal microbial loads, the air plate enumeration followed the same trend. Air plate colony counts for the formaldehyde treatment group were reduced while formaldehyde was turned on, yet when formaldehyde was turned off 12 h prior to the hatch being pulled, the treatments approached the same values.

Following successful laboratory trials evaluating the effects of formaldehyde application, *in ovo* injection's effect on chick intestinal tract microbiota was also evaluated in commercial

Ross 708 broiler hatching eggs. The experimental design was organized in a similar manner to Chapter III. Results concluded that the use of *in ovo* injection did not significantly affect the chick intestinal tract microbiota by 21 d of incubation. Data from Chapter IV demonstrated that in ovo injection significantly increased the total aerobic bacteria and total anaerobic bacteria on 20 d of incubation in Trial 1. Similarly, *in ovo* injection significantly increased the total aerobic bacteria on 20 d of incubation in Trial 2. However, on 21 d of incubation, the significant differences were lost as the no *in ovo* treatment's intestinal microbiota approached that of the *in ovo* treatment in Trial 1, and surpassed the enumeration values of the no *in ovo* injection hatcher in Trial 2. The results from this chapter concluded that *in ovo* injection is not the hatchery contamination method hypothesized. The values of the no *in ovo* injection enumeration are not near the limit of detection, suggesting the source of contamination is not related to *in ovo* injection.

Results from the eggshell membrane experiments demonstrated that White Leghorn fertile hatching egg's location of lay and collection did not significantly change the eggshell membrane microbiota for freshly collected eggs across all of the media used for enumeration. In addition to these findings, late-stage Ross 708 broiler hatching eggs were evaluated to determine the relationship between eggshell membrane and intestinal tract microbiota during the hatching process. Results concluded that when membrane microbiota increased, the intestinal tract microbiota also increased. Samples with a membrane enumeration below the LOD still had the potential to transfer viable colony forming units and replicate in pre- and post-hatch chick intestines.

Further research should be conducted to evaluate the effectiveness of these standard broiler production protocols to ensure application rates, time of transfer and hatchery sanitation practices are effective. Microbial loads, hatchling quality and hatchability should be further

assessed post chick-placement to study the effects of the treatments in Chapter III and Chapter VI. To further understand the relationship between membrane microbiota and its correlation to chick intestinal tract microbiota, further trials are needed to support the findings presented. More information on the innate egg defenses and their effectiveness into 21 d of incubation could aid in the optimal hatchery sanitation and mitigation strategies used in future microbial contamination defenses.

Hatching egg sanitation, handing, and vaccination are important steps in broiler production that greatly affect the economic status at the commercial level. Early and late stage hatching egg contamination can decrease hatchability, chick quality, and performance of poultry, in addition to post chick-placement cross contamination. These experiments, along with other hatchery sanitation procedures have the potential to decrease the amount of *Staphylococcus aureus, Enterococci, Streptococci, Escherichia coli*, and *clostridium* spp. in neonatal poultry. Shifts in animal agriculture have limited historical disease prevention with antibiotic use; however, sanitation and handling procedures traced back to the hatchery can greatly improve these odds.

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