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Development and Evaluation of Models for Hatchery-Mediated Infection of Neonatal Broiler Chicks

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Development and Evaluation of Models for Hatchery-Mediated Infection of Neonatal Broiler Chicks

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by

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Abstract

Formaldehyde fumigation has been used to control the microbial load in commercial hatch cabinets. The hatch cabinet environment promotes replication and dissemination of both apathogenic and pathogenic microorganisms. As the microbial load increases during the hatching phase, formaldehyde eliminates airborne microorganisms circulating in the hatch cabinet environment. Due to the hazardous properties of formaldehyde, non-toxic alternatives to formaldehyde fumigation to control the microbial bloom in hatch cabinets are needed. The objectives of the present dissertation were to develop challenge models using singular or multiple microorganisms associated with the microbial bloom to simulate contamination that occurs in commercial hatch cabinets. Initially, a challenge model was developed to mimic horizontal transmission of virulent *Escherichia coli* during the hatching phase. Seeder embryos were directly infected by *in ovo* injection into the amnion. Administration of the avian pathogenic *E. coli* (APEC) alone at 18 and 19 days of embryogenesis (DOE) was lethal to developing seeder embryos. However, seeder chick hatchability was improved when APEC were co-administered with tetracycline hydrochloride (272ug/mL). Exposure to APEC during the hatching phase significantly ($P < 0.05$) increased 7-day mortality compared to the non-exposed control group. Additionally, horizontal transmission of APEC reduced body weight gain (BWG) in 2/3 trials compared to the non-challenged control group. An alternative challenge model using wild-type (WT) *E. coli* strains that were previously isolated from colibacillosis field cases were selected to assess horizontal transmission of WT *E. coli* during the hatching phase. *In ovo* administration of either WT *E. coli* strain at DOE19 had minimal impact on seeder hatchability. As the seeder chicks hatched, the circulating airborne Gram-negative bacteria in the hatch cabinet increased. Gram-negative bacteria recovered from the GIT was

significantly ($P < 0.05$) increased for seeder and contact chicks compared to the non-challenged control group. As suspected, formaldehyde fumigation did not reduce seeder chick Gram-negative enteric colonization at day-of-hatch (DOH). However, formaldehyde fumigation effectively controlled the Gram-negative bacterial bloom in the hatch cabinets. The WT or APEC *E. coli* seeder challenge models could be used to assess the effect of candidate disinfectants or natural alternatives on the Gram-negative bacterial bloom and horizontal transmission during the hatching phase. Although the models using a singular challenge organism were validated, challenge with a singular species does not reflect real-world conditions in commercial hatcheries. Thus, a challenge model with multiple hatchery-relevant opportunistic pathogens was evaluated. Since the contents of non-viable embryonated eggs contain a plethora of microorganisms, an egg homogenate (EH) was derived from the contents of non-viable embryonated eggs at DOE18. To create the pathogen mix (PM) challenge, bacterial and fungal species were isolated from the EH to artificially replicate the contamination in commercial hatch cabinets. The PM consisted of two *E. coli* isolates, *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Enterococcus faecalis*, and one fungal isolate, *Aspergillus fumigatus.* EH or PM challenge was applied to the eggshell at DOE19 to determine which material be suitable for future investigations based on enteric bacterial recovery at DOH, bacterial and fungal recovery from fluff samples collect at DOH, chick rinses at DOH, or air samples collected from the hatch cabinet environment during the hatching phase. Based on overall microbial recovery and practicality, the PM challenge proved to be the more appropriate model to mimic microbial contamination in commercial hatch cabinets in a laboratory setting. These challenge models could be used to evaluate industry-applicable methods to control the microbial bloom in commercial hatch cabinets.

Dedication

I would like to dedicate my dissertation to my husband, Lucas Graham, and my dear friend and mentor, Marsha Williams. I love you both more than you will ever know.

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List of manuscripts

Graham, B., C. Selby, K. Teague, L. Graham, C. Vuong, J. Latorre, G. Tellez, and B. Hargis. 2019. Development of a novel *in ovo* challenge model for virulent *Escherichia coli* strains. *Poultry Science* 98:5330–5335. (Chapter III)

Graham, B., C. Selby, L. Graham, K. Teague, G. Tellez-Isaias, B. Hargis, and C. Vuong. 2021. Development of a wild-type *Escherichia coli* environmental bloom model to evaluate alternatives to formaldehyde fumigation in broiler chicken hatch cabinets. *Poultry Science* 100:100975. (Chapter IV)

Chapter I. Dissertation Introduction

Horizontal transmission of both apathogenic and pathogenic microorganisms occurs in commercial poultry hatcheries (Berrang et al., 1999; Osman et al., 2018). Commercial hatcheries serve as a source of contamination in vertically integrated production systems (Wales and Davies, 2020). During incubation, embryonated eggs become exposed to a plethora of fecal or environment-derived microorganisms capable of penetrating the eggshell (Williams et al., 1967; Williams et al., 1968; Cason et al., 1994; De Reu et al., 2006). As the chicks begin to hatch, the warm and moist environment in the hatch cabinet has been shown to facilitate microbial proliferation or a microbial bloom (Magwood, 1964; Sheldon and Brake, 1991). Perinatal colonization by opportunistic pathogens has been associated with elevated embryonic mortality (Williams and Brake, 2000). Moreover, these infertile or non-viable embryonated eggs may be potential reservoirs for opportunistic pathogens (Karunaranthna et al., 2020). However, infected chicks that successfully hatched horizontally transmitted pathogens to non-infected chicks in the hatch cabinet (Cox et al., 2000). Additionally, early exposure to opportunistic pathogens has been shown to increase the occurrence of omphalitis and 7-day flock mortality (Olsen et al., 2012; Walker et al., 2020).

Hatchery disinfection and sanitation practices are essential to mitigate cross-contamination of pathogens. The use of formaldehyde fumigation in commercial poultry hatcheries was initially reported in 1908 (Pernot, 1908). Due to its biocidal efficacy, formaldehyde fumigation suppressed the microbial bloom in the hatch cabinet environment (Whistler et al., 1988). However, formaldehyde fumigation has been shown to have damaging effects on the tracheal epithelium of neonatal chicks (Sander et al., 1995; Hayretdag et al., 2006; Maharjan et al., 2017). Additionally, formaldehyde fumigation does not promote colonization by

beneficial microorganisms. Considering that the neonatal GIT is rapidly colonized by microorganisms present in the environment at hatch, there is a need for alternatives to formaldehyde fumigation that promote enteric colonization by beneficial microorganisms.

Previously, laboratory challenge models have been used to simulate horizontal transmission that occurs in a commercial setting (Weinack et al., 1981; Montgomery et al., 1999; Jarquin et al., 2007). The objectives of the present dissertation were to develop reproducible horizontal challenge models using singular or multiple opportunistic pathogens associated with the microbial bloom to simulate the horizontal transmission that occurs in commercial hatch cabinets.

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Chapter II. Literature Review

Value and Limitations of Formaldehyde for Hatch Cabinet Applications: The Search for Alternatives

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This literature review is in preparation to be submitted as a review article.

Abstract

Pioneer colonization by beneficial organisms promote a shift in the composition of the gut microbiota, excluding opportunistic pathogens. Commercially, the horizontal transmission of both apathogenic and pathogenic organisms is common during the hatching phase. The microbial bloom occurs as the humidity rises during hatch, exposing naïve chicks to a plethora of potentially harmful microorganisms. Horizontal transmission or introduction of pathogens may occur as infected chicks hatch or during handling after hatch pull. Moreover, contaminated infertile or non-viable embryonated eggs can serve as reservoirs for pathogenic organisms and even rupture during incubation. The organisms within the contents of these eggs can penetrate the shell of the embryonated eggs and subsequently contaminate the entire cabinet. Formaldehyde fumigation is commonly applied during the hatching phase to control the microbial bloom in the environment, but does not penetrate the eggshell prior to hatch. Additionally, this fumigation technique eliminates microbial organisms in the environment at hatch, including beneficial species. Furthermore, prolonged exposure to formaldehyde can damage the tracheal epithelia of neonatal chicks increasing susceptibility to infection by opportunistic microorganisms. Laboratory challenge models that mimic the microbial bloom that occurs in commercial hatch cabinets can be used to evaluate effective alternatives to control the microbial bloom and promote colonization by beneficial bacteria without the use of formaldehyde fumigation.

Introduction

Horizontal transmission of pathogens during the neonatal period is a major concern to commercial poultry producers. In a commercial setting, viable eggs are removed from hens and transported to a hatchery for artificial incubation. Eggs from multiple source flocks are

frequently comingled during incubation which promotes both cross-contamination with pathogens as well as exposure to potential beneficial pioneer colonizing bacteria. At 18 days of embryogenesis (DOE), embryos are transferred from incubators to hatch cabinets with holding capacities exceeding 10,000 embryos. At approximately DOE20, or initiation of the hatching process, chicks begin to pip and break through the eggshell. As chicks pip, they are exposed to microorganisms on the surface of the eggshell (Cason et al., 1994). Fecal material on the surface of the eggshell may harbor potential pathogenic microorganisms capable of penetrating the eggshell and membranous layers during incubation (Cason et al., 1994). Eggshell contamination has been shown to negatively impact hatchability and hinder early performance (Scott et al., 1993). Additionally, these contaminated embryos serve as reservoirs that horizontally transmit pathogens during the hatching phase (Cox et al., 2000). As chicks hatch, the humidity in the hatching environment promotes replication of both apathogenic and pathogenic microorganisms. The composition of the microbial bloom during the hatching phase influences pioneer colonization of the neonatal gastrointestinal tract (GIT; Lu et al., 2003). As such, cross-contamination of primary poultry pathogens readily occurs in commercial hatcheries (Berrang et al., 1999). Prior to incubation, chemical sanitizers may be used to reduce the microbial load on the surface of the eggshell to prevent cross-contamination during embryogenesis (Brake and Sheldon, 1991; Scott et al., 1993; Spickler et al., 2011).

For over a century, formaldehyde fumigation has been utilized to control the dissemination of pathogens in some commercial hatcheries (Pernot, 1908; Graham & Michael, 1932). Although formaldehyde eliminates microorganisms in the hatching environment, it has been associated with tracheal epithelial damage and mucosal sloughing in neonatal chicks (Sander et al., 1995; Hayretdag et al., 2006; Maharjan et al., 2017). As a biocide, formaldehyde

effectively kills resistant forms of bacteria, fungi, and viruses (Swenberg et al., 2013), and likely eliminates airborne apathogenic and potentially beneficial microorganisms. Cost-effective and sustainable alternatives to formaldehyde fumigation to reduce the potentially pathogenic microbial load in the hatching environment are needed. However, a multi-faceted approach will be required to control the microbial bloom in the hatching environment and promote early colonization by beneficial microorganisms to improve poultry health.

Pioneer colonization of the GIT: critical timepoints during the neonatal period

Pioneer or initial colonizers of the neonatal GIT influence the diversity of the post-hatch intestinal microbiome (Wilson et al., 2019; Rodrigues et al., 2020a), promote functional development of the immune system (Smith and Anderson, 2005), and inhibit colonization by enteropathogenic bacteria (Frick and Autenrieth, 2013). Once established, the commensal microbiota inhibit pathogen invasion and colonization by forming a microbial barrier and by competing for nutrients and attachment sites (Turner et al., 2009). The commensal microbiota also modulate host immune development and maturation of the GIT (Smith and Anderson, 2005). The intestinal immune repertoire evolves to tolerate the resident microorganisms in the lumen of the GIT, which is critical for homeostasis (Belkaid and Harrison, 2017). Pioneer colonization of the neonatal intestinal tract occurs at birth (mammalian species) or hatch (avian species). For mammalian species, transfer of the maternal microbiota to progeny occurs during vaginal birth where the composition of the neonate's intestinal microbiota tends to resemble the vaginal microbiota (Dominguez-Bello, 2010). For avian species, transfer of the maternal microbiota occurs during oviposition (Gantois et al., 2009) and post-hatch due to coprophagic behavior or cloacal sampling of the nest or maternal environment. Cloacal sampling and uptake by retrograde transport of environmental antigens to the bursa of Fabricius has been shown to

stimulate immune development (Sovari et al., 1975; Ekino et al., 1985). Perhaps coprophagy and cloacal drinking amplify antigen exposure during the neonatal period before maternal immunity wanes. Additionally, cloacal drinking is known to transmit organisms directly to the ceca along with retrograde urine transport (Duke, 1989; Hu et al., 2004; McDougald and Fuller, 2005) and intracloacal administration of beneficial bacteria has been shown to be markedly more potent than oral administration with regard to exclusion of selected cecal pathogens (Cox et al., 1990; Corrier et al., 1991).

During incubation of eggs by hens, it has been shown that the number of pathogenic microorganisms on the eggshell decline during incubation, and resident microorganisms on the eggshell inhibit trans-shell invasion by pathogens (Cook et al., 2003; Cook et al., 2005). However, in commercial poultry operations, embryonated eggs immediately removed from the hen may be exposed to fecal or environmental microorganisms that adhere to and potentially penetrate the eggshell (Cason et al., 1994; De Reu et al., 2006). The risk of trans-shell invasion appears to be relative to the amount of contamination in the environment at the time of oviposition. Smeltzer et al. (1979) observed that floor eggs had more contamination and greater susceptibility to bacterial penetration than nested eggs. The increased contamination was likely associated with increased fecal debris on the surface of the eggshell of floor eggs. Preventing transmission of pathogens during the perinatal and postnatal periods is critical to improving poultry health and optimizing performance. For instance, early colonization by beneficial microorganisms during late embryonic development improved growth performance and immune system development (Pedroso et al., 2016; Pender et al., 2017). However, enteric pathogens, including *Salmonella enterica* serovar Typhimurium, capitalize on the host's inflammatory response to alter the composition of the commensal microbiota to enhance colonization of the

enteropathogen (Withanage et al., 2004; Stercher et al., 2007; Drumo et al., 2016). Moreover, the energetic costs related to the activation of inflammatory pathways by opportunistic pathogens have been shown to cause protein catabolism (Klasing and Austic, 1984). Thus, it is important to mitigate exposure to and transmission of pathogenic microorganisms in the hatchery to optimize poultry health and performance, but at present, mitigation efforts also destroy some eggshell defenses and reduce the opportunity for beneficial pioneer colonization.

Embryogenesis

The avian egg contains both physical and chemical defense mechanisms to inhibit microbial invasion and proliferation. The eggshell has four physical defense mechanisms: 1) the cuticle, 2) the shell, 3) inner shell membrane, and 4) outer shell membrane (Mayes and Takeballi, 1983). Chemical defenses within the developing embryo include antimicrobial properties of the albumen, alkaline pH, lysozyme, and conalbumin/ovotransferrin (Mayes and Takeballi, 1983). Potential contamination of the egg occurs both before oviposition (trans-ovarian route) or after oviposition (trans-shell route; Bruce and Drysdale, 1994). Environmental temperature and humidity are also known to impact the rate of microbial penetration of eggshells (McNally, 1954). High relative humidity is considered essential for trans-shell transmission of microorganisms because it promotes survival, growth and transport through eggshell pores (Board and Halls, 1973). As the egg cools after lay, a relative vacuum is generated and the negative pressure facilitates microbial penetration of the eggshell (Bruce and Drysdale, 1994). Additionally, the quality and thickness of the eggshell impact a microorganisms's ability to penetrate the eggshell (Sauter and Peterson, 1974). Comprehensive reviews describing microbial contamination of the egg and penetration of the eggshell have been published (Mayes and Takeballi, 1983; Bruce and Drysdale, 1994; Berrang et al., 1999).

The composition of the neonate's GIT microflora is thought to be predominantly influenced by fecal and environmental contaminants on the eggshell (Donaldson et al., 2017), but the composition may also be affected by microorganisms vertically transmitted from hen to offspring at oviposition. Luo et al. (2017) demonstrated that the hen's gastrointestinal tract microbiota influenced the composition of the chick's gut microbiota at hatch and there was a shared core microbial profile between the hen, embryo, and chick. There is further evidence of a partial transfer of the maternal oviduct microbiota to the embryo (progeny) during egg formation (Lee et al., 2019). However, introduction of environmentally-derived microbial contaminants may complicate findings when using DNA sequencing to assess microbial profiles in samples, especially when sample number is low. Nevertheless, pathogen transmission during the perinatal period, either maternal, fecal, or environmentally-derived, leads to potential horizontal transmission of pathogens at the hatchery level. If contaminated hatching eggs are not sanitized properly before incubation, these eggs serve as a primary source of contamination in commercial hatcheries (Brake and Sheldon, 1991; Scott and Swetnam, 1993; Spickler et al., 2011). Both culture-based methods and sequencing techniques (culture-independent methods) have been applied to evaluate microbial presence on the surface of the eggshell. Using conventional microbiological techniques or culture-based methods, it was determined that eggshell surface contained ~$1x10^3$ colony forming units (CFU) per egg (Sauter et al., 1979). The composition of the eggshell microbiota of hatching eggs can be altered by the breeder hen's fecal microbiota or the environment. Buhr et al. (1994) demonstrated that eggshell contamination negatively affected hatchability and surface sanitation of dirty eggs only marginally improved hatchability compared to non-sanitized dirty eggs. The eggshells of sanitized eggs have also been shown to harbor extensive numbers of microorganisms (Berrang et al., 1997). Thus, handling after the

sanitization process should be limited to prevent contamination or recontamination of the surface of the eggshell.

Although there are physical and chemical defense mechanisms to prohibit microbial penetration of the eggshell and endogenous replication during embryogenesis, certain microorganisms have developed the ability to more readily penetrate the eggshell and evade host defenses. Certain Gram-negative bacteria, such as *Salmonella* can replicate on the eggshell surface at suboptimal temperature for growth and without supplemental nutrients (Messens et al., 2006). At the time of lay, the eggshell may become contaminated with *Salmonella* by brief contact with contaminated nest box shavings (Padron, 1990). Contamination of the eggshell surface with fecal material, nest box shavings, or egg-derived debris increased cultivable aerobic bacteria compared to clean eggs (Olsen et al., 2017). Using 16S RNA amplicon sequencing, Olsen et al. (2017) showed that the eggshell surface microbiome of non-sanitized, dirty eggs and clean eggs were different, but variability between samples within the same group complicated the results. The authors suggested that environmental contaminants present on the eggshell could have influenced the results (Olsen et al., 2017). Furthermore, the composition of the microbiome depends on the bacterial DNA present at the time of sampling and cannot be used as a standalone metric to detect viable microorganisms (Emerson et al., 2017). In another study, 16S sequencing was used to compare the breeder hen's fecal microbiota to the eggshell microbiome in two independent flocks (Trudeau et al. 2020). Of the eggshells that were sampled, Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes contributed to 90% of the overall microbiota (Trudeau et al., 2020). Transfer of potentially pathogenic bacteria and those associated with spoilage from breeder hens to the eggshell surface, included *Salmonella, Escherichia coli,* and *Staphylococcus* spp. (Trudeau et al., 2020). Maki et al. (2020) showed that

source or exposure to only eggshell-derived, environment-derived, or to both eggshell and environment-derived microorganisms modulate the composition of intestinal tract microbiota and fecal microbiota post-hatch. The eggs that were only subjected to the environment-derived microorganisms were sterilized prior to incubation which could have negatively affected the eggshell cuticle integrity. Also, any maternal microbiota transferred during oviposition or that penetrated the eggshell may have confounded the results. Regardless, results published by Maki et al. (2020) do indicate that intestinal pioneer colonization of the GIT is readily affected by source of contamination during the neonatal period.

For decades, early exposure to probiotics or beneficial bacteria has been used to inhibit colonization of pathogenic bacteria by competitive exclusion (Nurmi and Rantala, 1973; Cox et al., 1992; Meijerhof and Hulet, 1997). In addition to competitive exclusion and performance benefits, beneficial bacteria may also have immunomodulatory effects on the host (Cox and Dalloul, 2015; Pedroso et al., 2016; Pender et al., 2017). However, the site of probiotic administration (air cell, amnion, allantoic sac), probiotic strain, dose, volume, and day of administration during embryonic development, all impact colonization efficiency and chick hatchability (De Oliveira et al., 2014). Early application by *in ovo* injection at DOE18 promotes uptake of the material (vaccine, probiotic, etc.) by the chick during the pipping process (Peebles, 2018). Teague et al. (2017) administered FloraMax-B11, a lactic acid bacteria (LAB)-based probiotic, into the amnion of embryonated broiler eggs at DOE18. *In ovo* application of the probiotic reduced *Salmonella* colonization, improved early performance, and had no impact on Marek's vaccine efficacy (Teague et al., 2017). Thus, *in ovo* administration could be utilized to promote early colonization by beneficial bacteria in domestic poultry neonates.

Migration and colonization by a non-pathogenic, bioluminescent *E. coli* was more efficient when administered by *in ovo* application at DOE18 into the amnion as compared to the air cell (Castaneda et al., 2019). Additionally, there was an increase in spleen weight at hatch related to *in ovo* administration into the amnion (Castaneda et al., 2019). The authors hypothesized this to be associated with an accelerated immune development compared to those that received *E. coli* via *in ovo* air cell injection (Castaneda et al., 2019). An increase in the weight of immune organs, including the spleen, was observed with probiotic supplementation has been reported and was attributed to improved immune stimulation (Kabir et al., 2004; Awad et al., 2009; Castaneda et al., 2020). A direct correlation between immunocompetence and the weight of the spleen has been described (John, 1994). Although probiotics have been shown to stimulate immune development (Cox and Dalloul, 2015; Pedroso et al., 2016; Pender et al., 2017) and suppress pathogen colonization or invasion when administered by *in ovo* application (Pender et al., 2016; Teague et al., 2017), certain microorganisms may be detrimental to embryonic development due to the rapid proliferation and accumulation of lethal byproducts within the embryo. For instance, *in ovo* administration with *Bacillus subtilis* negatively affected hatchability (Triplett et al., 2018). The authors hypothesized that *B. subtilis* produced enzymatic and metabolic byproducts that were detrimental to embryo development and contributed to the high percentage of late dead embryos compared to *Lactobacillus acidophilus* and *Bifidobacterium animalis* (Triplett et al., 2018). Alternatively, *in ovo* administration of Norum TM, a mixed *Bacillus* spp. culture containing vegetative cells of two *B. amyloliquefaciens* and one *B. subtilis* isolate at DOE18 did not affect hatchability, markedly reduced enteric Gram-negative bacterial colonization a day 3 and day 7 post-hatch, and significantly improved early performance compared to the non-treated challenged group

(Arreguin-Nava et al., 2019). *In ovo* administration of with *Bacillus* spp. may inhibit colonization of opportunistic pathogens without hindering livability and early chick performance. Future studies should be conducted with potential candidate organisms to confirm feasibility for perinatal application.

The effect of *in ovo* administration (amnion, DOE18) with apathogenic *Enterobacteriaceae* or LAB on the cecal microbiome and intestinal proteome in broiler chicks have been evaluated (Wilson et al., 2019; Wilson et al., 2020). In these studies, *in ovo* application of *Citrobacter* spp. or LAB differentially altered the cecal microbiome at DOH and potentially at 10 days-of-age (Wilson et al., 2019), and antioxidant effects were upregulated and inflammation was reduced in the GIT of chicks that received the LAB at day 18 of embryogenesis (Wilson et al., 2020). Though, *in ovo* administration with one strain of *Citrobacter* spp., but not both, increased oxidative stress and proinflammatory responses in the GIT at DOH (Wilson et al., 2020). Rodrigues et al. (2020) evaluated the effect of apathogenic *Enterobacteriaceae* or LAB on the ileal microbiome of 10-day-old broiler chickens. In contrast to LAB, pioneer colonization by *Enterobacteriaceae* postponed maturation of the ileal microbiome (Rodrigues et al., 2020a) and was associated with impaired intestinal immune function (Rodrigues et al., 2020b). Taken together, these studies suggest the pioneer colonizers of the GIT influenced the composition of the intestinal microbiome and modulated the host's enteric inflammatory response.

Postnatal or post-hatch period

The GIT is rapidly colonized by microorganisms present in the environment shortly after hatch and readily established 72h post-hatch (Apajalahti et al., 2004). The composition of the microbiota is impacted by the individual host and age of the host (Fujisaka et al., 2016). The

route of exposure (oral vs. environmental) to LAB at hatch influenced rate of colonization by beneficial pioneer colonizers and subsequent composition of the intestinal microbiome in broiler chickens (Baldwin et al., 2018). However, Stanley et al. (2013) documented significant inter-chicken variation in the composition of the cecal microbiome in broiler chickens perhaps associated with the lack of exposure to the maternal microbiota and sanitation procedures in commercial hatcheries (Stanley et al., 2013). To artificially mimic the transfer of maternal microbiota to progeny, the cecal microbiota was collected from 1, 3, 16, 28, or 42-week-old hens and orally administered at DOH to chicks followed by *Salmonella* Enteritidis challenge at day 2 (Varmuzova et al., 2016). Chicks that received cecal microbiota from 3, 16, 28, and 42-week-old of hens inhibited SE colonization in the ceca significantly compared to the non-treated, challenged control 4 days post-challenge (Varmuzova et al., 2016). However, administration of the cecal microbiota as a therapeutic treatment after oral challenge treatment with SE was not protective (Varmuzova et al., 2016). To investigate the rate of natural transfer of the maternal microbiota from hen to progeny, chicks were placed in contact with hens for 24h post-hatch (Kubasova et al., 2019). It was shown that exposure and transfer of the maternal microflora influenced the chick's cecal microbiota (Kubasova et al., 2019).

Administration of beneficial bacteria has been shown to inhibit pathogen colonization and reduce horizontal transmission of pathogenic bacteria (Wolfenden et al., 2007; Arreguin-Nava et al., 2020). Early establishment of beneficial pioneer colonizers is critical for pathogen exclusion since the GIT is rapidly colonized the initial microorganisms in the environment at hatch. The pioneer colonizers of the GIT influence immune and metabolic functions that regulate host resistance to pathogens and tolerance of the commensal microbiota. Since commercially-reared poultry neonates do not have any contact with the hen at hatch,

microorganisms present in fecal material or that predominate in the environment at the time of lay or hatch dictate the composition of the pioneer colonizers of the GIT. Artificial exposure to beneficial bacteria during the perinatal period may improve poultry health and wellbeing in integrated poultry production systems where prophylactics and therapeutics are more limited than ever due to multi-drug resistance and shift towards antibiotic-free production.

Opportunistic pathogens associated with commercial poultry hatcheries

In integrated poultry production systems, transfer of the maternal microbiota is limited. Commercially reared chicks are exposed to the plethora of environmental microorganisms in the hatchery. Cleaning and disinfection processes are implemented to control the microbial bloom in the hatchery, such as formaldehyde fumigation. Environmental contamination dictates the pioneer colonizers of the gastrointestinal tract, influences performance, and resistance to opportunistic pathogens throughout the life of the animal.

The composition of the microbial bloom can be impacted by placement of contaminated non-viable embryonated eggs in commercial hatch cabinets. As non-viable embryonated eggs incubate, the internal pressure increases within the egg and may rupture or explode. In doing so, the surface of viable embryonated eggs in proximity is contaminated with non-viable embryonated egg material, which also influences the level of environmental contamination that occurs during the hatching phase. Non-viable embryonated eggs have been shown to be predominantly contaminated with *Micrococcus* spp. and *Enterobacteriaceae* and the level of contamination directly affected embryonic development (Bruce and Johnson, 1978). Moreover, at DOE21, bacteria recovered from non-viable embryonated eggs was ~2.4 logs higher than the chicks that successfully hatch (Furata & Maruyama, 1981). In a more recent study, *E. faecalis* was shown to be the most abundant *Enterococcus* spp. recovered from non-viable

embryonated eggs, while 56% of the non-viable embryonated eggs contained both *E. faecalis* and *E. coli* (Karunarathna et al., 2017). Additionally, Karunarathna et al. (2020) demonstrated that non-viable embryonated eggs are potential reservoirs for enterococci and *E. coli*. In this study, antimicrobial resistance phenotypes were observed for up to 40% *E. faecalis* isolates and 37% of the *E. coli* isolates recovered from non-viable embryonated eggs (Karunarathna et al., 2020). Both *E. coli* and *E. faecalis* are a part of the commensal microflora, but co-infection with avian pathogenic *E. coli* (APEC) and *E. faecalis* may be associated with increased colibacillosis-related mortality in both chickens and turkeys (Walker et al., 2020). Recovery from the yolk sac suggests that the navel is a critical portal of entry for *E. faecalis* during the neonatal period (Walker et al., 2020). Reynolds et al. (2020) isolated *E. faecalis* from game birds in the United States. The ring-neck pheasant eggshells and embryos harbored pathogenic *E. faecalis* that have been shown to negatively impact hatchability (Reynolds et al., 2020). Transmission of opportunistic pathogens, including *E. faecalis* may occur via horizontal or vertical transmission. The inherent risk of vertical transmission of *E. faecalis* from broiler breeders to broiler chicks increased as the breeder hens aged (>42 weeks of age) which promoted horizontal transmission of *E. faecalis* during the hatching phase (Olsen et al., 2012). Moreover, antimicrobial-resistant *E. faecalis* strains have been isolated from broiler breeder hens (Noh et al., 2020). Thus, potentially pathogenic and antimicrobial-resistant *E. faecalis* may be vertically transmitted from breeder hens to progeny and subsequently horizontally transmitted to naïve chicks at hatch.

Methods to prevent vertical transmission of APEC from breeder hens to offspring are essential to prevent horizontal transmission at the hatchery level (Christensen et al., 2021). Portals of entry of APEC include the respiratory tract or translocation from the intestinal tract

during stress (Leitner & Heller, 1992). APEC strains cause primary and secondary extra-intestinal infections, however, successful colonization of the air sacs by APEC subsequently leads to a systemic infection. APEC strains contain virulence factors and proteins that promote adherence and colonization of that respiratory mucosa and air sacs (Dho-Moulin and Fairbrother, 1999) by evading host immune defenses (Mellata et al., 2002). Embryonic infection by APEC may or may not be lethal to a developing embryo. For instance, to evaluate vertical transmission of APEC, Giovanardi et al. (2005) isolated APEC from two broiler breeder flocks and their progeny. The APEC strains isolated from the breeders and progeny were genetically similar, which signifies the importance of APEC control at the breeder level (Giovanardi et al., 2005). APEC infection has also been associated with increased 7-day mortality related to airsacculitis and colisepticemia (Kemmet et al., 2014). Horizontal transmission of APEC during late embryogenesis has been replicated in small-scale hatch cabinets (Graham et al., 2019; Selby et al., 2021). Exposure to APEC post-lay or during embryogenesis may not always impact hatchability, but colonized chicks can serve as seeders to horizontally transmit the pathogen during the hatching process or production period.

Although *E. coli* and *E. faecalis* are frequently isolated from neonates, other presumptive pathogens must be considered. *Staphylococcus aureus* contamination in hatcheries has been shown to increases morbidity and mortality in chickens (Avens et al., 1975). There is evidence of *S. aureus* jumping from humans to poultry approximately 38 years ago due to an adaptation to increased resistance to host heterophils (Lowder et al., 2009). In 2009, *S. aureus* isolates recovered from poultry were predominantly related to a clonal complex relevant to humans (Lowder et al., 2009). Although *S. aureus* was not typically associated with disease in poultry ~50 years ago, there has been pressure to adapt, thus leading to the emergence of *S. aureus*-

associated diseases in poultry. Mobile genetic elements (MGEs) facilitate horizontal gene transfer and were identified in the *S. aureus* recovered from poultry sources, but were not present in the *S. aureus* strains recovered from humans (Lowder et al., 2009). Perhaps the unique MGEs are responsible for the host-specific pathogenesis of select *S. aureus* strains affecting commercial poultry. Additionally, severe *S. aureus* contamination in the hatchery may induce pneumonia further validating the need for control at the hatchery level (Smyth et al., 2001). Other investigators have also speculated that *S. aureus* on the hands of hatchery and parent flock personnel may contribute to increased *S. aureus*-associated skeletal diseases in broiler chickens (Rodgers et al., 1999).

Neonatal broiler chicks are far more susceptible to *Salmonella* colonization, with susceptibility decreasing as the GIT microflora mature. The first critical point for horizontal transmission of *Salmonella* to occur is at the hatchery level. As previously mentioned, *Salmonella* spp. readily penetrate the eggshell (Padron, 1990). Successful eggshell penetration by *Salmonella* does not necessarily have to occur during embryogenesis. For example, Cason et al. (1993) demonstrated that initial *Salmonella* recovery from yolk sacs, GIT, and chick rinses remained low until the onset of pipping (Cason et al., 1993). This suggests that oral ingestion of the bacterium during the pipping process was sufficient enough to cause infection. Although the oral route has been thought to be the primary route of infection for *Salmonella*, evidence suggests that the respiratory route should be considered as a viable portal of entry for *Salmonella* (Kallapura et al., 2014a; Kallapura et al., 2014b). This is critical because bioaerosols are generated throughout production in commercial poultry operations. Cason et al. (1994) demonstrated that horizontal transmission of *Salmonella* occurs during the hatching phase by comingling seeders embryos, or embryos directly inoculated

with *Salmonella* at DOE18, with non-challenged, naïve embryos in a hatch cabinet. *Salmonella* was recovered from air samples collected from the hatcher environment and the GIT of non-challenged contact chicks at hatch (Cason et al., 1994). Cross-contamination may also occur during the post-hatch phase during handling, transport, and placement at the farm. For example, in one study, infecting 5% of the population with 10^2 CFU of *Salmonella* Typhimurium (seeders/sentinels) at hatch was sufficient to contaminate 56.7% of the non-infected counterparts within the same pen (Byrd et al., 1998). This suggests that low-level *Salmonella* contamination at the hatchery level may increase the risk of horizontal transmission at the flock level. Furthermore, salmonellae have evolved mechanisms to evade host defenses to establish colonization and promote tolerance (Kogut and Arsenault, 2015). In the absence of stress, the infection can persist in asymptomatic carriers and remain undetectable. Although susceptibility to *Salmonella* infection decreases with age, stressful events, such as feed withdrawal, promote litter pecking and coprophagic behavior, increasing the prevalence of *Salmonella* in the crop of broiler chickens at processing (Corrier et al., 1999). Thus, it is imperative to limit horizontal transmission of *Salmonella* during the neonatal period.

Fungal contaminants, such as *Aspergillus* spp. are ubiquitous in commercial poultry hatcheries (Thermote, 2006; Gehan, 2009; Smith and Rehberger, 2018). *Aspergillus fumigatus* is the most common cause of aspergillosis in poultry (Arne et al., 2011). A single *Aspergillus fumigatus* hyphae produces thousands of hydrophobic conidia (spores) that are readily dispersed into the environment (Arne et al., 2011). Inhalation of *Aspergillus fumigatus* spores has been associated with respiratory mycosis, or brooder pneumonia (O'Meara and Chute, 1959; Sheldon and Brake, 1991). These fungi degrade the cuticle of the eggshell and increase the likelihood of invasion during embryogenesis (Board and Halls, 1973; Board and Tranter, 1986). Application

of *Aspergillus fumigatus* spores in a wet suspension or dry suspension increased embryo contamination and incidence of aspergillosis (Wright et al., 1960). Huhtanen and Pensack (1967) showed that washing eggs with water contaminated with *Aspergillus fumigatus* spores prior incubation markedly reduced hatchability. Moreover, *Aspergillus fumigatus* conidia can replicate in the air cell, which is inaccessible to any fungicidal compounds applied during the hatching phase (Williams et al., 2000). The egg yolk in non-viable embryonated eggs also serves as a nutritive source for *Aspergillus fumigatus* (Williams et al., 2000).

The 21-day embryonic period makes up 28% of the entire lifespan of a modern commercial 52-day-old broiler chicken. It is important to limit transmission of opportunistic pathogens during embryogenesis. Although the microbial bloom during the hatching phase has been controlled with formaldehyde, efficacious alternatives to formaldehyde are needed that favor colonization by beneficial bacteria and improve poultry health.

Formaldehyde fumigation

Formaldehyde is a byproduct of cellular metabolism and detoxification has been shown to be important for metabolic processes (Burgos-Barragan et al., 2017). However, exogenous formaldehyde is a colorless, irritant gas with cytotoxic activity. Due to its solubility in water and biocidal properties, formaldehyde is used as a disinfectant in commercial settings (Swenberg, 2013). The first published report of formaldehyde application in commercial hatcheries was in 1908 (Pernot, 1908). For decades, formaldehyde fumigation of hatching eggs has been recommended to control the microbial load in hatching environments (Funk and Irvin, 1955).

Formaldehyde fumigation has been shown to reduce the bacterial load on the surface of eggshells by 99% (Williams, 1970) and has been used to fog hatching eggs prior to incubation or applied into the hatch cabinet environment during late embryogenesis to control the microbial

bloom (Sheldon and Brake, 1991). The fumigant is typically applied by diffusion of 37% formalin alone or in combination with potassium permanganate inside the cabinet at a single time point or by controlled infusion (Steinlage et al., 2002). Steinlage et al. (2002) evaluated the application of 37% formalin applied as a constant rate infusion (CRI, 1mL/hour over 12h period) as compared to the traditional method of a single dose application of formaldehyde (12mL administered at one time point every 12h). The maximum concentration of formaldehyde in the environment was lower with CRI at 20ppm versus 102ppm with the single application of formaldehyde. The effects of each fumigation method on circulating aerobic bacteria in the hatch cabinet, hatchability, and early performance were evaluated and compared to a non-treated control, which received water in lieu of the fumigant In this study, both formaldehyde fumigation methods reduced circulating aerobic bacteria in the hatching environment at DOE20 compared to treatment with water, but the single application of formaldehyde markedly reduced aerobic bacteria in the hatching environment compared to the non-treated and CRI hatchers, and hatchability was improved as a result of formaldehyde fumigation (Steinlage et al., 2002). Although contamination increased because of *in ovo* injection in this study, formaldehyde fumigation reduced the microbial load in the hatching environment and potentially eliminated microorganisms capable of penetrating eggshells that are lethal to embryonic development. CRI of formaldehyde was effective and likely reduced peak exposure to formaldehyde for neonates and hatchery workers by 10.2-fold. Similar to these results published by Steinlage and coworkers (2002), formaldehyde applied by CRI in commercial hatch cabinets reduced circulating aerobic bacteria 4h before hatch pull at DOE21 more readily than a single administration of 37% formalin at transfer from incubator to hatch cabinet (Kim and Kim, 2010).

Formaldehyde fumigation reduced circulating coliforms in the hatching environment, which reduced horizontal transmission and enteric colonization at hatch (Graham et al., 2018; Graham et al., 2021). However, formaldehyde fumigation has been associated with tracheal epithelial damage and mucosal sloughing in neonatal chicks (Sander et al., 1995; Zulkifli et al., 1999; Hayretdag et al., 2006; Maharjan et al., 2017). At hatch, neonatal chicks are highly susceptible to colonization by respiratory pathogens due to the inherent architecture of the avian respiratory system because the bronchial-associated lymphoid tissue and the immune system do not functionally mature until at least 6 weeks-of-age (Reese et al., 2006). The avian respiratory tract has been suspected to be a portal of entry for enteric pathogens, including *Salmonella* enterica (Kallapura et al., 2014a; Kallapura et al., 2014b). Hence, an insult to the tracheal epithelium, when the neonatal chick is already predisposed to invasion and colonization by respiratory and enteric pathogens, should be avoided.

In 2011, formaldehyde was listed as a known carcinogen by the National Institute of Environmental Health and Safety. In addition to the potential carcinogenic properties of formaldehyde, other negative aspects have been identified (Sander et al., 1995; Zulkifli et al., 1999; Johnson, 2018). Although the application of formaldehyde during the hatching period effectively reduced aerobic bacterial contamination in commercial hatch cabinets (Kim et al., 2010; Graham et al., 2018), it has been shown that the efficacy of formaldehyde fumigation decreases as contamination increases (Magwood, 1964). Additionally, formaldehyde is not selective and eliminates both beneficial and pathogenic organisms. During late embryogenesis, the fumigant has a limited effect on endogenous microorganisms inside the egg (Williams, 1970; Graham et al., 2021). The impact of formaldehyde fumigation during late embryogenesis on performance has also been investigated. Zulkifli et al. (1999) demonstrated that feed conversion

was negatively affected due to formaldehyde exposure. Alternatively, CRI of formaldehyde or a single administration of formaldehyde every 12h marginally improved feed conversion ratio (FCR) but did not significantly affect body weight gain (BWG) from DOH to day 14 (Steinlage et al., 2002). Mahajan et al. (2017) also reported no effects of CRI of formaldehyde on early performance. Contradictory to previous reports, CRI of formaldehyde during late embryogenesis markedly reduced BWG from DOH to day 10 compared to the non-treated control group (Johnson, 2018).

Although formaldehyde effectively controls the circulating microorganisms in the hatching environment, there are no benefits for beneficial pioneer colonization. With the removal of antibiotic growth promoters and the rising concerns regarding antimicrobial resistance, a multifactorial approach to promote early colonization by beneficial microorganisms and control the microbial bloom in the hatching environment without the use of carcinogenic formaldehyde will be essential.

Methods to monitor hatchery sanitation

Controlling pathogens at the hatchery level is critical. Evidence of contamination at the farm level suggests that the hatchery could serve as a primary source of contamination (Wales and Davies, 2020). During the hatching phase, bioaerosols and dust are generated and dispersed by the ventilation system in the hatch cabinet (Mitchell et al., 2002). These bioaerosols circulate in the hatch cabinet, contaminating the environment, equipment surfaces, and fluff, as well as having the potential to affect late embryonic development and neonatal health. To prevent disease transmission and guarantee that disinfection measures are correctly conducted, routine hatchery hygiene monitoring must be implemented. Employee compliance can be improved by

using simple microbiological techniques, such as fluff sampling and swabbing of equipment surfaces.

Since the late 1950s, fluff samples have been collected from hatch cabinets to assess the efficacy of sanitization procedures in commercial hatcheries (Wright et al., 1959). During the hatching phase, fluff and dander accumulates in the hatching environment and have been shown to contain 4-8 logs of bacteria/g of fluff (Furata & Maruyama, 1981). Based on the microbial recovery from fluff samples, a rating system was developed to assess the quality of disinfection and fumigation procedures for a particular commercial hatchery (Wright et al., 1959). Magwood (1962) plated hatcher fluff samples in duplicates both pre and post-formaldehyde fumigation and applied Wright's rating system. Duplicates were plated to assess the level of variability within a single fluff sample and bacterial and fungal recovery from fluff samples were lower after formaldehyde fumigation. However, both pre- and post-fumigation, the microbial load in the hatcheries with unsatisfactory ratings remained significant (Magwood, 1962). The rating system developed by Wright (1959) to assess hatching sanitation practices has been utilized in other investigations (Magwood, 1962; Soucy et al., 1983). Other investigators also confirmed that fumigation of hatching eggs reduced microbial recovery from fluff collected from the hatch cabinet (Nichols et al., 1967).

The open-agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) as well as air sampling machines (Andersen, 1958) have been used to evaluate airborne contamination in the commercial hatcheries. For the open-agar plate method, the lid of the petri dish is simply removed, and the agar is exposed to the hatch cabinet environment for a short duration which differs based on the selective nature of the agar media used. Aerosol sampling machines have been investigated as alternatives to the conventional open agar plate method to

assess the quality of hatcher sanitation procedures (Chute and Gershman, 1960; Gentry, 1962). Gentry (1962) sampled various locations in a commercial hatchery using the open-agar plate method and the Anderson air sampler (Anderson, 1958) to compare the level of sensitivity for both bacterial and fungal recovery. For a 30 second period, the select environment was sampled using the Anderson air sampler (equated to 0.5 cubic ft) or open agar plates (Gentry, 1962). The Anderson air sampler proved to be the more sensitive method based on overall microbial recovery, specifically using non-selective agar. However, the increased volume of air was sampled with the Anderson sampler versus the inert surface of the agar when using the open-agar plate method, which was reflected by microbial recovery. The volume of air sampled using air sampling machines far exceeded the amount of volume sampled by the open-agar plate method when exposed to the environment for the same duration. These differences must be considered when comparing the two methods as increased time of exposure could negate sensitivity differences.

Magwood and Mar (1964) assessed the level of airborne and surface contamination in four commercial hatcheries to determine if aerosol and surface contamination was correlated in a commercial setting. The hatchery environment was sampled to determine airborne contamination, while surfaces in the hatchery, specifically the floors and tables, were swabbed and directly plated on agar media (Magwood and Mar 1964). The authors suggested that direct swabs of select surfaces in the hatchery would be as equally reflective of the level of sanitation as air or fluff samples and was a simpler technique to implement.

The microbial load within the hatch cabinet has been shown to increase with the rise in humidity as chicks or turkey poults begin to hatch (Magwood, 1964). In this study, it was determined that airborne contamination was reflected by eggshell and hatcher surface

contamination. Furthermore, it was shown that microbial recovery was lower for hatcheries with adequate sanitation practices while highly contaminated hatcheries had higher microbial loads from hatching cabinet sampling, (Magwood, 1964). These results indicate that horizontal surfaces could be sampled to assess hatchery sanitation procedures implemented to disinfect equipment and control the microbial load in the hatching cabinet. Berrang et al. (1995) reported that more salmonellae were recovered from commercial broiler chick hatch cabinets with the open agar plate enrichment method compared to the air sampling machine. However, recovery of *Enterobacteriaceae*, an indicator of fecal contamination, was increased in samples collected with the air sampling machine compared to the direct open-agar plate method without further enrichment (Berrang et al., 1995). Thus, sampling method, duration of sampling, sample port location, ventilation system, and type of media used for sampling influence microbial recovery from the hatching environment.

In one study, *Salmonella* was recovered from up to 75% of samples collected from commercial hatchery equipment or eggshell fragments recovered from the hatching cabinet (Cox et al., 1990). Shell membranes and chick rinses sampling has also been used to assess *Salmonella* Typhimurium contamination in an artificial challenge hatcher model using infected embryonated seeders (Cason et al., 1993). In this study, chick rinse samples remained *Salmonella*-negative until the onset of pipping at DOE19. Previous studies have shown that salmonellae are rarely isolated from eggs (Olesiuk et al., 1969), but the increased percentage of *Salmonella*-positive chicks at hatch suggest moderate replication and dispersion of the pathogen within the hatch cabinet environment. Bailey and coworkers (1998) showed that placement of artificially infected seeder eggs (3 of 200 eggs total, 1.5%) resulted in the colonization of 98% of non-challenged contacts with *Salmonella* at 7 days-of-age. Even though salmonellae presence

may appear to be minimal based on microbiological sampling at DOH, infected chicks horizontally transmit the pathogen when comingled with non-infected counterparts (Byrd et al., 1998).

The incidence of *Salmonella* in commercial hatcheries for other gallinaceous species, including geese, has been documented. Chao et al. (2007) collected fluff samples, hatch cabinet surface swabs, and shell membranes post-hatch from goose hatcheries and recovered *Salmonella* from ~36% of the fluff samples, 27% from hatch cabinet swabs, and 86% from shell membranes post-hatch. Alternatively, shell membrane samples collected from commercial chicken hatcheries had a significantly lower incidence of *Salmonella* (Chao et al., 2007). The authors postulated that the use of formaldehyde in the chicken hatcheries was associated with a greater level of sanitation observed compared to the other poultry hatcheries evaluated. In another study, Zhao et al. (2019) isolated *E. coli* from 47 fluff samples collected from commercial hatcheries that contained less virulence-associated genes than the 20 APEC isolates evaluated (Zhao et al., 2019). However, these samples were collected from formaldehyde-fumigated hatch cabinets and do not provide insight regarding the natural level of contamination in the absence of formaldehyde fumigation.

If hatchery disinfection and sanitation practices are not effective, it will be reflected by hatchability and overall chick quality. Extensive contamination at the hatchery level promotes cross-contamination of strict and opportunistic pathogens during the hatching phase and at the farm. Transmission at the hatchery level can be costly to poultry producers due to reduced performance and potential transmission of foodborne pathogens to consumers. Thus, sampling of the hatching environment (agar plates, aerosol sampling machines, equipment surfaces) and waste generated during the hatching process (fluff, eggshell fragments, post-mortem chick

rinses) can provide insight regarding sanitation procedures. These techniques can be utilized to evaluated potential alternatives to formaldehyde fumigation to control the microbial load in the hatching environment.

Alternatives to formaldehyde fumigation

Research efforts to identify alternatives to formaldehyde to mitigate pathogen transmission of pathogens in poultry hatcheries have been reviewed (Berrang et al., 2000). Alternatives to formaldehyde fogging or fumigation of hatch cabinets should have minimal effects on eggshell integrity and hatchability and also inhibit penetration or replication of microorganisms on the eggshell or within the hatching environment. Eggshell surface contaminants obtained at the breeder facility or during transport should be eliminated prior to incubation to prevent cross-contamination in the hatchery. Whistler and Sheldon (1989) demonstrated that ozone fumigation reduced bacterial growth similar to formaldehyde fumigation when applied for 2 minutes in a prototype setter. Another potential sanitizer, hydrogen peroxide, reduced the microbial load on the surface of the eggshell with minimal effects on structural integrity of the eggshell (Scott et al., 1993; Sander and Wilson, 1999). Bailey et al. (1996) showed that a hydrogen peroxide mist at a concentration of 2.5% limited cross-contamination of *Salmonella* during late embryogenesis compared to UV light and ozone treatment. In this study, the incidence of *Salmonella*-positive eggshells collected at hatch and cecal samples at 7 days-of-age was reduced compared to ozone, UV light, and the challenged control. In a follow up study, efficacy of hydrogen peroxide improved when applied by immersion compared to spray application to the eggshells, but effectiveness was diminished if applied after sufficient *Salmonella* contamination occurred regardless of application method (Cox et al., 1998). More recently, application of 30% hydrogen peroxide by vaporization

reduced total aerobic bacterial recovery from the eggshell and did not impact hatchability or early performance (Keita et al., 2016). Thus, contamination prior to treatment should be limited. Additionally, aerosolized application of sanitizers would be more feasible than immersion in commercial hatchery operations.

Eggshell surface contamination was reduced after application of hydrogen peroxide in conjunction with UV light exposure, referred to as an Advanced Oxidation Process (Wells et al., 2010; Gottselig et al., 2016). The combined treatment only reduced the incidence of *Salmonella* on the surface of the eggshell, and did not prevent bacterial penetration of the eggshell (Gottselig et al., 2016). The incidence of *Salmonella* in the GIT of chicks and early performance were not reported in this study. However, Rehkopf et al. (2017) showed that UV light exposure and hydrogen peroxide treatment to eggshell surfaces prior to incubation reduced *Salmonella* enteric colonization at DOH and at 14 days-of-age. More recently, Melo et al. (2019) evaluated UV irradiation, ozone fumigation, hydrogen peroxide spray, or peracetic acid spray as potential alternatives to paraformaldehyde fumigation for hatching eggs. UV treatment and spray application of peracetic acid more effectively reduced total aerobic bacteria on eggshells compared to all treatment groups, including formaldehyde (Melo et al., 2019). However, both UV and peracetic acid treatment actually increased total aerobic bacteria and *Enterobacteriaceae* recovered from yolk samples 24h post-hatch as compared to non-treated controls and formaldehyde treated group (Melo et al., 2019). Another alternative sanitizer, chlorine dioxide was applied at a concentration of 0.3% to hatching eggs at 18 days of embryogenesis but did not effectively reduce the microbial load on the eggshell compared to formaldehyde and had no effect on performance (Maharjan et al., 2017). Introduction of an artificial challenge and

additional sampling would provide more insight as to the effectiveness of candidate disinfectants.

Some additional naturally-derived candidates have also been evaluated. Eggshells were treated by spray application of grain alcohol, clove essential oil, or an ethanolic extract of propolis, a component of bee hives, and compared to sanitizing eggshell with paraformaldehyde prior to incubation (Oliveira et al., 2020a). In this study, application of the ethanolic extract of propolis negatively impacted hatchability of fertile eggs and significantly increased late embryonic mortality compared to the other treatment groups, which was likely associated with impaired gas exchange and moisture loss during incubation. Similar to paraformaldehyde fumigation, spray application of clove essential oil eliminated *Enterobacteriaceae* on the eggshell surface and had no apparent effect on integrity of the eggshell (Oliveira et al., 2020a; Oliveira et al., 2020b). Pyrazines are naturally-occurring organic nitrogen-containing ring structures which can be chemically synthesized or obtained by microbial fermentation (Mortzfeld et al., 2020). Alkyl pyrazines are typically used as flavoring agents or as fragrances) and have been shown to have antimicrobial activity (Schock et al., 2018). Application of a volatile organic compound, an alkylated pyrazine (5-isobutyl-2,3-dimethylpyrazine), reduced viable microorganisms on the surface of the eggshell (Kusstatscher et al., 2017). However, since overall eggshell contamination was low and the effects of the treatment on eggshell quality and chick viability were not assessed, future studies are required to validate efficacy and feasibility of alkylated pyrazine.

The effect of spray application of probiotics into commercial hatch cabinets as a potential replacement for formaldehyde fumigation has also been preliminarily investigated. Although the Gram-negative bacterial bloom was elevated in probiotic-treated hatchers,

probiotic application effectively reduced GIT coliforms of neonatal chicks compared to chicks placed in formaldehyde fumigated hatch cabinets (Graham et al., 2018). Compared to formaldehyde fumigation, probiotic-application would not be expected to inhibit the microbial bloom in the hatching environment, but could perhaps displace the opportunistic pathogens in the hatching environment thereby promoting colonization by beneficial bacteria.

In future studies, the ability of candidate alternatives should be evaluated under artificial challenged conditions to assess the impact on microbial load in the hatching environment and enteric colonization at hatch. Sampling the environment in the hatch cabinet during the hatching phase would provide insight on the microbial load compared to traditional formaldehyde fumigation. Furthermore, eggshell quality may be compromised due to treatment and have detrimental effects on embryonic development and should be evaluated. Although chemically and naturally-derived sanitizers reduced the microbial load on the eggshell and potentially limited horizontal transmission of pathogens in the hatchery setting, these compounds lack the ability to competitively exclude pathogens. Since formaldehyde non-selectively acts on microorganisms on surfaces or in the environment eliminating both beneficial and pathogenic microorganisms, artificial introduction of probiotic candidates during the hatching phase may be a promising method to enhance enteric colonization by beneficial pioneer colonizers.

Conclusion

Formaldehyde effectively controls the microbial load on the surface of eggshells and in the environment, but identification of alternatives to formaldehyde represent an opportunity for improving the health and performance of postnatal chicks. Exposure to opportunistic pathogens during the neonatal period can be costly to poultry producers and reduction of infection and impact remains a worthy goal. Since the level of natural contamination is inherently variable,

reproducible laboratory challenge models are essential for development and validation of alternatives to formaldehyde fumigation to control the microbial load in commercial hatch cabinets. Artificial challenge models to simulate exposure to hatchery-relevant pathogens during the neonatal period have been employed, including direct application of the challenge to eggshells (spray, immersion, etc.), *in ovo* application, and horizontal transmission models. Additionally, prophylactic use of antibiotics in the feed has previously been used to control bacterial infections and improve growth performance. Emergence of multi-drug resistant strains of bacteria and concern for human health has limited the use of antibiotics in commercial poultry production. Thus, a multifaceted approach to control the microbial bloom in the hatching environment and promote pioneer colonization by beneficial organisms that is applicable to the poultry industry is a major unmet opportunity.

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Chapter III. Development of a novel *in ovo* challenge model for virulent *Escherichia coli* strains

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Abstract

During the hatching process, chicks are exposed to opportunistic and/or pathogenic organisms, such as virulent or avirulent *Escherichia coli.* Virulent *E. coli* strains have not been feasible for induction of neonatal colibacillosis via *in ovo* challenge due to high embryonic mortality. In this manuscript, we describe the addition and co-administration of the bacteriostatic antibiotic tetracycline to a virulent *E. coli* challenge culture, improving hatchability and livability of seeder chicks while allowing robust horizontal transmission in the hatching cabinet to contact chicks. Experiment 1 consisted of 3 trials. Experiment 1, trial 1 was conducted to determine an effective ratio of *E. coli* challenge and tetracycline dose to be utilized in the seeder model. Trial 2 and 3 were conducted to evaluate the transmission of *E. coli* from seeder to contact chicks. Experiment 2 consisted of 3 independent 7-day trials where body weight gain (BWG), mortality, and selected enteric bacterial recovery was evaluated. In trials 1-3, significantly ($P < 0.05$) more Gram-negative bacteria were recovered from whole gut samples (GIT) vs. negative controls on day-of-hatch, from both seeder and contact chicks. At d7 in trial 1, contact chicks had significantly ($P < 0.05$) more Gram-negative bacteria recovered from the GIT than the negative control, but not in trials 2 and 3. Presumptive lactic acid bacterial recovery was elevated in contact and seeder chicks compared to the negative control in all 3 trials. Contact-challenge caused a significant ($P < 0.05$) reduction in BWG in 2 out of 3 trials at d7 and there was a significant ($P < 0.05$) increase in mortality as compared to the negative controls in all trials. These data suggest that co-administration of a virulent *E. coli* strain with tetracycline allows for hatch of direct challenged chicks and effective horizontal transmission to contact chicks during the hatching process, as evidenced by reduced d7 performance and altered selected enteric bacterial recovery.

Key words: *Escherichia coli, in ovo,* broiler, hatchers, model

Introduction

Escherichia coli, a Gram-negative bacterium present in the gastrointestinal tract of poultry, can result in substantial economic losses in the industry depending on strain virulence (Kabir et al., 2010). Chicks may be exposed to opportunistic pathogens, such as *E. coli,* in commercial hatcheries (Thermote et al., 2006). Pathogens can be vertically transferred by contamination of eggs at oviposition (Berchieri et al., 2001) and bacteria present in the hatcher setting can penetrate the eggshell during incubation (Williams et al., 1967; Williams et al., 1968), ultimately resulting in colonization and horizontal transfer of the bacterial organism during the commonly-observed microbial bloom during hatching. Colonization by avian pathogenic *E. coli* (APEC) strains can result in septicemia, omphalitis, and high mortality in the field (Moulin et al., 1999). Thus, exposure to virulent *E. coli* strains during the incubation and hatching process can be detrimental to the health of neonatal chicks. Infected, or directly-challenged chicks (seeders) have previously been used to horizontally transmit challenge to non-challenged chicks (contacts) within the same environment (Weinack et al., 1981; Montgomery et al., 1998; Jarquin et al., 2007) modeling commercial conditions.

In a preliminary experiment, a virulent *E. coli* strain administered by *in ovo* injection in 200μL saline containing $1x10^2$ cfu/embryo or $1x10^3$ cfu/embryo at d18, 19, or 19.5 of embryogenesis, resulted in 60-90% embryonic mortality in challenged embryos on day-of-hatch (data not shown). Considering tetracyclines are bacteriostatic antibiotics that can inhibit protein synthesis of *E. coli* (Chopra et al., 2001; Schnappinger et al., 1996), we evaluated co-administration of a tetracycline and *E. coli in ovo* in a seeder challenge model in the present manuscript.

Materials and Methods

E. coli culture and challenge

A lactose negative *E. coli* strain serotype O2 known to cause respiratory disease and mortality in both chickens and turkeys (Huff et al., 1998; Huff et al., 2002; Huff et al., 2003) was used in these experiments. In these studies, 100µL of *E. coli* was removed from a frozen aliquot and added to 10mL of tryptic soy broth (Tryptic soy broth, cat. no. 90000-378, VWR, Suwanee, GA 30024). The culture was incubated at 37C for 18 h. Post incubation, bacterial cells were washed with sterile 0.9% saline by centrifugation at 1,800 × *g* for 15 min and reconstituted in saline. The wash procedure was completed 3 times. *E. coli* colony-forming units (cfu) enumeration was determined by serial dilution and plating on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024) to determine stock concentration and then cells were held overnight at 4C. Approximately 16 h later, the culture was serially diluted to desired cfu concentration for *in ovo* challenge (d 19 of embryogenesis) with the selected dose of tetracycline hydrochloride (tetracycline hydrochloride, cat. no. 64755, Sigma, St. Louis, MO 63103). Actual *E. coli* challenge dose (cfu/mL) was confirmed as described above and reported. Approximate minimal inhibitory concentrations of tetracycline were determined *in vitro* (data not shown) and then adjusted in subsequent trials based on *in vivo* results.

Enumeration of Bacteria

For experiment 1 and 2, the whole gut (ventriculus to cecum) was aseptically removed and collected into sterile bags. Samples were weighed and homogenized and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold dilutions of each sample, from each group, were made in a sterile 96 well Bacti flat bottom plates and the diluted samples were plated on culture media to evaluate total number of lactic acid bacteria (LAB) on Man Rogosa Sharpe agar (Difco

Lactobacilli MRS Agar, cat. no. 90004–084, VWR, Suwanee, GA 30024) and Gram-negative bacteria, specifically lactose negative colony morphology (challenge strain is a non-lactose fermenter), on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024). All plates were incubated at 37C for 18 h and bacterial counts were expressed as Log_{10} cfu/g of sample.

Development of an in ovo seeder challenge model for co-administration of virulent E. coli strains and tetracycline. Experiment 1

The objective of experiment 1 was to determine the appropriate *E. coli* challenge and tetracycline dose for *in ovo* co-administration without excessive embryonic mortality but with effective horizontal transmission between seeder and contact chicks during the hatching phase. Experiment 1 consisted of 3 trials. In each trial, embryonated Ross 308 broiler hatching embryos were candled at 18d of incubation and placed into separate hatchers (G.Q.F. Manufacturing 1602N Hova-Bator Incubator with a circulating air fan kit) based on treatment group. On d 19 of embryogenesis, embryos were inoculated with 200µL of respective *E. coli* and tetracycline treatment into the amnion. On d21, dry chicks were removed from hatchers and hatchability and pre-hatch pull mortality was determined. In each trial, chicks alive at hatch pull (up to n=12 per group) were euthanized to evaluate MacConkey agar-recoverable bacteria as described above. In trial 1, selected concentrations of *E. coli* and tetracycline (200µL) were inoculated into the amnion of 19 d embryos (n=20/combo). *E. coli* and tetracycline combinations (3.7×10^2 cfu/mL *E. coli* + 1µg/mL tetracycline; 3.7×10^3 cfu/mL *E. coli* + 1µg/mL tetracycline; 3.7×10^3 cfu/mL *E. coli* + 9µg/mL tetracycline; 1.4×10^4 cfu/mL *E. coli* + 9µg/mL tetracycline; 1.4×10^4 cfu/mL *E. coli* + 90µg/mL tetracycline) were based on preliminary *in vitro* results (data not shown). In trial 2, 2 dose combinations (6.6×10^3 cfu/mL *E. coli* + 90µg/mL tetracycline; 6.6×10^3 cfu/mL

E. coli + 272µg/mL tetracycline) were evaluated (n=25 contact embryos, n=5 seeder embryos/treatment). In trial 3, 2 combinations (1.4 x 10^4 cfu/mL *E. coli* + 90µg/mL; 1.4 x 10^4 cfu/mL *E. coli* + 272µg/mL tetracycline) were evaluated (n=25 contact embryos, n=5 seeder embryos/treatment) with 2 replicate hatchers per *E. coli*/tetracycline combination. In both trial 2 and 3, seeders (n=5 seeders/hatcher or 16.67%/hatcher) were *in ovo* challenged with respective *E. coli*/tetracycline combinations on d19 and segregated into mesh bags (reusable mesh nylon netting, IDS, Amazon) in a hatcher containing non-challenged embryos (n=25).

Evaluation of effects of virulent E. coli exposure within the hatching environment on body weight gain, mortality, and bacterial colonization of the gastrointestinal tract. Experiment 2

The objectives of experiment 2 were to evaluate 7-day BWG, mortality (%), and bacterial colonization (day-of-hatch (DOH) and d7) of non-challenged controls and contact challenged chicks. Experiment 2 consisted of 3 replicate trials. In each trial, 18d Ross 308 embryos were candled and placed into separate hatchers (G.Q.F. 1550 Digital Cabinet Egg Incubator) based on treatment group. Hatchers (n=2/treatment) were housed separately to prevent possible cross contamination between treatments during hatch. On d19 of embryogenesis, seeder embryos (n=18 seeders/hatcher or 8.45%/hatcher) were inoculated with the respective *E. coli*/tetracycline treatment or 0.9% sterile saline (vehicle) via *in ovo* injection into the amnion and segregated into mesh hatching bags as described above (Experiment 1). On d21, dry chicks were removed from hatchers and hatchability was determined. In each trial, 12 chicks per group were euthanized to evaluate gastrointestinal composition on selective media as described above. Seeder challenge dose for trial 1 was 1x10^4 cfu/mL *E. coli* + 272µg/ml tetracycline, dose for trial 2 was 4.5x10^4 cfu/mL *E. coli* + 272µg/mL tetracycline, and dose for trial 3 was 3x10^5 cfu/mL *E. coli* + 272µg/ml tetracycline. In trial 1, both the negative control and contact challenge chicks were

weighed and allocated into 16 pens (n=20/pen). In trial 2 and 3, the negative control chicks were weighed and allocated into 8 pens (n=20/pen) and the contact challenged chicks were allocated into 16 pens (n=20/pen). Weight allocation on DOH was performed to normalize BW and prevent initial treatment effect on BW. Pen BW was determined at placement and on d7 to calculate BWG. Mortality was recorded for the duration of each trial (7-day trial period). Chickens were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean diet meeting the nutritional requirements for broilers recommended by Aviagen. All experiments and animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

Statistical Analysis

All data were subjected to one-way analysis of variance as a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). Data are expressed as mean ± standard error (SE). Significant differences ($P < 0.05$) among the means were further separated using Tukey's multiple range test for Gram-negative bacterial recovery (Experiment 1, Trial 1 only) or Student's t test for DOH and d7 data (Experiment 1, Trial 2, 3; Experiment 2, Trial 1, 2, 3) with pen as the experimental unit for BW data. Mortality was compared using the chi-squared test of independence to determine the significance ($P < 0.001$) for these studies (Zar, 1984).

Results and Discussion

Virulent *E. coli* strains, such as APEC, can invade the host via the respiratory tract, leading to septicemia and airsacculitis (Mouhlin et al., 1999). *In vitro* results show that APEC strains can invade chicken heterophils and macrophages by evading host immune defenses through virulence mechanisms (Mellata et al., 2002) suggesting involvement in immunosuppression. Under commercial conditions, chicks may be exposed to virulent *E. coli*

strains during hatch, indicating a need for a laboratory model allowing for evaluation of the effects of exposure during the hatching process. To determine the appropriate dose of tetracycline and *E. coli* for *in ovo* application, selected combinations of a virulent *E. coli* strain and tetracycline were applied *in ovo* (into the amnion) at d19 of embryogenesis (Experiment 1, Trial 1). On DOH, there was a significant increase in Gram-negative bacterial recovery from GIT samples from the low (1μg/mL) to high (100μg/mL) concentrations of tetracycline administered with varying concentrations of *E. coli* applied at d19 of embryogenesis (Table 1). The 1.4 x 10^4 cfu/mL *E. coli* + 90μg/mL tetracycline and 3.7 x 10^2 cfu/mL *E. coli* + 1μg/mL tetracycline treatment groups had 90% hatchability with significantly more presumptively Gram-negative bacteria recovered from the 1.4 x 10^4 cfu/mL *E. coli* + 90μg/mL tetracycline group (Table 1). The observed increase in Gram-negative enteric colonization suggests that residual tetracycline levels, probably due to dilution during administration into the amnion, and/or further dilution upon ingestion, is not a limiting factor for this model. The increased livability at hatch pull may be attributed to the higher inclusion of tetracycline allowing for ample colonization of the *E. coli* strain, whereas the lower inclusion of tetracycline resulted in diminished livability at hatch pull and lower Gram-negative recovery at hatch. Tetracycline, a bacteriostatic antibiotic (Schnappinger et al., 1996; Chopra et al., 2001), prevents active replication of *E. coli* at certain concentrations without having bactericidal effects. We hypothesize that through this mechanism, dilution of the tetracycline possibly occurs within the developing embryo, reducing concentrations below the minimal inhibitory concentration, thus allowing the challenge strain of *E. coli* to replicate. These data are consistent with the hypothesis that the higher selected tetracycline dose delayed *E. coli* replication until later in embryogenesis, allowing for improved hatchability, livability at hatch pull, yet allowing for increased enteric colonization on DOH.

Based on these data, two dose combinations, 6.1 x 10^3 cfu/mL *E. coli* + 90μg/mL tetracycline and 6.1 x 10^3 cfu/mL *E. coli* + 272μg/mL tetracycline, were evaluated in trial 2 (Table 2). A higher *in ovo* dose of tetracycline was evaluated as we hypothesized that it would positively affect seeder livability at hatch pull. In trial 2, no significant effects on seeder livability were observed when 272μg/mL tetracycline was co-administered with *E. coli* as compared to the 90μg/mL tetracycline + *E. coli* group. In trial 3, 2 doses (2 replicates/dose) of 1.4 x 10^4 cfu/mL *E. coli* + 90μg/mL tetracycline and 1.4 x 10^4 cfu/mL *E. coli* + 272μg/mL tetracycline, were evaluated with 2 replicate hatchers per treatment (Table 3). There was a numerical improvement in seeder hatchability in the 272μg/mL tetracycline group compared to the 90μg/mL tetracycline group and no effect on contact hatchability in trial 3. Additionally, contact chicks in the 90μg/mL tetracycline group had more Gram-negative bacteria recovered from GIT samples than the contacts in the 272μg/mL group. Due to the improvement in seeder livability, we chose to move forward with the 272μg/mL tetracycline dose in experiment 2. Seeder percentage within the hatchers was higher in experiment 1 (16.67%) than experiment 2 (8.45%) which is reflected in the variation in Gram-negative bacterial colonization between experiments.

In experiment 2, all trials exhibited a significant ($P < 0.05$) difference in Gram-negative (specifically lactose negative) recovery from GIT samples between the negative control and challenged chicks (seeders and contacts) on DOH (Table 4). On d7, there were more Gram-negative bacteria recovered from the contact-challenged group ($P < 0.05$) than the negative control only in trial 1. In trials 2 and 3, the lack of difference in Gram-negative recovery may be due to respiratory transmission that may have occurred as both the negative control and contact-challenged groups were housed in the same environment, although this was not specifically

evaluated. There was significantly ($P < 0.05$) more presumptive lactic acid bacteria recovered from contact-challenged GIT compared to the negative control group on DOH in all 3 trials (Table 4). A similar trend was observed where *in ovo* administration of *Citrobacter freundii* 97A4 at d18 of embryogenesis resulted in a significant increase in total lactic acid bacteria recovered from the lower portion of gastrointestinal tract 3 days post-hatch (Wilson et al., 2018).

Exposure to virulent *E. coli* strains can result in mortality up to 20% in the field (Moulin et al., 1999). In these trials, mortality was significantly ($P < 0.001$) higher in the contact-challenged chicks compared to the negative control group (Table 5). The elevated mortality in the contact-challenged is due to exposure to the virulent *E. coli* strain by seeder chicks during the hatching phase. However, negative control chicks in 2 of the 3 trials may have been colonized by the challenge strain due to respiratory exposure after placement. Elevated mortality in the negative control group may have been observed if the experimental period had been extended.

It has previously been shown that respiratory challenge at 7 days-of-age, with this *E. coli* strain, resulted in reduced body weight gain (BWG) in challenge controls compared to the negative controls (Huff et al., 2006). In the present study, only a significant ($P < 0.05$) difference in BWG was observed at d7 in 2 of the 3 trials (Table 5). It is well known that the eggshell can harbor extensive amounts of microorganisms (Gentry et al., 1972) resulting in potential exposure to pathogens during the hatch. The embryos used in these experiments were not from a specific pathogen free source flock, therefore, this may account for variation observed in BW between trials.

By co-administering *E. coli* and tetracycline *in ovo*, seeder chicks harboring the virulent *E. coli* strain were able to hatch and effectively transmit challenge to contact chicks within the hatching environment. This exposure resulted in elevated Gram-negative bacterial recovery at

hatch, decreased BWG, and increased 7-day mortality in the contact-challenge group compared to negative control group. Since the challenge strain used in these experiments is known to induce respiratory disease in poultry (Huff et al., 1998; Huff et al., 2002; Huff et al., 2003), histopathological evaluation of sections of the respiratory system and the gastrointestinal tract are being assessed. Additionally, in commercial hatcheries, chicks are exposed to Gram-negative bacteria and thus colonized on DOH (Graham et al., 2018). Studies aimed at identification of potential alternative methods of controlling horizontal transmission in the hatching environment are currently underway.

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Tables

Table 1. Effect of an *in ovo* challenge with selected *E. coli* and tetracycline combinations on hatchability and Gram-negative bacterial recovery (Experiment 1, Trial 1)

***E. coli* dose[1]**	**Tetracycline dose[1]**	**Hatch from shell (%)**	**Alive at hatch pull (%)**	**Gram-negative bacterial recovery (Log_{10})[2]**
3.7×10^2 cfu/mL	1µg/mL	18/20 (90)	7/20 (35)	7.78 ± 0.12^{b}
3.7×10^3 cfu/mL	1µg/mL	11/20 (55)	5/20 (25)	7.94 ± 0.15^{ab}
3.7×10^3 cfu/mL	9µg/mL	14/20 (70)	7/20 (35)	8.45 ± 0.22^{ab}
1.4×10^4 cfu/mL	9µg/mL	16/20 (80)	12/20 (60)	8.50 ± 0.15^{a}
1.4×10^4 cfu/mL	90µg/mL	18/20 (90)	12/20 (60)	8.56 ± 0.12^{a}

[1]200µL of respective *in ovo* treatment applied into the amnion at d19 of embryogenesis
[2]Gram-negative bacterial recovery consistent with challenge strain morphology. Data are expressed as Log_{10} cfu/g mean ± SE
[a, b] Indicates significant difference between doses ($P < 0.05$)
Hatched from shell and alive at hatch pull percentage reported in parentheses
n=20/treatment, although GIT samples were only collected from chicks alive at hatch

Table 2. Effect of a virulent *E. coli in ovo* seeder challenge on hatchability and horizontal transmission within the hatcher (Experiment 1, Trial 2)

Treatment[1]	Hatched from shell (%)	Alive at hatch pull (%)	Gram-negative bacterial recovery[2]
Contacts – no treatment	24/25 (96)	24/25 (96)	6.77 ± 0.59
6.1 x 10^3 cfu/mL *E. coli* + 90μg/mL tetracycline seeders	5/5 (100)	5/5 (100)	8.38 ± 0.36
Contacts – no treatment	22/25 (88)	22/25 (88)	6.53 ± 0.57
6.1 x 10^3 cfu/mL *E. coli* + 272μg/mL tetracycline seeders	5/5 (100)	5/5 (100)	8.10 ± 0.30

[1]Seeders: 200μL of respective *in ovo* treatment administered into the amnion at d19 of embryogenesis

[2]Gram-negative bacterial recovery consistent with challenge strain morphology. Data are expressed as Log_{10} cfu/g mean ± SE

Hatched from shell and alive at hatch pull percentage reported in parentheses

Contact (n=25) and seeder (n=5) embryos placed in same hatch cabinet

Table 3. Effect of a virulent *E. coli in ovo* seeder challenge on hatchability and horizontal transmission within the hatcher (Experiment 1, Trial 3)

Treatment[1]	Hatched from shell (%)	Alive at hatch pull (%)	Gram-negative bacterial recovery[2]
Contacts – no treatment	50/50 (100)	50/50 (100)	7.88 ± 0.39
1.4 x 10^4 cfu/mL *E. coli* + 90μg/mL tetracycline seeders	8/10 (80)	7/10 (70)	8.67 ± 0.07
Contacts – no treatment	49/50 (96)	49/50 (96)	5.88 ± 0.37
1.4 x 10^4 cfu/mL *E. coli* + 272μg/mL tetracycline seeders	10/10 (100)	9/10 (90)	8.33 ± 0.10

[1]Seeders: 200μL of respective *in ovo* treatment administered into the amnion at d19 of embryogenesis
[2]Gram-negative bacterial recovery consistent with challenge strain morphology. Data are expressed as Log_{10} cfu/g mean ± SE
Hatched from shell and alive at hatch pull percentage reported in parentheses
n=2 replicates/treatment; 25 contacts and 5 seeders/replicate

Table 4. Effect of a virulent *E. coli in ovo* seeder challenge on Gram-negative bacteria and presumptive lactic acid bacteria recovered from GIT (Experiment 2)[1, 2]

Treatment	Gram-negative recovery DOH (Log_{10} cfu/g)	Presumptive lactic acid bacteria DOH (Log_{10} cfu/g)	Gram-negative recovery d7 (Log_{10} cfu/g)
Trial 1			
Negative Control	0.00 ± 0.00 [b]	0.25 ± 0.25 [b]	1.66 ± 1.09 [b]
In ovo Challenge Contacts	2.30 ± 0.90 [a]	3.87 ± 1.00 [a]	6.76 ± 0.24 [a]
In ovo Challenge Seeders	7.34 ± 0.78 [a]	2.06 ± 0.84 [b]	ND
Trial 2			
Negative Control	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	7.22 ± 0.17 [a]
In ovo Challenge Contacts	4.32 ± 0.91 [a]	5.17 ± 1.01 [a]	7.43 ± 0.12 [a]
In ovo Challenge Seeders	8.07 ± 0.29 [a]	8.32 ± 0.26 [a]	ND
Trial 3			
Negative Control	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	5.64 ± 0.79 [a]
In ovo Challenge Contacts	3.91 ± 0.81 [a]	1.84 ± 0.84 [a]	6.34 ± 0.33 [a]
In ovo Challenge Seeders	8.51 ± 0.36 [a]	0.58 ± 0.58 [b]	ND

[1]Data expressed as mean ± SE

[2]ND-Indicates no data as seeders were not placed at hatch

[a, b] Indicates significant difference between negative control and treatment group within columns ($P < 0.05$)

Table 5. Effect of a virulent *E. coli* seeder challenge on BW, BWG, and 7-day mortality in broiler chickens (Experiment 2)[1]

Treatment	Average BW DOH	Average BW d7	BWG d0-d7	Mortality (%)
Trial 1				
Negative Control	46.05 ± 0.04 [a]	201.13 ± 2.18 [a]	155.08 ± 2.19 [a]	0/320 (0)
In ovo Challenge Contacts	45.65 ± 0.04 [b]	198.77 ± 2.23 [a]	153.12 ± 2.24 [a]	24/320 (7.5) [*]
Trial 2				
Negative Control	40.27 ± 0.07 [a]	171.15 ± 2.71 [a]	130.61 ± 2.69 [a]	2/160 (1.25)
In ovo Challenge Contacts	40.03 ± 0.07 [b]	164.56 ± 2.52 [a]	116.93 ± 2.63 [b]	50/320 (15.63) [*]
Trial 3				
Negative Control	41.01 ± 0.03 [b]	175.89 ± 1.97 [a]	134.60 ± 1.88 [a]	1/160 (0.63)
In ovo Challenge Contacts	41.30 ± 0.03 [a]	161.31 ± 2.68 [b]	111.81 ± 1.91 [b]	51/320 (15.94) [*]

[1]Data expressed as mean ± SE
[a,b] Indicates significant difference between columns ($P < 0.05$)
[*] Indicates significant differences in mortality ($P < 0.001$)
Mortality percentage reported in parentheses

Chapter IV. Development of a wild-type *Escherichia coli* environmental bloom model to evaluate alternatives to formaldehyde fumigation in broiler chicken hatch cabinets

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Abstract

Horizontal transmission of opportunistic *Escherichia coli (E. coli)* during hatch can have detrimental effects on early performance, particularly as pioneer colonizers. Commercially, formaldehyde is often applied in the U.S. to combat the bacterial bloom that occurs inside of the hatching environment. The purpose of these experiments was to develop a replicable *E. coli* horizontal challenge model to evaluate alternatives to formaldehyde sanitation applied to the hatching environment. In Exp 1 two trials were conducted for two wild-type (WT) *E. coli* isolates (I1 or I2) to determine the appropriate *in ovo* challenge dose and day of embryogenesis (DOE) for challenge administration. In Exp 1 Trial 1, the most appropriate inoculation dose and time point were determined to be 10^2 CFU/embryo on DOE19. Exp 1 Trial 2 evaluated whether placement of seeder (direct-challenged) embryos with contact (indirect-challenged) embryos during hatch affected contact hatchability. Trial 2 showed no differences in hatchability between groups. A 7-day experiment (Exp 1 Trial 2) was conducted to evaluate the effects of I1 or I2 on horizontal transmission, Gram-negative bacterial (GNB) recovery from the gastrointestinal tract (GIT), and impact on body weight gain (BWG). Compared to the negative control, seeder and contact chicks challenged with I1 or I2, we observed increased ($P < 0.05$) GNB recovered from GIT on day-of-hatch. There was a marked ($P < 0.05$) reduction in 7-day BWG between the I1 indirect-challenged group and the negative control group. To further validate the model, two 7-day trials (Exp 2, Exp 3) were conducted to evaluate the effects of formaldehyde fumigation on coliform recovery from the hatching environment and on early performance using I1 for the challenge. I1 positive control hatchers had increased levels of circulating coliforms compared to the negative control and formaldehyde-treated hatchers, although there was no significant impact on performance induced by challenge or formaldehyde treatment in Exp 2 or Exp 3. These data

provide a potential model for investigations related to horizontal transmission of WT *E. coli* at a low-dose at DOE19 to promote simulated commercially-relevant bacterial blooms under laboratory conditions.

Keywords: *Escherichia coli, in ovo,* broiler, hatchers, model

Introduction

Commercial hatcheries serve as microbial reservoirs and promote the proliferation of opportunistic pathogens, such as antimicrobial-resistant *Escherichia coli* (Osman et al., 2018). Vertical transmission between the flock (breeder) and progeny (broiler) has been correlated for avian pathogenic *E. coli* (APEC) strains (Giovanardi et al., 2005). Exposure to apathogenic microorganisms may have little risk; however, APEC strains cause extra-intestinal lesions resulting in systemic disease associated with elevated 7-day morality (Kemmett et al., 2013).

Previously, *in ovo* inoculation into the chorioallantois sac with a nalidixic-resistant *E. coli* (36-55 cfu/100uL/embryo) at 12 days of embryogenesis negatively impacted hatchability and BWG, and increased mortality compared to the negative control group (Montgomery et al., 1999). Furthermore, Montgomery and co-authors (1999) indicated that the placement of seeder (direct-challenged) eggs had little effect on contact (indirect-challenged) chick hatchability and BW over the 21-day experimental period. This suggests that chicks exposed to microorganisms during oviposition or the hatching period can serve as reservoirs for opportunistic pathogens during the grow-out period. Recently, it was shown that the highest prevalence of select antimicrobial resistance *E. coli* was associated with the neonatal period suggesting the opportunistic pathogens could be transmitted vertically or horizontally (Apostolakos et al., 2019). Additionally, non-viable embryos have also been implicated as potential reservoirs for antimicrobial resistant *E. coli* (Karunarathna et al., 2020).

During hatch relative humidity increases markedly, promoting the proliferation of the microbial bloom within hatching environment. Thus, hatchery sanitation is crucial. To evaluate circulating bacteria (such as total aerobic bacteria, presumptive lactic acid bacteria, and Gram-negative bacteria) within the commercial hatching environment, the open agar plate method was previously employed to compare environmental treatments with spray application of a probiotic to conventional formaldehyde fumigation (Graham et al., 2018). Formaldehyde application inhibits proliferation of opportunistic pathogens within the hatching environment (Whistler et al., 1988), but is a potential carcinogen (Swenberg et al., 2014) and has been shown to have negative effects on the tracheal epithelium of poultry (Sander et al., 1995). As a result, development of both virulent (Graham et al., 2019) and wild-type *E. coli* challenge models are of importance for the investigation of mitigative strategies, other than formaldehyde, to control the microbial bloom. The purpose of the present study was to develop a consistent *E. coli* horizontal challenge model to evaluate formaldehyde fumigation alternatives under laboratory conditions. A commercially-relevant laboratory model for simulating *E. coli* horizontal transmission is needed.

Materials and Methods

E. coli culture and challenge

Two wild-type *E. coli* isolates were evaluated in these experiments: isolate 1 (I1) and isolate 2 (I2). Both isolates were recovered post-mortem from diseased chicks and identification was confirmed using the API 20E kit (cat. no. 95060-674, VWR, Suwanee, GA 30024). Aliquots of each isolate, consisting of 30% glycerol, were stored at -80C for long-term preservation. For the challenge culture, 100µL of *E. coli* was removed from a frozen aliquot and added to 10mL of tryptic soy broth (Tryptic soy broth, cat. no. 90000-378, VWR, Suwanee, GA 30024). The culture was incubated at 37°C for 18h. Post-incubation, bacterial cells were washed three times with sterile

0.9% saline by centrifugation at 1,800 × *g* for 15m and reconstituted in saline. *E. coli* colony-forming units (cfu) enumeration was determined by serial dilution and plating on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024) to determine the stock concentration and then cells were held overnight, approximately 16h, at 4C. The culture was then serially diluted to desired cfu concentration for *in ovo* administration. Actual *E. coli* challenge dose (cfu/mL) was confirmed as described above and reported in Table 1.

Enumeration of Bacteria

For Exp 1, Trial 2, Exp 2, and Exp 3 the gastrointestinal tract (proventriculus to the ileocecal junction including ceca) was aseptically removed post-mortem and collected into sterile bags. Samples were weighed and homogenized, and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold dilutions of each sample, from each group, were made in sterile 96-well Bacti flat-bottom plates and the diluted samples were plated on culture media to evaluate presumptive Gram-negative bacteria on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024). Plates were incubated at 37C for 18h and bacterial counts were expressed as Log_{10} cfu/g of sample. Additionally, the open-agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) was used for enumeration of circulating presumptive Gram-negative bacteria within the hatching environment. Up to three agar plates (with the lids removed) were placed open side up on the top tray of the hatchers (GQF 1550 Digital Cabinet Egg Incubator) for 1m or 5m. The plates were incubated at 37°C for 18h to enumerate presumptive Gram-negative bacteria present in the hatching cabinets; data expressed as cfu/plate.

Animal Source

For all experiments, eighteen-day-old Ross 308 embryos were candled, randomly allocated, and placed in separate hatchers based on treatment group. Mortality was recorded for the duration of

each trial (7-day trial period). Chicks were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean diet meeting the nutritional requirements for broilers recommended by Aviagen (Aviagen, 2018). All experiments and animal handling procedures complied with Institutional Animal Care and Use Committee at the University of Arkansas.

Exp 1 Design

Exp 1 consisted of two trials. In Exp 1 Trial 1, I1 or I2 (10^2 or 10^3 cfu/200uL/embryo) was administered into the amnion via *in ovo* inoculation at day 18, 19, or 19.5 of embryogenesis (n=37-40/treatment). The impact of late embryogenesis challenge was compared to a negative control group (no treatment, n=240) to determine optimal administration time point and appropriate challenge dose for future studies. Each hatcher contained three trays capable of holding 80 eggs per tray. For Exp 1 Trial 1, the negative control eggs were housed in one hatcher (n=80/tray) and both doses for each day of challenge were allocated as follows: tray one, day 18 challenge; tray two, day 19 challenge; tray three, day 19.5 challenge. I1 and I2 challenge groups were placed in separate hatchers. In Exp 1 Trial 2, seeder embryos (n=15 seeders/hatcher or n=50 seeders/hatcher) were inoculated with I1 or I2 at 10^2 cfu/200uL/embryo via *in ovo* injection into the amnion and segregated into mesh hatching bags (reusable mesh nylon netting, IDS, Amazon) to evaluate the impact of horizontal transmission of *E. coli* on contact chick hatchability. In Exp 1 Trial 2, and Exp 2, the open-agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) was utilized to evaluate circulating coliforms within the hatching environment at select time points during the hatch. A MacConkey's agar plate, a selective media for Gram-negative bacteria, was placed on the top tray of the hatcher for 1m (Exp 1 Trial 2 only) or 5m at ~80% hatch (PM of day 20 of embryogenesis) or immediately prior to hatch pull. Exp 1 Trial 2 was the initial evaluation of the open-agar plate method under these

specific challenge conditions, and one plate was placed per hatcher (n=1 hatcher/treatment) per time point. At day 21 of embryogenesis (day-of-hatch, DOH), dry chicks were removed from the hatching environment. Additionally, Gram-negative enteric colonization (n=12/treatment) was evaluated on DOH, d3, and d7 and BW was recorded on DOH, d3, and d7. Chicks were neck-tagged and allocated into separate pens with fresh pine shavings on DOH. BW of I1 and I2 seeder treatment groups (n=15 seeders/cabinet) were not evaluated because of low animal numbers (i.e., 15 total chicks for placement). Six replicate pens per treatment were placed (n=15 chicks/pen), except seeders of the I2 50% group, which had five replicate pens (n=15 chicks/pen).

Exp 2 and Exp 3 Design

Based on Exp 1 results, I1 was selected as the challenge strain for Exp 2 and Exp 3. There were two hatchers/treatment (n=210 eggs/hatcher; n=15 seeders/hatcher or 7.14%), and three replicate MacConkey plates were placed in the respective hatcher for 5m for Gram-negative bacterial recovery. The hatchers were sampled at four time points during the hatching phase: ~20% hatch, ~50% hatch, ~80% hatch, and prior to hatch pull at DOH. For the formaldehyde-treated hatch cabinets, fumigation was performed via a drip application of 6mL of formalin every three hours following transfer from the incubator to the hatching cabinet and ceased 12h prior to hatch pull. At DOH, dry chicks were removed from the hatching environment. Gastrointestinal tract samples were collected post-mortem for presumptive Gram-negative bacterial recovery, as described above on DOH and d7 (n=12/treatment). For both trials, weight allocation on DOH was performed to normalize BW and prevent the initial treatment effect on BW. Pen BW was determined at placement and on d7 to determine BWG with 12 replicate pens per treatment

(n=20 chicks/pen). Hatchability and 7-day mortality were not impacted as a result of the challenge (data not shown).

Statistical Analysis

All data were subjected to one-way analysis of variance using JMP Pro 13 (SAS, 2016). Data are expressed as mean ± standard error (SE). Significant differences ($P < 0.05$) among the means were further separated using Tukey's multiple range test for Gram-negative bacterial recovery with individual bird (Exp 1 Trial 2) or pen (Exp 2 or Exp 3) as the experimental unit for BW data. Mortality was compared using the chi-squared test of independence to determine the significance ($P < 0.05$) for these studies (Zar, 1984).

Results

Challenge dose(s) for each experiment are reported in Table 1. *In ovo* administration of I1 or I2 at 10^2 or 10^3 cfu/embryo on day 18 of embryogenesis negatively impacted hatchability, with I2 10^3 cfu/embryo being the most lethal, and I1 10^2 cfu/embryo having less of an impact compared to the I2 challenge (Table 2). Exp 1 Trial 1 data suggest that *in ovo* challenge with I1 or I2 at 10^2 cfu/embryo on day 19 of embryogenesis did not negatively affect development since hatchability was 90%, although the I1 10^2 cfu/embryo, when administered at day 19.5 of embryogenesis, had no effect on hatchability.

Horizontal transmission of I1 or I2 between the seeder chicks (direct-challenged) at a level of 7.14% or 50% of the population did not affect the hatchability of the contact (indirect-challenged) chicks (Table 3). However, Gram-negative bacteria recovered from gastrointestinal tract samples on DOH, of both seeder and contact chicks, were higher ($P < 0.05$) than the negative control group as a result of *in ovo* challenge (Table 3). There was no statistical difference in Gram-negative bacterial recovery between all groups at d3 or d7 (Table 3). DOH

BW was not impacted by treatment, although seeder chicks of the I2 – 50 treatment group were markedly ($P < 0.05$) lighter at d3 and d7 (Table 4). The d7 BW and 7-day BWG were significantly ($P < 0.05$) reduced for the contact chicks of the I1 – 7.14% treatment group compared to the negative control but was not different from the other treatment groups (Table 4). Additionally, there was 10.75% mortality over the 7-day trial period in the I2 – 50 seeder group, yet not statistically different than the negative control (Table 4).

As expected, exposing a MacConkey's agar plate to the hatching environment for 5m resulted in higher recovery of Gram-negative bacteria compared to sampling the air within the hatch cabinet for 1m (Table 5). Moreover, inoculating 50% of the embryos at d19 of embryogenesis as compared to 7.14% of the embryo increased the total number of colonies recovered (80% hatch cfu/plate + DOH cfu/plate) on MacConkey's agar compared to the negative control (Table 5). In Exp 2 and Exp 3, there was a significant difference ($P < 0.05$) in Gram-negative bacterial recovery between the negative control and contact chicks of the I1 + formaldehyde treatment group compared to the positive control contact and seeder chicks and the seeders of the I1 + formaldehyde treatment group on DOH (Table 6). There was a significant ($P < 0.05$) difference in d3 Gram-negative bacterial recovery between the negative control and contact chicks of the positive control group only in Exp 2 (Table 6). No differences were observed for Gram-negative bacterial recovery between treatment groups by d7 (Table 6). Seeder challenge increased coliform recovery from the hatching environment, and formaldehyde fumigation effectively controlled the artificial microbial bloom (Table 7). No differences were observed in DOH or d7 BW or 7-day BWG between all treatments in Exp 2 and Exp 3 (Table 8). In Exp 2 and Exp 3, 7-day mortality was not impacted as a result of challenge (data not shown).

Discussion

Colibacillosis is one of the leading causes of morbidity and mortality in poultry and is of significant economic importance to the industry (Kabir et al., 2010). Commercial hatch cabinet temperatures and humidity levels promote the proliferation of opportunistic pathogens, such as *E. coli* during hatch (Thermote, 2006). Hatchery sanitation methods, including formaldehyde fumigation, are employed to prevent the spread of pathogens. Formaldehyde fumigation is commonly implemented as a precautionary measure in commercial hatcheries due to its biocidal efficacy regardless of the research that has been conducted for decades to evaluate potential carcinogenicity (Swenberg et al., 2014). Fumigation can affect the tracheal epithelial integrity of chicks exposed to formaldehyde during the late hatching phase, such as a reduction in cilia and extensive mucus accumulation (Fauziah et al., 1996; Zulkifli et al., 1999). In addition to chemical treatments, a probiotic application to control the microbial loads during hatch has been investigated as a formaldehyde fumigation alternative (Graham et al., 2018). Probiotic application versus chemical application would expose the chicks to presumptive beneficial pioneer colonizers and reduce formaldehyde exposure for the hatching chicks and hatchery employees. Hence, the rationale for developing an *in ovo* seeder challenge model to investigate formaldehyde fumigation methods imitating commercial horizontal transmission and the microbial bloom within the hatcher.

In the present study, *in ovo* inoculation with ~10^2 or 10^3 cfu/embryo at d18 of embryogenesis with wild-type *E. coli* negatively impacted hatchability, but hatchability improved when challenge administration was delayed to d19 or 19.5 of embryogenesis. This suggests that the *in vivo* replication of the bacteria, when 10^3 cfu/embryo or less was administered at d19 of embryogenesis, reduced lethality to the chick. As such, previous research

indicates that the doubling time of *E. coli in vitro* is between 22 and 40 minutes (Helmstetter 1968). Thus, a later *in ovo* challenge during embryogenesis reduced the time for the *E. coli* to replicate within the developing embryo.

Horizontal challenge models, which consist of comingling seeder (challenged) and contact (non-challenged) chicks, have been developed to mimic natural challenge conditions (Weinack et al., 1981; Montgomery et al., 1999; Jarquin et al., 2007; Graham et al., 2019). Previously, a low dose (<100 cfu/embryo) *in ovo* inoculation with a nalidixic acid-resistant *E. coli* at day 12 of embryogenesis negatively affected the hatchability of directly challenged chicks, although there was no significant effect on contact chick hatchability (Montgomery et al., 1999). Exp 1, Trial 1 results suggested that day 19 administration of ~100 CFU of *E. coli* was not damaging to the developing embryo, and contact chick hatchability was also not impacted as a result of seeder challenge. Additionally, Gram-negative bacterial recovery was increased in the contact chicks compared to the negative control on DOH, but no differences were observed at day three or day seven post-hatch. The lack of difference in Gram-negative bacterial recovery between the negative control and the treatment groups on day three and day seven can be attributed to the presence of commensal *E. coli* within the gastrointestinal tract. Since wild-type *E. coli* strains were used for the challenge, differentiation between lactose-fermenting colonies (commensal and challenge strain) on MacConkey agar was not possible using the employed culture methods.

Although transmission via the fecal-oral route has been considered the primary route of infection for *Salmonella*, respiratory transmission has also been noted as a portal of entry (Kallapura et al., 2014) and fluff circulating in the cabinet during hatch can harbor pathogenic organisms with respiratory tropisms. Presently, formaldehyde fumigation effectively reduced the

number of Gram-negative bacteria in the hatching environment and in the GIT of contact chicks on DOH compared to the non-treated challenged control group. However, horizontal transmission of *E. coli* or formaldehyde treatment did not alter 7-day performance compared to controls. These results are similar to those reported by Zulkifli et al. (1999), where no significant effects on overall (41 days) performance as a result of formaldehyde fumigation were observed. While not directly compared or evaluated in these studies, the common coliform blooms and effects on horizontal transmission during commercial hatch primarily cause relatively subtle effects on overt 7-day mortality and performance, similar to the findings of these experiments. The primary purpose of the model was to be able to compare alternatives to formaldehyde during hatch. *E. coli* are predominantly involved in secondary infections, and perhaps why there was not a consistent impact on early performance. However, this model could be used to evaluate formaldehyde fumigation alternatives to control wild-type *E. coli* bloom within the hatching environment. Further research is being conducted to determine the effects of a multi-pathogen horizontal transmission model on the microbial load within the hatching cabinet, hatchability, and post-hatch morbidity/mortality.

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Tables

Table 1. Confirmed *in ovo* challenge doses by isolate and group for each experiment

Experiment	Isolate 1		Isolate 2	
	Group	**confirmed cfu/200uL/embryo**	**Group**	**confirmed cfu/200uL/embryo**
Exp 1 Trial 1	d18 10^2 d18 10^3 d19, d19.5 10^2 d19, d19.5 10^3	6 x 10^1 6 x 10^2 7 x 10^1 7 x 10^2	d18 10^2 d18 10^3 d19, d19.5 10^2 d19, d19.5 10^3	3 x 10^1 3 x 10^2 3 x 10^1 3 x 10^2
Exp 1 Trial 2	d19 10^2	1.5 x 10^2	d19 10^2	2.1 x 10^2
Exp 2	d19 10^2	1.00 x 10^2	-	-
Exp 3	d19 10^2	1.12 x 10^2	-	-

Table 2. Effect of *in ovo* administration of select wild-type *E. coli* during late embryogenesis on hatchability (%) – Exp 1 Trial 1

Challenge	Hatchability (%)			
	DOE18 inoculation	**DOE19 inoculation**	**DOE19.5 inoculation**	**Total hatchability by challenge**
I1 – 10^2	20/37 (54.05)	36/40 (90)	39/39 (100)	95/116 (81.89)
I1 – 10^3	16/39 (41.03)	37/40 (92.50)	39/40 (97.50)	92/119 (77.31)
I2 – 10^2	8/37 (21.62)	36/40 (90)	33/38 (86.84)	69/115 (60)
I2 – 10^3	4/37 (10.81)	28/40 (70)	34/40 (85)	66/117 (56.41)

n=1 hatcher per isolate with each inoculation day receiving its own tray level in its respective hatch cabinet (n=240 eggs for negative control hatcher; n=37-40 eggs/challenge, n=3 trays/cabinet)
Negative control hatchability; 238/240 (99.17%)

Table 3. Effect of horizontal transmission of wild-type *E. coli* on hatchability and Gram-negative enteric colonization at DOH, d3, and d7 – Exp 1 Trial 2

Treatment - % seeder embryos	Contact or Seeder	Hatchability (%)	DOH Log_{10} CFU/g	d3 Log_{10} CFU/g	d7 Log_{10} CFU/g
Negative Control	-	209/210 (99.52)	2.80 ± 0.94 [b]	8.28 ± 0.16	6.65 ± 0.17
I1 – 7.14	Contact	195/195 (100)	7.60 ± 0.77 [a]	8.04 ± 0.16	7.02 ± 0.24
	Seeder	15/15 (100)	8.38 ± 0.13 [a]	-	-
I1 – 50	Contact	104/105 (99.05)	8.19 ± 0.39 [a]	7.88 ± 0.12	6.95 ± 0.26
	Seeder	104/105 (99.05)	8.54 ± 0.12 [a]	7.64 ± 0.12	6.94 ± 0.18
I2 – 7.14	Contact	195/195 (100)	6.65 ± 0.65 [a]	8.09 ± 0.14	6.54 ± 0.14
	Seeder	15/15 (100)	8.18 ± 0.41 [a]	-	-
I2 – 50	Contact	105/105 (100)	8.02 ± 0.33 [a]	7.78 ± 0.18	6.65 ± 0.22
	Seeder	103/105 (98.10)	8.29 ± 0.39 [a]	8.07 ± 0.21	6.62 ± 0.17

[a,b] Indicates significant ($P < 0.05$) difference between treatments
Data expressed as mean ± standard error
n=210 total eggs placed/hatcher (7.14%: n=15/hatcher, 50%: n=105/hatcher)
For Gram-negative recovery, n=12/treatment

Table 4. Effect of horizontal transmission of wild-type *E. coli* on average BW, BWG, and 7-day mortality of contact and seeder chicks – Exp 1 Trial 2

Treatment - % seeder embryos	Contact or Seeder	BW (g) DOH	BW (g) d3	BW (g) d7	BWG (g) d0-d7	Mortality (%)
Negative Control	-	42.78 ± 0.35	81.31 ± 0.77 [a]	171.84 ± 2.72 [a]	129.13 ± 2.70 [a]	0/90 (0.00)
I1 – 7.14	Contact	42.76 ± 0.28	77.86 ± 0.87 [ab]	158.87 ± 2.83 [b]	116.60 ± 2.76 [b]	0/90 (0.00)
I1 – 50	Contact	42.64 ± 0.31	80.97 ± 0.74 [a]	164.59 ± 2.59 [ab]	121.71 ± 2.59 [ab]	0/90 (0.00)
	Seeder	41.92 ± 0.33	78.02 ± 0.85 [ab]	160.40 ± 3.12 [ab]	118.52 ± 3.07 [ab]	3/90 (3.33)
I2 – 7.14	Contact	42.49 ± 0.38	79.24 ± 0.82 [ab]	167.73 ± 3.07 [ab]	125.46 ± 3.01 [ab]	0/90 (0.00)
I2 – 50	Contact	42.02 ± 0.38	78.22 ± 0.85 [ab]	159.66 ± 2.80 [ab]	117.97 ± 2.74 [ab]	0/90 (0.00)
	Seeder	42.27 ± 0.36	76.94 ± 1.31 [b]	155.16 ± 3.91 [b]	113.16 ± 3.75 [b]	8/75 (10.75)

[a,b] Indicates significant differences between treatments groups within columns ($P < 0.05$)
Data expressed as mean ± standard error
n=6 pens/treatment, n=15 chicks/pen all groups except for I2 – 50 seeder group, n=5 pens/treatment, n=15 chicks/pen
Low n for I1 and I2 – 7.14 seeder chicks – chicks were not placed

Table 5. Gram-negative bacterial recovery from hatching environment at ~80% hatch and DOH – Exp 1 Trial 2

Treatment - % seeder embryos	Sampling duration	80% hatch CFU/plate	DOH CFU/plate	Total (80% hatch + DOH CFU/plate)
Negative Control	1m	19	0	19
	5m	46	8	54
I1 – 7.14	1m	35	24	59
	5m	126	76	202
I1 – 50	1m	140	71	211
	5m	632	224	856
I2 – 7.14	1m	4	8	12
	5m	30	27	57
I2 – 50	1m	50	16	66
	5m	760	92	852

n=1 hatcher/treatment
n=1 MacConkey agar plate/sample time point

Table 6. Effect of horizontal transmission of *E. coli* and formaldehyde fumigation during hatch on Gram-negative enteric colonization at DOH, d3, and d7 – Exp 2 and Exp 3

		DOH Log_{10} CFU/g		d3 Log_{10} CFU/g		d7 Log_{10} CFU/g	
Treatment	**Contact or Seeder**	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**
Negative Control	-	1.13 ± 0.71 [b]	0.56 ± 0.56 [b]	7.18 ± 0.19 [b]	7.98 ± 0.12	6.40 ± 0.18	6.75 ± 0.22
I1	Contact	6.32 ± 0.94 [a]	5.96 ± 0.38 [a]	8.18 ± 0.19 [a]	8.34 ± 0.11	6.48 ± 0.31	7.07 ± 0.23
	Seeder	7.56 ± 0.39 [a]	7.89 ± 0.09 [a]	-	-	-	-
I1 + formaldehyde	Contact	1.70 ± 0.92 [b]	1.65 ± 0.93 [b]	7.67 ± 0.21 [ab]	8.09 ± 0.20	6.72 ± 0.21	6.58 ± 0.25
	Seeder	7.22 ± 0.62 [a]	7.15 ± 0.41 [a]	-	-	-	-

[a,b] Indicates significant differences between treatments groups within columns ($P < 0.05$)
Data expressed as mean ± standard error

Table 7. Gram-negative bacterial recovery from hatching environment (DOE20 and DOH) – Exp 2 and Exp 3

	~20% hatch CFU/plate		~50% hatch CFU/plate		~80% hatch CFU/plate		DOH CFU/plate	
Treatment	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**
Negative Control	2.5	0	1.5	0	0.5	2	0.5	0
I1	1	7	22.5	20	18	112	120	7
I1 + formaldehyde	0	0	0	3	2	1	0	0

n=2 hatchers/treatment
n=3 MacConkey plates/sample time point

Table 8. Average BW and BWG – Exp 2 & Exp 3

	BW (g) DOH		BW (g) d7		BWG (g) d0-d7	
Treatment	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**	**Exp 2**	**Exp 3**
Negative Control	43.01 ± 0.03	42.59 ± 0.03	142.52 ± 1.77	138.97 ± 1.71	99.51 ± 1.76	96.39 ± 1.73
I1	42.93 ± 0.03	42.74 ± 0.02	144.86 ± 1.56	140.68 ± 1.98	101.94 ± 1.56	97.96 ± 1.98
I1 + formaldehyde	42.91 ± 0.04	42.69 ± 0.03	141.04 ± 1.80	140.08 ± 2.05	98.12 ± 1.81	97.37 ± 2.05

Data expressed as mean ± standard error
n=12 pens/treatment, n=20 chicks/pen

Chapter V. Development of an environmental contamination model to simulate microbial contamination in commercial hatcheries under laboratory conditions

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Abstract

Microbial blooms that emerge during hatch consist of apathogenic and pathogenic microorganisms, including *Escherichia coli, Enterococcus faecalis,* and *Aspergillus fumigatus.* Commercially, horizontal transmission of these organisms occurs throughout hatch. Objectives of the present study included development of a multi-pathogen laboratory challenge model to mimic commercial conditions and optimization of sampling methods to quantify bacterial and/or fungal presence within the hatch cabinet. The pathogen challenge mix (PM) was recreated from select bacterial or fungal isolates recovered from an egg homogenate (EH) derived from the contents of infertile eggs and late embryonic mortalities. Isolates selected for PM included *Enterococcus faecalis* (~10^8 CFU/egg)*, Staphylococcus aureus* (~10^7 CFU/egg)*, Staphylococcus chromogenes* (~10^7 CFU/egg)*, Aspergillus fumigatus* (~10^6 spores/egg)*,* and two *Escherichia coli* (~10^8 CFU/egg) isolates. Challenge (100µL of PM or EH) was administered to a 28mm area on the blunt end of the eggshell at day 19 of embryogenesis (DOE) (3 hatchers/trt, n=225/hatcher). In three experiments, microbiological data was collected from environmental hatcher samples (open-agar plate method), fluff samples, post-mortem whole-body chick rinse samples, and gastrointestinal tract (GIT) samples to evaluate select bacteria and fungi circulating within the hatch cabinet and colonization of GIT. Cumulative bacterial and fungal recovery from the PM hatching environment from DOE20 to hatch was higher compared to the non-challenged group and EH group at an average of 989 and 2555 CFU respectively. The reduction in CFU recovered from the EH hatching environment could be attributed to decline in viability of the EH during storage whereas the PM challenge was freshly prepared for each experiment. Bacterial recovery from GIT, fluff, and chick rinse samples were similar for the PM and EH group in Exp 1. However, *Aspergillus fumigatus* recovery from fluff and chick rinse samples of PM group was

significantly higher than the non-challenged control and EH group. In Exp 2 and 3, PM challenge significantly ($P<0.05$) increased Gram-negative bacterial recovery from the GIT, fluff, and chick rinse samples compared to both the non-challenged control and EH group. These data suggest this innovative multi-species environmental contamination model using PM could be utilized to evaluate strategies to mitigate microbial contamination in commercial hatch cabinets in a laboratory setting.

Keywords: hatchery, challenge, model, pathogen, broiler

Introduction

Establishment of a beneficial microbial niche during the neonatal phase more be important to ensure proper development and maturation of the gastrointestinal tract (GIT). In nature, neonatal chicks are exposed to the hen's microbiota. The maternal microbiota is transferred to neonatal chicks within 24 hours post-hatch and the direct contact with the hen or maternal microbiota influences composition of the chick's cecal microbiome (Kubasova et al., 2019). In a commercial setting, there is no physical contact between the hen and chick at hatch. As a result, naive neonates are exposed to a variety of fecal or environment-derived apathogenic and pathogenic microorganisms during embryonic development and the hatching phase.

The cuticle layer of the eggshell serves as a protective barrier (Board et al., 1973), but fluctuation in temperature post-lay may accelerate penetration by certain microorganisms present on the surface of the eggshell (Lock et al., 1992). Contaminated embryos, or non-viable embryonated broiler chicken eggs, have the potential to explode during incubation due to microbial overgrowth and may harbor pathogens, such as antimicrobial resistant *Escherichia coli* and *Enterococcus* spp. (Karunaranthna et al., 2020). If non-viable embryonated eggs are not removed at transfer, the eggs could rupture during late embryogenesis and contaminate the

environment and adjacent eggs in the hatch cabinet. As the chicks begin to hatch, any microorganisms that had penetrated the eggshell and effectively replicated within the developing the embryo during incubation will be transmitted to the non-infected chicks in the hatch cabinet. Cason et al. (1993) demonstrated that *Salmonella* contamination, as determined by post-mortem whole-body rinses, occurred after the eggshell had been pipped. This suggests that, while contamination within the developing embryo may not have occurred, contamination of the chick occurs when the eggshell is breached during pipping. The humidity in the hatching environment rises when the chicks hatch, boosting microbial proliferation (Sheldon and Brake, 1991). These microorganisms serve as are pioneer colonizers and are the first to colonize the GIT and other mucosal associated lymphoid tissues.

Pioneer colonizers influence the composition of the enteric microbiota and modulate intestinal immune development in broiler chickens (Rubio, 2019). Pioneer colonization by opportunistic pathogens, such as *E. coli* and *E. faecalis,* has been associated with elevated flock mortality (Olsen et al., 2012). Additionally, avian pathogenic *E. coli* (APEC) and *E. faecalis* have been isolated from the yolk sac of chicks with omphalitis signifying presence of both microorganisms at the hatchery level (Walker et al., 2020). However, other potential opportunistic pathogens must be considered. For instance, *Staphylococcus aureus* infections in chickens have become more common with most of the strains recovered being genetically similar to *S. aureus* strains that principally infect humans (Lowder et al., 2009). Additionally, severe *S. aureus* contamination in the hatchery can induce pneumonia, further validating the need for control at the hatchery level (Smyth and McNamee, 1999; Rodgers et al., 1999). In mice, toxin production by *S. aureus* prevented elimination by the host immune system, which supported replication of Gram-negative bacteria in the lung and subsequent systemic infection

(Cohen et al., 2016). More recently, Wu et al. (2021) demonstrated that *S. chromogenes* colonization in the upper respiratory tract of chickens promoted infection by *Avibacterium paragallinarum,* the etiologic agent of infectious coryza. These findings suggest that pioneer colonizers of the upper respiratory tract may facilitate infection and disease caused by opportunistic pathogens. Aside from bacterial infections, fungal diseases, like aspergillosis are a major concern as *Aspergillus fumigatus* is frequently recovered from commercial hatch cabinets (Thermote, 2006). *Aspergillus fumigatus* conidia or spores can penetrate the eggshell and replicate in the air cell within the egg, which is inaccessible to any fungicidal compounds applied during the hatching phase (Williams and Brake, 2000). Although colonization by microorganisms during embryogenesis may not always be fatal, the number of microorganisms circulating in the environment will rise as the infected chicks hatch, exposing the naïve chicks.

Our laboratory previously developed horizontal challenge models for wild-type and virulent *E. coli* to model the seeding phenomenon where a small number of contaminated chicks horizontally transmit the challenge at hatch to the non-infected chicks (Graham et al., 2019; Selby et al., 2021; Graham et al., 2021). The horizontal challenge models required *in ovo* or spray application of *E. coli* to a small number of the embryos (<10% of population deemed seeders) at DOE19 to seed the environment and expose the naïve contact chicks during the hatching period. We recently demonstrated that exposure to *E. coli* during the hatching phase increased enteric coliform recovery from naïve contact chicks (Graham et al., 2021). The purpose of the current proposed model was to simulate bacterial and fungal contamination in commercial hatch and evaluate culture-dependent microbiological methods to monitor the microbial load in small-scale hatch cabinets. Additionally, we assessed eggshell surface

contamination, rather than *in ovo* administration or spray application, to reflect environmental exposure that occurs in the presence of exploder eggs or during severe microbial contamination in commercial hatch cabinets.

Materials and Methods

Experimental Design

Three experiments were conducted (Exp 1-3). For each experiment, a total of 2,025 fertile eggs (n=224-225 per hatcher x 3 hatchers per treatment x 3 treatments) were placed in separately assigned hatcher cabinets. The treatments included: 1) non-challenged control (NC), 2) egg homogenate (EH) challenge, and 3) pathogen mix (PM) challenge. Hatch cabinets were setup in different rooms within the same building to prevent any potential cross-contamination between cabinets during the hatching phase. The PM challenge consisted of microorganisms recovered from a homogenate prepared from contaminated infertile eggs and late embryonic mortalities removed at transfer at DOE18, including two wild-type *Escherichia coli* isolates, *Staphylococcus aureus, Staphylococcus chromogenes, Enterococcus faecalis,* and *Aspergillus fumigatus.* The bacterial isolates were identity confirmed with 16S sequencing. The fungal isolate was speciated as *Aspergillus fumigatus* based on colony morphology. Application of challenge in hatchers was conducted by applying 100µL of the EH challenge (homogenate from non-viable eggs) or PM challenge (amplified species recovered from EH) to the blunt end of the egg's surface. The material was distributed over a 28mm area, or ~half the size of the air cell, using a sterile disposable loop at 19 DOE (9:30am) simulating the "exploder" phenomenon that occurs commercially. To ensure viability of the challenge material after application, the EH challenge consisted of 50% EH prepared from infertile eggs and 50% 2X tryptic soy broth (TSB, cat. no. 90000-378, VWR, Suwanee, GA) supplemented with 0.01% xanthan gum. The PM challenge

material was resuspended in 2X TSB supplemented with 0.01% xanthan gum to obtain the desired CFU/egg (for bacterial species) or conidia or spores/egg (*Aspergillus fumigatus*). This particular vehicle of 2X TSB supplemented with 0.01% xanthan gum has been previously evaluated and does not alter the viability of the challenge organisms or affect chick hatchability (unpublished data). The NC did not receive any treatment. Following hatch, percent hatchability was be recorded and a composite sample of the chick fluff (~1g) was collected from the hatching environment. Chicks were immediately euthanized and samples were collected, and included body rinse for surface bacteria and fungi, as well as gastrointestinal tracts (GIT) collected for enumeration of relevant enteric pathogens. Each sample was homogenized with sterile saline by stomaching, 10-fold serially diluted, and plated onto different selective agar plates to enumerate population changes of various bacteria or fungi present in the different treatments as described below. Hatch cabinet components were thoroughly disinfected, allowed to dry, and then fumigated with formaldehyde between each experiment.

Challenge Preparation

Bacterial isolates

To prepare the PM challenge for each experiment, 1mL of each *E. coli* isolate, *S. aureus, S. chromogenes,* or *E. faecalis* was removed from a frozen aliquot and added to 100mL of tryptic soy broth. The cultures were incubated aerobically at 37C for ~18h. Each *Staphylococcus* spp. culture was placed on an orbital shaker during incubation, whereas the *E. coli* and *E. faecalis* cultures were incubated statically. Post-incubation, bacterial cells were washed three times with sterile saline (0.9% NaCl) by centrifugation at 1,800 × *g* for 15m. Colony-forming units (CFU) were determined by serial dilution and plating on respective agar media to determine the stock concentration. Cells were then held approximately 16h at 4C. On the day of challenge, a specific

volume of each challenge organism was concentrated by centrifugation based on the target CFU concentration for application. The pelleted bacterial cells and *Aspergillus fumigatus* spores were combined and resuspended with the vehicle to achieve the pre-determined concentration of each organism for the actual challenge. The EH challenge was simply prepared by removing a frozen aliquot of the material recovered from non-viable embryonated eggs and combined 1:1 with the vehicle. Actual CFU/egg or spores/egg for each microorganism, for PM and EH challenge, was confirmed by spread plating in triplicates on the relevant media described below.

Fungal isolate

From a thawed aliquot, *Aspergillus fumigatus* was directly swabbed onto Sabouraud dextrose agar (SDA, cat. no. 95021-184, VWR, Suwanee, GA) supplemented with chloramphenicol 50mg/L. The methods used to recover and enumerate the *Aspergillus fumigatus* spores was derived from Sala et al. (1972) and National Institute of Health standard operating procedures for model for invasive Aspergillosis (NIH-NIAID-N01-AI-30041 Version 1.10). *Aspergillus fumigatus* spores/100uL/egg was confirmed using a hemacytometer and spread plating on SDA supplemented with chloramphenicol 50mg/mL.

Enumeration of Bacteria and Fungi

Environmental sampling

The open-agar plate method (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018) was used to enumerate select airborne microorganisms circulating in the hatching environment. For each media used, three agar plates (with the lids removed) were placed open side up on the top tray of the hatchers (G.Q.F. 1550 Digital Cabinet Egg Incubator) using a modified sample port as previously described (Graham et al., 2021) to evaluate Gram-negative bacteria (MacConkey agar, cat. no. 89429–342, VWR, Suwanee, GA 30024), *Staphylococcus* spp. (mannitol salt agar,

MSA agar, cat. no. 89405-680, VWR, Suwanee, GA), *Enterococcus* spp. (Chromagar Orientation, CO agar, RT412, DRG International, Springfield, NJ), or *Aspergillus fumigatus* presence in the hatching environment. The open agar plates were placed in the hatch cabinet environment for either 1m (minute) or 5m durations based on type of media. A 5m sampling duration was selected for MacConkey agar (Graham et al., 2021) and for SDA based off of preliminary data (results not shown). However, CO agar, MSA agar, tryptic soy agar (TSA, cat. no. 90002-700, VWR, Suwanee, GA) plates were placed in the hatching environment for 1m at each sampling time point. The hatch cabinet environment was sampled at four time points during the hatching phase: DOE20 8:00am (~20% hatch), DOE20 2:00pm (~50% hatch), DOE20 5:00pm (~80% hatch), and DOE21/DOH 7:00am (~100% hatch, DOH). Post-sampling, the agar plates were incubated aerobically at 37C for 18h to enumerate total aerobic bacteria, Gram-negative bacteria, and *Enterococcus* spp. However, select agar plates were incubated for 48h to determine *Staphylococcus* spp. and *Aspergillus fumigatus* presence in the hatching cabinets.

Gastrointestinal tract sampling

For all experiments, the GIT samples (n=5 chicks/hatcher, n=15 chicks/treatment) were aseptically removed from chicks after the whole-body rinse samples were collected. The GIT (ventriculus to the cecum) was collected into sterile bags. GIT samples were weighed and homogenized, and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold dilutions of each sample, from each group, were made in a sterile 96-well Bacti flat bottom plates and the diluted samples were plated to evaluate Gram-negative bacteria, *Staphylococcus* spp., and *Enterococcus* spp. on the media described above. All plates were incubated aerobically at 37C. MacConkey and CO agar plates were incubated at 37C for 18h. MSA plates were incubated for 48h. Bacterial counts were expressed as Log_{10} CFU/g of sample.

Fluff and chick rinse sampling

At hatch, ~1g of fluff was collected from each hatch cabinet (n=1 composite sample/hatch, n=3 samples/treatment). During the collection process, gloves were changed between each hatch cabinet and eggshell fragments were avoided. Fluff samples were weighed, diluted with sterile 0.9% saline at a 1:50 w/v dilution, and homogenized prior to drop plating samples onto MacConkey agar, TSA, CO agar, MSA agar, and SDA plates. The chick rinse samples were collected post-mortem where five chicks per hatcher (n=15 per treatment) were placed in a sterile whirl pack bag with 50mL of sterile saline. The exterior of the chick was gently massaged with the sterile saline for 30s as previously described (Bailey et al., 1994). Samples were drop plated as described above to enumerate select microorganisms present on the surface of the chick.

Animal Source

For all experiments, eighteen-day-old Ross 308 embryos were candled, randomly allocated, and placed into separate hatchers based on treatment group. All experiments and animal handling procedures complied with Institutional Animal Care and Use Committee at the University of Arkansas, protocol #20017.

Statistical Analysis

Hatchability and microbial recovery (GIT, fluff, chick rinse) data were subjected to analysis of variance using JMP Pro 13 (SAS, 2016). GIT, fluff, and chick rinse means for select bacterial and fungal recovery were further separated using Tukey's multiple range test (Table 4, 5, and 6). In Table 3, the air sampling data obtained using the open-agar plate method were reported as an average of three agar plates per media for each collection time point and experiment.

Results

Hatchability

In all experiments, application of 100uL of EH or PM challenge to a 28mm surface on the blunt end of the eggshell at DOE 19 did not impact hatchability (Table 2). There were no significant ($P > 0.05$) differences in hatchability across treatment groups by experiment (Table 2).

Bacteria and fungi recovered from the hatching environment (DOE20-DOH)

There were four collection time points as described above and in Table 3. The time of sampling was held constant across experiments. Select bacterial and fungal recovery for each time point and experiment is shown in Table 3. In Exp 1, there was low-level contamination associated with the embryo source indicated by the increased bacterial recovery (>100 CFU) from the NC hatching environment at all time points. However, DOE18 embryonated eggs were randomized prior to placement in hatch cabinets to account for naturally acquired contamination. Application of EH or PM challenge increased the amount of select bacteria circulating during the hatching phase as compared to the NC in all experiments. Bacterial and fungal recovery from the hatching environment was more consistent for the PM challenge group compared to the EH challenge group. Gram-negative bacterial recovery from the hatching environment was increased by Exp 2 and 3 for both the EH and PM hatchers, although the PM hatchers had a 2.2-4.5-fold increase in cumulative circulating coliforms compared to the EH hatchers (Table 3). The reduction in CFU recovered from the EH hatching environment could be attributed to decline in viability of the EH during storage at -80C whereas the PM challenge was freshly prepared for each experiment or perhaps due to small fluctuations in humidity in the hatcher rooms/hatch cabinets across experiments. Regarding fungal recovery, the increase in *Aspergillus fumigatus* recovered from the PM hatchers compared to EH hatchers could be is associated with the ~2-3 log difference in *Aspergillus fumigatus* between the PM and EH challenge treatment that was applied to the eggshell surface at DOE 19 (Table 1).

GIT samples at hatch

The mean bacterial recovery from the whole GIT (Log_{10} CFU/g) at hatch by experiment in presented in Tables 4-6). Gram-negative bacterial recovery from the GIT at hatch was markedly (P<0.0001) higher for the PM group compared to NC in all experiments and compared to EH for Exp 2 and Exp 3 (Table 4-6). *S. aureus* recovery from the GIT was significantly increased due to PM or EH challenge compared to NC in Exp 1 and Exp 3, P=0.0025 and P=0.0035, respectively (Table 4-6). Enteric recovery of non-mannitol fermenting staphylococci was only observed in Exp 1, although there were no significant differences across treatments (Table 4). Application of EH or PM to the eggshells at DOE19 significantly (P<0.0004) increased *Enterococcus* spp. recovery from the GIT at hatch compared to the NC group in all experiments (Tables 4-6).

Fluff samples at hatch

For all experiments, the mean for select bacterial and fungal recovery (Log_{10} CFU/g) from composite fluff samples collected at hatch is reported in Tables 4-6. In Exp 1, application of EH or PM to eggshells at DOE19 significantly (P<0.0001) increased Gram-negative, total aerobic bacteria, *S. aureus,* and *Enterococcus* spp. recovery from fluff samples compared to NC in all three trials (Table 4). In Exp 2 and 3, PM challenge markedly (P<0.0001) increased Gram-negative bacterial recovery from fluff samples compared to both the EH and NC group (Tables 5 and 6). Recovery of non-mannitol fermenting staphylococci was significantly (P<0.0001) higher in fluff samples collected from the EH hatchers in only Exp 2 (Table 6). PM application significantly (P<0.0001) increased *Aspergillus fumigatus* recovery compared to NC and EH groups in Exp 1 and Exp 3 (Table 4 and 6).

Chick rinse samples at hatch

The mean bacterial and fungal recovery (Log_{10} CFU/mL) from post-mortem chick rinse samples at hatch for Exp 1-3 is presented in Tables 4-6. In Exp 1, challenge with PM or EH significantly (P<0.0001, except P=0.0021 for *Enterococcus* spp. recovery) increased all non-selective and selective bacterial recovery compared to the NC (Table 4). PM challenge markedly (P<0.0001) increased *Aspergillus fumigatus* recovery from chick rinse samples compared to groups NC and EH in Exp 1 only (Table 4). In Exp 2, EH and PM application significantly (P<0.0001) increased total aerobic bacteria and *Enterococcus* spp. recovery from chick rinse samples at the time of hatch compared to the NC (Table 5). However, there were no differences in total aerobic bacteria and *Enterococcus* spp. recovery between the two challenged groups (Table 5). In Exp 2, *S. aureus* and Gram-negative bacterial recovery was numerically increased for PM compared to the EH, but there were no differences in *Aspergillus fumigatus* recovery across treatment groups (Table 5). EH challenge significantly (P<0.0001) increased non-mannitol fermenting *Staphylococcus* spp. recovery compared to EH and NC (Table 5). In Exp 3, EH and PM chick rinse samples had statistically (P<0.0001) more Gram-negative bacteria, total aerobic bacteria, *S. aureus,* and *Enterococcus* spp. recovery compared to group C (Table 6). Alternatively, there were no differences in non-mannitol fermenting *Staphylococcus* spp. or *Aspergillus fumigatus* recovery across all treatment groups (Table 6). However, PM treatment significantly (P<0.0001) increased *S. aureus*, total aerobic bacteria, and Gram-negative bacterial recovery from chick rinse samples compared to the EH treatment (Table 6).

Discussion

Neonatal chicks may be exposed to circulating apathogenic and pathogenic microorganisms during the hatching phase. Certain bacteria, such as *Salmonella* spp. are capable

of penetrating the eggshell post-lay (Berrang et al., 1999). Non-viable embryonated eggs not removed at the time of transfer may explode due to microbial overgrowth within the egg and contaminate the adjacent eggs and environment in the hatch cabinet (Karunarathna et al., 2017). Chicks may become exposed to the contaminated material on the exterior of the eggshell as they begin to pip. Moreover, as the humidity rises during the hatching phase, chicks are further exposed to the plethora of microorganisms that rapidly proliferate in the environment (Thermote, 2006). Since the relative humidity associated with hatching dictates the onset and proliferation of microbial bloom in the hatch cabinet environment (Magwood, 1964), any variation in microbial recovery between experiments in the present study could be attributed to the natural fluctuation in timing of hatch or proliferation of naturally-acquired microorganisms. Nevertheless, the purpose of the present study was to develop a reproducible multi-pathogen challenge model to mimic the microbial bloom present in commercial hatch cabinets and validate methods to assess the impact of artificial contamination in small-scale hatch cabinets.

Magwood (1964) determined that the microbial load in the hatching environment was most elevated at the time of hatch. In another study, the observed increase in bacterial load was specifically associated with the onset of hatch (pipping) at DOE19 (Sander and Wilson, 1999). Chick fluff accumulates in the environment during hatch and can be used as a proxy to determine the bacterial and fungal load in a hatch cabinet as a feasible and cheap method to monitor hatchery sanitation (Magwood, 1962). Additionally, storage for up to a week did not alter the level of contamination recovered from the fluff samples (Magwood, 1962). Muira and coworkers (1964) also showed that fluff samples stored at room temperature for four years remained positive for *Salmonella* (up to 10^{4-6} CFU/g). Fluff sampling and periodic air sampling of the hatch cabinet environment were investigated as methods to assess airborne contamination

in a hatch cabinet (Magwood, 1964). Results published by Magwood and Marr (1964) indicate that there is a direct relationship between the level of air contamination, as measured by fluff and air sampling, and surface contamination in commercial hatcheries. Taken together, these findings suggest that some microorganisms may be capable of remaining dormant in organic matter for an extended period of time. Thus, complete removal of debris and disinfection is important to avoid inadvertent contamination of embryonated eggs and the hatch cabinet environment.

Air sampling methods have been used by the commercial poultry industry to assess hatchery sanitation (Berrang et al., 1995; Kim et al., 2010; Graham et al., 2018). The open-agar plate method and air sampling machines have been used to determine the microbial load in the hatching environment (Berrang et al., 1995). Berrang et al. (1995) collected air samples from the hatching environment on DOE20, or approximately 50% hatch, to determine the level of contamination in a commercial hatchery. To enumerate *Enterobacteriaceae* over a 2m sampling period, two methods were used: 1) a surface air sampling machine (CFU/180L, 2m) or 2) the open agar plate method (CFU, 2m) (Berrang et al., 1995). The authors attributed to the 0.71 log increase in *Enterobacteriaceae* recovery when using the air sampling machine to the higher volume of air that was sampled as compared to the open agar plate method. However, there were no differences between the sampling methods for *Salmonella* recovery. In the current study, a 5m sampling duration was deemed to be sufficient for enumeration of Gram-negative bacteria and SDA for the particular hatch cabinets. The additional media were placed in the hatch cabinet environment for 1m based off of preliminary results (data not shown).

Alternative microbiological techniques have been explored to quantify culturable microorganisms from fluff samples collected from hatchery settings. For instance, Warren et al.

(2016) collected 25 fluff samples from 25 commercial hatcheries to evaluate bacterial and fungal load using the pour plate method or the Petrifilm technique and observed no meaningful differences between the two techniques. However, in the present study, a single composite fluff sample was collected from each hatch cabinet immediately after hatch pull. The samples were serially diluted and drop plated in triplicates to evaluate the effect of EH or PM challenge on the level of bacterial and fungal contamination in fluff samples as compared to the non-challenged control. The plating technique was simple for quantifying select bacterial and fungal in fluff samples collected from small-scale hatch cabinets.

In addition to fluff sampling, bacterial and fungal recovery from whole-body chick rinses was evaluated in the present study. Whole-body chick rinse sampling has been used to assess *Salmonella* contamination in hatchery settings (Bailey et al., 1994). Although there were no differences observed for *Salmonella* recovery from egg shells or whole-body chick rinses, there was a strong correlation between the two sampling methods (Bailey et al., 1994). *Salmonella* has also been recovered from air samples collected from the hatching environment and GIT samples of non-challenged contact chicks (Cason et al., 1994). Cross-contamination can occur between infected and naïve, non-infected chicks during the neonatal period. Infecting 5% of the population with 10^2 CFU of *Salmonella* Typhimurium at hatch was sufficient to contaminate 56.7% of the non-infected counterparts within the same pen (Byrd et al., 1998). This suggests that low level contamination at the hatchery level can increase the risk of horizontal transmission of opportunistic pathogens at the flock level. Thus, for the present study, it was important to assess contamination in the hatching environment using multiple methods, including the open-agar plate method, chick rinse sampling, and fluff sampling since both the EH and PM challenge treatments contained multiple microorganisms.

As previously stated, the microbial load in the hatching environment is affected by the composition of microorganisms present and the relative humidity in the environment. Naturally-acquired contamination was observed in Exp 1 based on the overall bacterial and fungal recovery from samples collected from the NC group. Moreover, *S. aureus* recovered from the hatching environment (DOE20 to DOH) and GIT at hatch was numerically higher for all treatment groups compared to the other experiments. Even though *S. aureus* recovery from the hatching environment and GIT declined in Exp 2 and Exp 3, *S. aureus* recovery from fluff and chick rinse samples of the PM group was elevated compared to both the EH and NC group. The PM challenge more consistently increased Gram-negative bacteria recovery from fluff, chick rinse, and GIT samples compared to the NC and EH group. Furthermore, there were more Gram-negative bacteria recovered from the hatching environment of the PM group compared to the EH and NC groups at ~20%, ~50%, and ~80% hatch across all experiments. Application of challenge, whether via EH or PM, similarly increased *Enterococcus* spp. recovery from fluff, chick rinse, or GIT samples in all experiments. As expected, the increased challenge dose of *Aspergillus fumigatus* for the PM group increased recovery from the hatching environment and fluff samples collected at hatch compared to EH. These data suggest that the recreated PM is the more appropriate multi-species model to reproduce microbial contamination in commercial hatch cabinets in a laboratory setting.

Since it is not practicable to evaluate and compare novel methods to control the microbial bloom in a commercial hatchery, extensive testing in a laboratory setting is generally required before large-scale application. Several methods were evaluated in the present study to assess the effect of eggshell application "exploder" derived bacteria and fungi as a model to simulate the microbial bloom present in commercial hatch cabinets under laboratory conditions. Moreover,

challenge models using a singular challenge organism do not truly reflect contamination in commercial hatcher settings. The PM described herein contained multiple microorganisms associated with hatchery contamination and application to eggshells at DOE19 increases the microbial load in small-scale hatch cabinets. In future studies, the PM model will be utilized to evaluate alternative methods to formaldehyde fumigation to control the microbial load in the hatch cabinet environment and methods to introduce beneficial pioneer colonizers to displace colonization by potential opportunistic pathogens in neonatal broiler chicks.

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Tables

Table 1. Microbial recovery from EH or PM material by experiment

Colony morphology	EH[1] Exp 1	EH[1] Exp 2	EH[1] Exp 3	PM Exp 1	PM Exp 2	PM Exp 3
Gram-negative bacteria (lactose fermenter)	5.00×10^5	6.33×10^5	2.00×10^5	5.00×10^8	1.13×10^8	1.07×10^8
Gram-negative bacteria (lactose non-fermenter)	3.67×10^6	2.67×10^6	3.67×10^6	0	0	0
Enterococcus spp.	1.57×10^7	1.27×10^7	1.03×10^7	1.00×10^8	2.00×10^8	4.67×10^7
Staphylococcus aureus (mannitol fermenter)	2.00×10^6	7.67×10^5	6.67×10^5	3.23×10^7	8.67×10^7	7.67×10^7
Staphylococcus spp. (mannitol non-fermenter)	3.00×10^6	5.00×10^6	3.33×10^6	0	0	0
Total aerobic bacteria	1.93×10^7	2.80×10^8	2.33×10^7	4.67×10^8	2.67×10^8	3.33×10^8
Aspergillus fumigatus	7.00×10^3	1.00×10^4	4.00×10^3	1.00×10^7	1.00×10^6	1.00×10^6

[1]EH: Egg Homogenate; PM: Pathogen Mix

Dose (CFU or spores/100uL/egg) reported as an average of three replicate agar plates

Table 2. Percent hatchability (Exp 1-3)

Treatment[1]	Exp 1	Exp 2	Exp 3
NC	97.70 ± 0.007	96.70 ± 0.007	97.70 ± 0.009
EH	98.00 ± 0.015	97.00 ± 0.010	97.70 ± 0.003
PM	98.30 ± 0.003	98.30 ± 0.012	98.70 ± 0.003
p-value	0.893	0.489	0.422

[1]NC: Negative Control; EH: Egg Homogenate; PM: Pathogen Mix
Data reported as mean percent hatchability ± standard error
n=3 hatchers/treatment, n=225/hatcher
Note: Exp 3 EH n=224 for one replicate hatcher

Table 3. Microbial recovery (CFU/plate) from the hatching environment at DOE20 (~20%, ~50%, or ~80% hatch) or at DOH immediately prior to hatch pull (Exp 1-3)

		Gram-negative bacteria				***Staphylococcus aureus***				***Enterococcus* spp.**				**Total aerobic bacteria**				***Aspergillus fumigatus***			
Exp	Trt[1]	20[2]	50	80	DOH	20	50	80	DOH	20	50	80	DOH	20	50	80	DOH	20	50	80	DOH
Exp 1	NC														78	48	74	3	3	2	2
	EH	1	24	43	54	2	158	173	149	56	107	128	291	50	294	542	424	2	3	3	2
	PM	43	35	85	46	25	126	90	96	132	121	455	351	124	270	415	431	25	56	66	100
Exp 2	NC	0	0	0	1	74	0	0	0	0	1	0	6	3	3	2	6	14	5	4	1
	EH	4	209	13	38	13	24	64	22	22	38	166	229	79	89	268	318	13	13	9	36
	PM	18	454	211	495	17	5	18	47	8	37	51	151	15	112	196	154	17	30	39	14
Exp 3	NC	0	0	0	1	1	0	0	0	0	0	0	0	0	0	5	4	1	0	1	0
	EH	17	38	68	548	0	1	2	25	1	9	20	105	8	20	23	62	0	1	1	1
	PM	184	300	784	239	15	50	43	133	46	68	200	117	43	172	467	159	15	24	22	8

[1]NC: Negative Control; EH: Egg Homogenate; PM: Pathogen Mix

[2]CFU reported for ~20% hatch, ~50% hatch, ~80% hatch, or immediately prior to hatch pull at DOH as an average of three replicate plates for each time point

n=3 replicate hatchers/treatment

n=3 replicate agar plates/media were exposed to the hatch cabinet environment for 1m or 5m based on the type of media

Non-mannitol fermenting *Staphylococcus* spp. data not shown

Darker shaded areas are related to higher CFU recovery for ease of interpretation

Table 4. Effect of EH or PM challenge application at DOE19 on select bacterial and fungal recovery from the GIT, fluff, or chick rinse samples at DOH (Exp 1)

GIT (Log_{10} CFU/g)	**NC[1]**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	3.06[b]	4.62[ab]	6.71[a]	0.54	0.0185
Staphylococcus aureus	0.22[b]	1.98[a]	2.95[a]	0.34	0.0025
Staphylococcus spp. (non-mannitol fermenter)	0.30[a]	1.82[a]	1.22[a]	0.53	0.0885
Enterococcus spp.	0.73[b]	6.53[a]	5.91[a]	0.57	<0.0001
Fluff (Log_{10} CFU/g)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	1.34[b]	6.03[a]	6.79[a]	0.57	<0.0001
Staphylococcus aureus	0.63[b]	6.13[a]	7.38[a]	0.65	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	2.58[b]	7.37[a]	4.42[ab]	0.61	0.0031
Enterococcus spp.	2.16[b]	6.45[a]	6.47[a]	0.45	<0.0001
Total aerobic bacteria	3.50[b]	6.89[a]	6.66[a]	0.37	<0.0001
Aspergillus fumigatus	0.41[b]	1.34[b]	4.92[a]	0.46	<0.0001
Chick Rinse (Log_{10} CFU/mL)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0.40[b]	3.32[a]	2.93[a]	0.34	0.0002
Staphylococcus aureus	0[b]	4.09[a]	4.94[a]	0.36	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	1.00[b]	4.85[a]	3.68[a]	0.33	<0.0001
Enterococcus spp.	2.00[b]	5.42[a]	4.94[a]	0.45	0.0021
Total aerobic bacteria	2.06[b]	5.03[a]	5.09[a]	0.27	<0.0001
Aspergillus fumigatus	0.25[b]	0[b]	2.53[a]	0.26	<0.0001

[a,b] Means across rows with different superscripts indicate significance at $P < 0.05$
[1]NC: Negative Control; EH: Egg Homogenate; PM: Pathogen Mix
Sample size: For fluff, n=3 composite samples/hatcher plated on respective media in triplicates, so n=9/treatment group. For chick rinse and GIT, n=5/hatcher or n=15 samples/treatment group

Table 5. Effect of EH or PM challenge application at DOE19 on select bacterial and fungal recovery from the GIT, fluff, or chick rinse samples at DOH (Exp 2)

GIT (Log_{10} CFU/g)	**NC[1]**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0.60^{b}	1.62^{b}	6.67^{a}	0.58	<0.0001
Staphylococcus aureus	0^{a}	0.75^{a}	0.66^{a}	0.15	0.09
Staphylococcus spp. (non-mannitol fermenter)	0	0	0	-	-
Enterococcus spp.	0^{b}	4.19^{a}	3.94^{a}	0.52	0.0004
Fluff (Log_{10} CFU/g)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0^{c}	2.78^{b}	6.31^{a}	0.55	<0.0001
Staphylococcus aureus	0.41^{c}	3.06^{b}	5.82^{a}	0.54	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	0^{b}	5.36^{a}	0^{b}	0.53	<0.0001
Enterococcus spp.	2.65^{b}	6.38^{a}	5.73^{a}	0.42	<0.0001
Total aerobic bacteria	3.10^{b}	6.51^{a}	6.62^{a}	0.41	<0.0001
Aspergillus fumigatus	0.82^{a}	1.98^{a}	2.58^{a}	0.39	0.1968
Chick Rinse (Log_{10} CFU/mL)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0^{b}	1.03^{b}	3.21^{a}	0.31	<0.0001
Staphylococcus aureus	0^{c}	2.27^{b}	3.83^{a}	0.31	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	0^{b}	3.59^{a}	0.20^{b}	0.28	<0.0001
Enterococcus spp.	0.54^{b}	4.05^{a}	3.97^{a}	0.31	<0.0001
Total aerobic bacteria	0.20^{b}	5.20^{a}	5.20^{a}	0.37	<0.0001
Aspergillus fumigatus	0^{a}	0.36^{a}	0.36^{a}	0.11	0.3499

[a-c] Means across rows with different superscripts indicate significance at $P < 0.05$
[1]NC: Negative Control; EH: Egg Homogenate; PM: Pathogen Mix
Sample size: For fluff, n=3 composite samples/hatcher plated on respective media in triplicates, so n=9/treatment group. For chick rinse and GIT, n=5/hatcher or n=15 samples/treatment group

Table 6. Effect of EH or PM challenge application at DOE19 on select bacterial and fungal recovery from the GIT, fluff, or chick rinse samples at DOH (Exp 3)

GIT (Log_{10} CFU/g)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0^{c}	3.34^{b}	6.29^{a}	0.57	<0.0001
Staphylococcus aureus	0^{b}	0.18^{b}	1.14^{a}	0.15	0.0035
Staphylococcus spp. (non-mannitol fermenter)	0	0	0	-	-
Enterococcus spp.	0^{b}	3.19^{a}	4.82^{a}	0.53	0.0003
Fluff (Log_{10} CFU/g)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0^{c}	4.37^{b}	6.64^{a}	0.63	<0.0001
Staphylococcus aureus	0.93^{b}	4.61^{a}	6.10^{a}	0.51	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	0	0	0	-	-
Enterococcus spp.	0.41^{b}	5.92^{a}	6.25^{a}	0.54	<0.0001
Total aerobic bacteria	1.49^{b}	6.53^{a}	7.16^{a}	0.54	<0.0001
Aspergillus fumigatus	0^{b}	0.41^{b}	3.73^{a}	0.38	<0.0001
Chick Rinse (Log_{10} CFU/mL)	**NC**	**EH**	**PM**	**SEM**	***p*-value**
Gram-negative bacteria	0^{c}	2.25^{b}	3.51^{a}	0.29	<0.0001
Staphylococcus aureus	0^{c}	0.93^{b}	3.43^{a}	0.26	<0.0001
Staphylococcus spp. (non-mannitol fermenter)	0^{a}	0.40^{a}	0^{a}	0.09	0.1287
Enterococcus spp.	0^{b}	2.67^{a}	3.61^{a}	0.29	<0.0001
Total aerobic bacteria	0^{c}	3.38^{b}	4.91^{a}	0.33	<0.0001
Aspergillus fumigatus	0^{a}	0^{a}	0.40^{a}	0.07	0.3499

[a-c] Means across rows with different superscripts indicate significance at $P < 0.05$

[1]NC: Negative Control; EH: Egg Homogenate; PM: Pathogen Mix

Sample size: For fluff, n=3 composite samples/hatcher plated on respective media in triplicates, so n=9/treatment group. For chick rinse and GIT, n=5/hatcher or n=15 samples/treatment group

Chapter VI. Concluding Remarks

In the United States, the microbial bloom in commercial hatch cabinets has been frequently controlled with formaldehyde fumigation. The seeder challenge models using a singular organism (i.e. *E. coli*) demonstrated how readily horizontal transmission occurs in a hatch cabinet when less than 10% of the embryos were infected. APEC colonization during embryogenesis tends to be detrimental to the developing embryo, but was mitigated when tetracycline hydrochloride was co-administered with APEC at DOE19 (Chapter III). We hypothesized that co-administration of tetracycline hydrochloride delayed replication of APEC, which allowed the seeder chick to hatch and disseminate APEC into the environment. Horizontal transmission of APEC during the hatching phase increased GIT colonization for naïve contact chicks at hatch. Exposure to APEC also reduced early performance and markedly increased 7-day mortality compared to the non-exposed controls. This model could be used to evaluate the impact of horizontal transmission of other APEC strains during the neonatal period and potential strategies to mitigate cross-contamination post-hatch.

A similar approach, as described above, was used to evaluate the horizontal transmission of wild-type *E. coli* during the hatching phase (Chapter IV). But, embryonic infection with wild-type *E. coli* $<10^3$ CFU/embryo at DOE19 or DOE19.5 did not affect hatchability compared to APEC. As the infected seeder chicks hatched, there was an increase in circulating airborne Gram-negative bacteria in the hatching environment. Although exposure to wild-type *E. coli* during the hatching phase increased enteric Gram-negative bacterial recovery at DOH, it did not impact early performance nor enteric bacterial recovery at d3 or d7 post-hatch. Formaldehyde reduced the Gram-negative bacterial load in the hatch cabinet and limited horizontal transmission during the hatching phase. However, formaldehyde fumigation did not

affect seeder chick enteric colonization, which suggested that the fumigant did not readily penetrate the eggshell. This model could be used to assess the ability of alternatives to formaldehyde to mitigate the Gram-negative bacterial bloom and horizontal transmission during the hatching phase.

A single challenge organism was used for each challenge model described in Chapters III and IV. Most challenge models with a singular challenge organism prove to be too harsh or do not truly reflect contamination in commercial hatch cabinets. Therefore, a multi-pathogen challenge model was developed using multiple opportunistic pathogens frequently isolated from hatch cabinets as described in Chapter V. Contents were removed from DOE18 non-fertile eggs to create a homogenate to use for the EH challenge. However, the viability of the EH would be expected to decline during storage over time, so there was a need to assess a more replicable method using an artificial challenge that included bacterial and fungal pathogens. Direct administration of EH or PM to the eggshell at DOE19 was done to mimic contamination by ruptured non-viable embryonated eggs during incubation. Hatch cabinet environment samples, fluff samples, and post-mortem chick rinse samples were collected and used to assess bacterial and fungal presence in the hatch cabinet at DOH. GIT samples were collected at DOH to evaluate the impact of EH or PM challenge on enteric colonization by Gram-negative bacteria, *Staphylococcus* spp., and *Enterococcus* spp. PM challenge increased Gram-negative bacterial recovery and *Aspergillus fumigatus* recovery compared to the EH and NC groups. Application of PM challenge produced a more consistent microbial bloom in the hatch cabinet environment and is the most practical challenge model for simulating the "exploder" phenomenon. In future studies, this multi-species contamination model will be used to assess

alternatives to formaldehyde fumigation and to evaluate the effect of the PM challenge model on the enteric microbiome with and without probiotic intervention.

The horizontal transmission or environmental contamination models developed and evaluated in the present dissertation artificially replicate microbial contamination ongoing in commercial hatch cabinets in a laboratory setting. These models can be used to assess alternative methods to control microbial proliferation in commercial hatch cabinets and to promote early colonization by beneficial microorganisms in a contaminated hatchery setting.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	February 5th, 2018
Subject:	IACUC Approval
Expiration Date:	February 1st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18079:** *Development of Escherichia coli/Salmonella in ovo seeder model in the hatching environment with and without GRAS probiotic treatment.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 1st, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Brittany Danielle-Mahaf, Callie McCreery, Amanda Wolfenden, Lucas Graham, and Kyle Teague. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Billy Hargis
Date:	September 5th, 2019
Subject:	IACUC Approval
Expiration Date:	August 29th, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **20017:** *Development of a natural challenge "bloom" model to evaluate probiotic efficacy in a hatching cabinet*..

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond August 29th, 2022 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Christine Vuong, Callie Selby (McCreer, Danielle Graham, Cheryl Lester, Makenly Coles, Lucas Graham, and Kyle Teague. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

CURRICULUM VITAE
Danielle Mahaffey Graham

Education

PhD	University of Arkansas – Fayetteville, Poultry Diseases Advisor: Dr. Billy Hargis	2018-2021
MS	University of Arkansas – Fayetteville, Poultry Diseases Advisor: Dr. Billy Hargis	2015-2018
BS	University of Arkansas – Fayetteville, Animal Science	2011-2015

MS Thesis
Evaluation of the Efficacy of a Candidate Turkey Dermatitis/Cellulitis Oil Emulsion Vaccine on Immune Response, Morbidity, and Mortality under Laboratory and Commercial Conditions

PhD Dissertation
Development and Evaluation of Models for Hatchery-Mediated Infection of Neonatal Broiler Chicks

Professional Experience

University of Arkansas, Fayetteville, AR, USA August 2018-Present
Program Associate/Graduate Student

- Projects:
  - Development of environmental challenge models simulating commercial hatching conditions to evaluate alternatives to formaldehyde fumigation (B. D. Graham, PhD Dissertation)
  - Development of a candidate bioshuttle coccidiosis vaccination program for the turkey industry (Led by B. D. Graham)
  - Anticoccidial sensitivity, speciation, and phylogenetic analysis of *Eimeria* spp. recovered from wild turkey feces (Led by B. D. Graham)
  - Optimization of *Clostridium septicum* antigenic output to improve immunogenicity of a candidate Clostridial Dermatitis vaccine for commercial turkeys (B. D. Graham, major leadership role)
  - Application of *Eimeria* spp. sporulation assays to assess alternatives to potassium dichromate (B. D. Graham, project supervisor)
- Duties: coordinating and overseeing BSL-2 research, experimental design, interfacing with collaborators, managing and mentoring students and lab technicians, maintaining laboratory accounts/budget, scientific writing (grants, reports, manuscripts for publication)

University of Arkansas, Fayetteville, AR, USA May 2015-August 2018
Research Technician/Graduate Student

- Developed a candidate Clostridial Dermatitis oil emulsion vaccine for commercial turkeys
- Involved with research related to intestinal health and mitigation of enteric disease
- Managed disease research for turkeys, broilers, and leghorns
- Collected and analyzed data for contract trials

University of Arkansas, Fayetteville, AR, USA March 2014-May 2015
Undergraduate Lab Technician

- Conducted research evaluating the effects of a probiotic on intestinal permeability and earlyperformance in broiler chickens
- Investigated the use of bacteriophages to control *Salmonella* in mechanically separated chicken
- Interacted with research scientists to construct projects and collect data
- Involved in prepping materials for contract trials

Professional Affiliations

- World Poultry Science Association, 2017-Present
- Poultry Science Association, 2015-Present
- UA Poultry Science Graduate Association, 2015-Present

Academic Collaborations/Workshops/Unofficial Course Audits

- Workshop: ForBio K-mer: Using k-mer-based approaches to analyze genomic sequence data, September 2021
- Audit: Principles of Parasitology PATH3040, Dr. John Barta, University of Guelph, Spring 2021
- Training: Turkey *Eimeria* lesion scoring, Dr. John Barta, University of Guelph, June 2019
- Collaboration: *Escherichia coli* challenge model, Dr. Lisa Bielke, Ohio State University, October 2018

Honors/Awards

- World Poultry Congress Youth Program, Paris, France, 2021 *(awarded 2020, postponed to 2022)*
- Recipient of Poultry Federation Scholarship, 2019-2021
- SCAD Poster Award "Evaluation of the effects of a turkey cellulitis/dermatitis oil emulsion vaccine on immune response and mortality under commercial conditions", 2018
- Jones-Hamilton Co. Undergraduate Travel Award, 2015

Industry Funding (*$377,346*)

Evaluation of the effects of select Amlan products on coccidiosis control in broiler chickensPI:
Dr. Billy Hargis, Co-PI(s): Dr. Guillermo Tellez-Isaias, Danielle Graham*
Oil-Dri Corporation of America, 9/13/21-9/12/22 ($93,871.20)
*Protocol design and implementation, contract development and management

Effect of an encapsulated Jefo feed additive and/or select product formulations on *Salmonella* colonization in broiler chickens and on *Eimeria* spp. oocyst sporulation efficiency *in vitro*
PI: Dr. Billy Hargis, Co-PI(s): Danielle Graham*
Jefo Nutrition Inc., 9/13/21-9/12/22 ($90,000)
*Principle contact, protocol design and implementation, and contract facilitator

Evaluation of the effect of a feed additive against histomoniasis in turkeys
PI: Dr. Billy Hargis, Co-PI(s): Danielle Graham*
BioVet S.A., 7/12/21-7/11/22 ($25,000)
*Principle contact, protocol design and implementation, and contract facilitator

Titration, comparison, and validation of various micro-particles for replicating the ascitesinducing effect caused by cellulose CM-32 micro-particles when intravenously injected
PI: Dr. Billy Hargis, Co-PI(s): Lucas Graham, Danielle Graham
Cobb-Vantress, 3/30/21-3/29/22 ($38,780)

Effect of different probiotic application routes on neonatal colibacillosis
PI: Dr. Billy Hargis, Co-PI(s): Dr. Christine Vuong, Danielle Graham*
Perdue Agribusiness, 1/4/21-12/31/21 ($17,520)
*Principle contact, protocol design and implementation, and contract facilitator

Evaluation of the effect of select feed additives against a multi-species *Eimeria* challenge in broiler chickens
PI: Dr. Billy Hargis, Co-PI(s): Callie Selby, Danielle Graham*
Vetagro, 10/1/20-9/30/21 ($38,000)
*Principle contact, protocol design and implementation, and contract facilitator

Effect of feed additives on performance and *Salmonella* colonization in broiler chickensPI:
Dr. Billy Hargis, Co-PI(s): Dr. Christine Vuong, Danielle Graham*
Cobb-Vantress, 3/16/20-4/16/21 ($45,435)
*Principle contact, protocol design and implementation, and contract facilitator

Evaluating probiotic administration routes during *Salmonella* or *E. coli* challengePI:
Dr. Billy Hargis, Co-PI(s): Dr. Christine Vuong, Danielle Graham* Perdue Agribusiness, 10/3/19-10/4/20 ($28,740)
*Principle contact, protocol design and implementation, and contract facilitator

Grants (*$109,053*)

Realistic multi-species challenge model to evaluate treatment methods able to prevent colonization of pathogenic bacteria in the hatching environment
PI: Dr. Christine Vuong, Co-PI(s): Dr. Billy Hargis, Danielle Graham US Poultry & Egg Association, 6/2021-6/2023 ($79,053)

Isolation of drug sensitive turkey *Eimeria* spp. from wild-turkey feces
PI: Dr. Billy Hargis, Co-PI(s): Dr. Christine Vuong, Danielle Graham USDA Animal Health Award, 11/11/20-11/10/2022 ($30,000)

Service

Frontiers – Co-Editor of Research Topic — Begins January 2022
Poultry Science – Reviewer — February 2020-present

Invited Seminars/Symposium Lectures

- Turkey Dermatitis/Cellulitis: where are we headed? (virtual). D. Graham. Midwest PoultryConference. May 18-21, 2021.

- Update on Clostridial Dermatitis and blackhead research from UA PHL (virtual). D. Graham, T. L. Barros. Poultry Symposium for Production and Processing. April 14-15, 2021.

- Emergence and sources of *Salmonella enterica* serovar Reading contamination of processed turkey from infected flocks (virtual). D. Graham, B. Hargis. Poultry Federation's 8th AnnualFood Safety Conference. March 29-21, 2021.

- Raising healthy chickens in a challenging world (virtual). D. Graham, T. L. Barros. Jefo, November 24, 2020

Publications (24: 4 first author, 20 co-author)

1. **B. D. Graham**, C. Selby, L. Graham, K. Teague, G. Tellez-Isaias, B. Hargis, and C. Vuong.Development of a wild-type *Escherichia coli* environmental bloom model to evaluate alternatives to formaldehyde fumigation in broiler chicken hatch cabinets. *Poultry Science*, March 2021, 100(3):100975.

2. **B. D. Graham**, C. M. Selby, K. D. Teague, L. E. Graham, C. N. Vuong, J. D. Latorre, G. Tellez, and B. M. Hargis. Development of a novel *in ovo* challenge model for virulent *Escherichia coli* strains. *Poultry Science*, November 2019, 98(11), 5330–5335.

3. **B. D. Graham**, K. M. Robbins, K. D. Teague, L. E. Graham, R. Merino-Guzman, G. Tellez,and B. M. Hargis. Evaluation of the efficacy of a candidate turkey cellulitis/dermatitis oil emulsion vaccine on immune response and mortality under laboratory and commercial conditions. *Journal of Applied Poultry Research,* December 2019, 28(4):818-825.

4. **B. D. Mahaffey**, L. E. Graham, N. Calhoun, R. Merino-Guzman, K. D. Teague, M. F. A. Baxter, J. D. Latorre, A. D. Wolfenden, X. Hernandez-Velasco, L. R. Bielke, B. M. Hargis, and G. Tellez. Evaluation of a lactic acid bacteria-based probiotic on leaky gut and microbiome associated with *Salmonella* Enteritidis infection and feed restriction in broiler chickens. *Approaches in Poultry Dairy & Vet Sciences,* September 2017, 1(1).

5. L. C. Beer, **B. D. Graham**, T. L. Barros, J. D. Latorre, G. Tellez-Isaias, A. L. Fuller, B. M. Hargis, and C. Vuong. Evaluation of live-attenuated *Histomonas meleagridis* isolates as vaccine candidates against wild-type challenge. Accepted. *Poultry Science.*

6. G. Tellez-Isaias, C. N. Vuong, B. D. **Graham**, C. M. Selby, L. E. Graham, R. Seas-Cuesta, T. L. Barros, L. Beer, M. E. Coles, A. J. Forga, J. Ruff, X. Hernandez-Velasco, and B. M. Hargis.Developing probiotics, prebiotics, and organic acids to control Salmonella spp. in commercialturkeys at the University of Arkansas USA. *German Journal of Veterinary Research*, May 2021, 1(3):7-12.

7. M. Bansal, T. Alenezi, Y. Fu, A. Almansour, H. Wang, A. Gupta, R. Liyanage, B. D. **Graham**, B. M. Hargis, and X. Sun. Specific Secondary Bile Acids Control Chicken Necrotic Enteritis. *Pathogens*. August 2021, 10(8):1041.

8. A. M. Ashcraft, M. E. Coles, L. C. Beer, B. D. **Graham**, G. Tellez-Isaias, B. Wooming, and B. M. Hargis. Research note: Fate and dissemination of Salmonella enterica serovar Reading in turkeys at processing using an oral gavage challenge model. *Poultry Science*, July 2021, 100(7):101114.

9. K. M. Chasser, K. McGovern, A. F. Duff, **B. D. Graham**, W. N. Briggs, D. R. Rodrigues, M.Trombetta, E. Winson, and L. R. Bielke. Evaluation of day of hatch exposure to various Enterobacteriaceae on inducing gastrointestinal inflammation in chicks through two weeks ofage. *Poultry Science*, July 2021, 100(7):101193.

10. M. E. Coles, A. J. Forga, R. Señas-Cuesta, **B. D. Graham**, C.M. Selby, Á. J. Uribe, B. C. Martínez, J. A. Angel-Isaza, C. N. Vuong, X. Hernandez-Velasco, B. M. Hargis, and G. Tellez-Isaias. Assessment of Lippia origanoides Essential Oils in a *Salmonella* typhimurium, *Eimeria maxima*, and *Clostridium perfringens* Challenge Model to Induce Necrotic Enteritis in Broiler Chickens. *Animals*, April 2021; 11(4):1111.

11. C. Selby, **B. D. Graham**, L. Graham, K. Teague, G. Tellez-Isaias, B. Hargis, and C. Vuong. Research Note: Application of an *Escherichia coli* spray challenge model for neonatal broilerchickens. *Poultry Science*, April 2021, 100(4):100988.

12. L. Laverty, R. Senas-Cuesta, S. Martinez-Gonzalez, C. Selby, G. Tellez Jr., X. Hernandez-Velasco, C. Vuong, B. Hargis, G. Tellez-Isaias, and **D. Graham**. Evaluation of oocyst shedding of *Eimeria maxima* and *Eimeria acervulina* in broiler chickens. *Abanico Veterinario*, December 2020, 10:1-11.

13. M. A. Arreguin-Nava, **B. D. Graham**, B. Adhikari, M. Agnello, C. M. Selby, X. Hernandez-Velasco, C. N. Vuong, B. Solis-Cruz, D. Hernandez-Patlan, J. D. Latorre, G. Tellez, B. M. Hargis, and G. Tellez-Isaias. *In ovo* Administration of Defined Lactic Acid Bacteria PreviouslyIsolated From Adult Hens Induced Variations in the Cecae Microbiota Structure and *Enterobacteriaceae* Colonization on a Virulent *Escherichia coli* Horizontal Infection Model in Broiler Chickens. *Frontiers in Veterinary Science*, August 2020, 7:489.

14. J. Ruff, T. L. Barros, G. Tellez, J. Blankenship, H. Lester, **B. D. Graham**, C. M. Selby, C. N. Vuong, S. Dridi, E. S. Greene, X. Hernandez-Velasco, B. M. Hargis, and G Tellez-Isaias. Evaluation of a heat stress model to induce gastrointestinal leakage in broiler chickens. *PoultryScience,* March 2020, 9(3):1687-1692.

15. A. F. Duff, M. F. A. Baxter, **B. D. Graham**, B. M. Hargis, and L. R. Bielke. Mode of action ofdietary dexamethasone may not be dependent upon microbial mechanisms in broilers. *Microorganisms,* September 2019, 7:346.

16. M. A. Arreguin-Nava, **B. D. Graham**, B. Adhikari, M. Agnello, C. M. Selby, X. Hernandez- Velasco, C. N. Vuong, B. Solis-Cruz, D. Hernandez-Patlan, J. D. Latorre, G. Tellez, B. M. Hargis, and G. Tellez-Isaias. Evaluation of *in ovo Bacillus* spp. based probiotic administrationon horizontal transmission of virulent *Escherichia coli* in neonatal broiler chickens. *Poultry Science*, December 2019, 98(12):6483-6491.

17. L. Beer, L. Butler, A. Gautier, **B. D. Graham**, K. Teague, V. Wilson, and S. Dridi. Xylanasesupplementation in wheat-based diets and the influence on necrotic enteritis in broilers: A translational model for human malnutrition. *Adv Food Technol Nutr Sci Open J*, November 2018,4(2): e4-e5.

18. Graham, L. E., K. D. Teague, J. D. Latorre, Y. Yang, M. F. A. Baxter, **B. D. Mahaffey**, X. Hernandez-Velasco, L. R. Bielke, B. M. Hargis, and G. Tellez. Use of probiotics as an alternative to formaldehyde fumigation in commercial broiler chicken hatch cabinets. *The Journal of Applied Poultry Research,* September 2018, 27(3):371–379.

19. J. D. Latorre, B. Adhikari, Si Hong Park, K. D. Teague, L. E. Graham, **B. D. Mahaffey**, M. F.A. Baxter, X. Hernandez, Y. M. Kwon, S. C. Ricke, L. R. Bielke, B. M. Hargis, and G. Tellez. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. *Frontiers in Veterinary Science,* August 2018, 5:199.

20. K. D. Teague, L. E. Graham, J. Dunn, H. Cheng, N. Anthony, J. D. Latorre, A. Menconi, R. Wolfenden, A. Wolfenden, **B. D. Mahaffey**, M. F. A. Baxter, X. Hernández-Velasco, R. Merino-Guzman, L. R. Bielke, B. M. Hargis, and G. Tellez. *In ovo* evaluation of FloraMax®- B11 on Marek´s disease HVT vaccine protective efficacy, hatchability, microbiota composition,morphometric analysis, and *Salmonella* Enteritidis infection in broiler chickens. *Poultry Science*, July 2017, 96(7):2074–2082.

21. Y. Yang, J. D. Latorre, B. Khatri, Y. M. Kwon, B. Kong, K. D. Teague, L. E. Graham A. Wolfenden, **B. D. Mahaffey**, M. F. A. Baxter, X. Hernández-Velasco, R. Merino-Guzman, B. M. Hargis, and G. Tellez. Characterization and evaluation of lactic acid bacteria candidates forintestinal epithelial permeability and *Salmonella* Typhimurium colonization in neonatal turkeypoults. *Poultry Science*, February 2018, 97(2):515-521.

22. R. Delgado, X. Hernandez-Velasco, R. Merino-Guzmán, J. D. Latorre, A. Wolfenden, K. Teague, L. Graham, **B. Mahaffey**, M. Baxter, B. Hargis, and G. Tellez. Comparison of totalIgA levels in different samples in Leghorn or broiler chickens. *Asian Pacific Journal of Tropical Biomedicine,* February 2017, 7(2):116–120.

23. M. F. A. Baxter, R. Merino-Guzman, J. D. Latorre, **B. D. Mahaffey**, Y. Yang, K. D. Teague, L. E. Graham, A. D. Wolfenden, X. Hernandex-Velasco, L. R. Bielke, B. M. Hargis, and G. Tellez. Optimizing fluorescein isothiocyanate dextran measurement as a biomarker in a 24-h feed restriction model to induce gut permeability in broiler chickens. *Frontiers in Veterinary Science*, April 2017, 4:56.

24. E. Morales-Barrera, N. Calhoun, J. L. Lobato-Tapia, V. Lucca, O. Prado-Rebolledo, X. Hernandez-Velasco, R. Merino-Guzman, V. M. Petrone-García, J. D. Latorre, **B. D. Mahaffey**, K. D. Teague, L. E. Graham, A. D. Wolfenden, M. F. A. Baxter, B. M. Hargis and G. Tellez. Risks involved in the use of Enrofloxacin for *Salmonella* Enteritidis or *Salmonella* Heidelberg in commercial poultry. *Frontiers in Veterinary Science*, August 2016, 3:72.

Refereed Abstracts (30: 8 first author, 22 co-author)

1. B. Danielle **Graham**, Callie M. Selby, Lucas E. Graham, Christine N. Vuong, Guillermo Tellez-Isaias, and Billy M. Hargis. "Development of a bacterial and fungal bloom model simulating commercial hatching conditions to evaluate formaldehyde fumigation alternatives."World Poultry Congress. *Postponed to August 7-11, 2022.* Paris, France. ID334 poster currently available online.

2. B. Danielle **Graham**, Callie M. Selby, Lucas E. Graham, Kyle D. Teague, Christine N. Vuong,Guillermo Tellez-Isaias, and Billy M. Hargis. "Development of a wild-type *Escherichia coli* environmental bloom model to evaluate alternatives to formaldehyde fumigation." InternationalPoultry Scientific Forum. January 27-28, 2020. Atlanta, Georgia.

3. B. Danielle **Graham**, Callie M. Selby, Lucas E. Graham, Kyle D. Teague, Christine N. Vuong,Guillermo Tellez-Isaias, and Billy M. Hargis. "Effect of norepinephrine on *Salmonella* Enteritidis growth *in vitro* and infectivity in broiler chickens." World Veterinary Poultry Association Congress. September 16-20, 2019. Bangkok, Thailand.

4. B. Danielle **Graham**, Callie M. Selby, Kyle D. Teague, Lucas E. Graham, Christine N. Vuong, Juan D. Latorre, Guillermo Tellez, and Billy M. Hargis. "Development of an *in ovo* colibacillosischallenge model utilizing sub-lethal bacteriostatic tetracycline co-administered with virulent *Escherichia coli*." International Conference on Poultry Intestinal Health. April 2-5. 2019. Rome, Italy.

5. B. Danielle **Graham**, Callie M. Selby, Kyle D. Teague, Lucas E. Graham, Christine N. Vuong, Juan David Latorre, Guillermo Tellez, and Billy M. Hargis. "Utilization of sub-lethal bacteriostatictetracycline co-administration for development of a novel *in ovo* challenge model for virulent *Escherichia coli* strains" International Poultry Scientific Forum February 11-13, 2019. Atlanta, Georgia.

6. B. Danielle **Graham**, Kabel Robbins, Lucas Graham, Kyle Teague, Amanda Wolfenden, Guillermo Tellez, Billy Hargis. "Evaluation of the effects of a turkey cellulitis/dermatitis oil emulsion vaccine on immune response and mortality under commercial conditions" InternationalPoultry Scientific Forum January 29-30, 2018. Atlanta, Georgia. Poultry Science Vol. 97 (E-suppl.1): P243.

7. Brittany D. **Mahaffey**, Kabel M. Robbins, Neil R. Pumford, Marion J. Morgan, Ruben Merion-Guzman, Lucas E. Graham, Kyle D. Teague, Mikayla F. A. Baxter, Juan D. Latorre, Amanda D. Wolfenden, Guillermo Tellez, and Billy M. Hargis. "Evaluation of the effects of an autogenous turkey dermatitis/ cellulitis oil emulsion vaccine on immune response and mortality in commercialturkeys." Poultry Science Association. New Orleans, Louisiana, July 11-14, 2016. Poult. Sci. Vol. 95 (E-suppl. 1): 1.

8. Brittany D. **Mahaffey**, Lucas E. Graham, Kyle D. Teague, Amanda D. Wolfenden, Johel C. Bielke,Billy M. Hargis, and Lisa R. Bielke. "Application of selected bacteriophages to control *Salmonella*in mechanically separated chicken." Poultry Science Association. July 27-30, 2015. Louisville, Kentucky. Poult. Sci. 94 (E-Suppl. 1): 35.

9. Guillermo Tellez-Isaias, Daniel Hernandez-Patlan, Bruno Solis-Cruz, Victor Petrone-Garcia, AnaLeyva-Diaz, Christine Vuong, Danielle **Graham**, Callie Selby, Juan Latorre, and Billy Hargis. "Studies conducted at the University of Arkansas to evaluate curcumin as a feed additive to control bacterial and protozoal infections and reduce aflatoxicosis severity in poultry." Symposium on GutHealth. October 31-November 3, 2021. St. Louis, Missouri. 105

10. Ying Fu, Mohit Bansal, Ayidh Almansour, Tahrir Alenezi, Hong Wang, Danielle **Graham**, Billy M. Hargis, and Xiaolun Sun. "Optimization of a *Clostridium perfringens* sporulation vaccine toreduce necrotic enteritis." Poultry Science Association. July 19-22, 2021. Virtual meeting.

11. Aaron J. Forga, B. Danielle **Graham**, Makenly Coles, Lesleigh Beer, Callie Selby, Lucas E. Graham, Roberto S. Cuesta, Jared Ruff, Guillermo Tellez-Isaias, Billy M. Hargis, and Christine Vuong. "Effect of *Clostridium septicum* hemolytic activity units on Clostridial dermatitis (cellulitis) bacterin/toxoid humoral immunogenicity in turkeys." Poultry Science Association. July19-22, 2021. Virtual meeting. 349P

12. Callie Selby, Danielle **Graham**, Lucas Graham, Aaron Forga, Mikayla Baxter, Guillermo-Tellez-Isaias, Billy M. Hargis, and Christine Vuong. "Effect of oral or intracloacal challenge with *Escherichia coli* or *Salmonella* Enteritidis on enteric colonization and early performance in broilerchickens." Poultry Science Association. July 19-22, 2021. Virtual meeting. 350P

13. Makenly E. Coles, Roberto Señas-Cuesta, B. Danielle **Graham**, Callie M. Selby, Álvaro J. Uribe,Blanca C. Martínez, Jamie A. Angel-Isaza, Christine N. Vuong, X. Hernandez-Velasco, Billy M.Hargis, and Guillermo Tellez-Isaias. "Assessment of essential oil formulations on growth performance, intestinal barrier integrity, and lesions in broiler chickens using a necrotic enteritis laboratory challenge model." International Poultry Scientific Forum. January 25-26, 2021. Virtualmeeting. P170.

14. Mohit Bansal, Ying Fu, Ayidh Almansour, Tahrir Aleneji, Mussie Abraha, Danielle **Graham**, Anamika Gupta, Hong Wang, Rohana Liyanage, Billy Hargis, and Xiaolun Sun. "Evaluation of bile acids against chicken necrotic enteritis." Poultry Science Association. July 20-22, 2020. Virtual Meeting.

15. Mohit Bansal, Ying Fu, Ayidh Almansour, Tahrir Aleneji, Mussie Abraha, Danielle **Graham**, Anamika Gupta, Hong Wang, Billy Hargis, and Xiaolun Sun. "Mammalian target of rapamycin signaling modulates chicken necrotic enteritis." Poultry Science Association. July 20-22, 2020. Virtual Meeting.

16. Ying Fu, Mohit Bansal, Ayidh Almansour, Tahrir Aleneji, Mussie Abraha, Hong Wang, Danielle **Graham**, Billy Hargis, and Xiaolun Sun. "*Clostridium perfringens* vaccine prevents chicken necrotic enteritis." Poultry Science Association. July 20-22, 2020. Virtual Meeting.

17. Lucas E. Graham, Kyle D. Teague, B. Danielle **Graham**, Sam J. Rochell, and Billy M. Hargis."Isolation and selection of *Bacillus* spp. as candidate direct-fed microbials based on in vitro utilization of indigestible non-starch polysaccharides and oligosaccharides in soybean meal." International Poultry Scientific Forum. January 27-28, 2020. Atlanta, Georgia.

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