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Evaluation of Two Neonatal Challenge Models for Broiler Chickens and their Effects on Early Performance Parameters

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Evaluation of Two Neonatal Challenge Models for Broiler Chickens
and their Effects on Early Performance Parameters

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by

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Abstract

Formaldehyde fumigation in poultry hatch cabinets has been utilized for sanitation of hatching eggs for over a century. Formaldehyde is utilized to minimize pathogenic microbes on the surface of the egg as well as the microbial bloom during the hatching process. While formaldehyde is effective, its use is regulated in the United States and Europe due to its carcinogenic nature. Formaldehyde has been shown to damage the cuticle of the egg, cause embryonic death, and damage the epithelial lining of the respiratory tract of freshly hatched chicks, predisposing them for respiratory infection. Alternatives for formaldehyde fumigation must be identified and investigated. Testing potential alternatives in commercial settings is not feasible as new technologies must be invented and integrators do not want to risk economical loss. Moreover, reliable challenge models must be developed to simulate the microbial bloom that occurs during hatch. The purpose of these experiments was to evaluate two neonatal challenge models and their effects on early performance parameters for broiler chickens. In Chapter 2, the efficacy of a spray challenge model is investigated. Utilizing this model, on d20 of embryogenesis, selected chicks, called seeders, were sprayed with a virulent *Escherichia coli* and placed back into the hatch cabinet to horizontally spread the pathogen. On day of hatch, selected contact chicks were utilized for gastrointestinal tract sampling, while the rest were weighed and randomly allocated into pens to evaluate performance parameters. For two 7-day experiments, the efficacy of transmission was evaluated via enteric bacterial recovery, body weight gain (BWG), and mortality. For Exp 1 and Exp 2, significantly ($P<0.0001$) more Gram-negative bacteria were recovered from the seeder and contact gastrointestinal samples compared to the negative control samples on day-of-hatch (DOH). Additionally, there was a reduction ($P<0.05$) in 7-day BWG and significantly ($P<0.0001$) higher mortality in the contact-challenged chicks

compared to the negative control chicks in both Exp 1 and Exp 2. These data suggest that this challenge model could be utilized to evaluate different methods of controlling the bacterial bloom that occurs in the hatching environment.

In Chapter 3, the use of an innovative multi-pathogen challenge model's effects on early performance is evaluated. In a companion paper, the model is developed and compared to an egg homogenate challenge. In this manuscript, the multi-pathogen challenge is utilized as the challenge control group and applied to a second group to evaluate the effects of formaldehyde fumigation. Over the course of three experiments, significant differences were not consistently observed when evaluating performance parameters. However, significant differences ($P<0.05$) were observed in gastrointestinal tract colonization when comparing the negative control, challenge control, and formaldehyde treated groups. These data indicated that both challenge models are innovative ways to evaluate formaldehyde alternatives and their effects on early chick performance.

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Dedication

This thesis is dedicated to my husband, Owen Selby and his late mother, Gina. Owen, you have been my rock for the past seven years. You have sacrificed and supported me to continue my education. Thank you for your constant encouragement. Gina brought us dinner on the nights I was up studying and tidied the house when I was too overwhelmed. I am so thankful and blessed to have known you.

List of Published Papers

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Chapter 1

Introduction

Formaldehyde is a colorless, noxious gas that is soluble in water and utilized throughout many industries. Formaldehyde appears in construction, automotive, clothing, healthcare, and animal industries. Within the poultry industry, it has been utilized for its ability to control microbial blooms within the hatch cabinets of commercial poultry hatcheries and to prevent the transmission of pathogens from feed to bird. Low level formaldehyde exposure during the hatching process allows for the control and elimination of opportunist pathogens affecting commercial poultry. While formaldehyde can diminish potential pathogens, it also has adverse health effects on neonatal poultry as well as humans exposed to the gas. Exposure to formaldehyde has been shown to reduce tracheal ciliary function, possibly predisposing neonates for respiratory problems later in life (Sanders et al. 1995). Moreover, formaldehyde exposure can result in a significant increase in mucus production, vacuolization, and mitochondrial swelling (Zulkifli et al. 1999; Hayretadğ and Kolankaya, 2006). In humans, formaldehyde exposure causes irritation of the eyes, throat, and is considered a carcinogen (National Cancer Institute, 2011; National Toxicology Program 2011). While it does pose a threat to the health of humans and food producing animals, formaldehyde is an effective, affordable disinfectant to control pathogens affecting neonatal poultry. Selected researchers have been working on effective alternatives to formaldehyde that do not pose a threat to human or animal health.

In 2018, researchers found that the spray application of probiotic in commercial hatcheries can be as effective as formaldehyde without adverse health effects. By applying the selected probiotic isolates within a commercial hatch cabinet, chicks treated with the probiotic had

significantly less Gram-negative bacteria colonization of the gastrointestinal tract in comparison with those treated with formaldehyde fumigation (Graham, 2018).

While alternatives to formaldehyde have shown to be effective, there is no published data on their efficacy under laboratory challenge conditions. To evaluate formaldehyde alternatives, reliable, laboratory challenge models that simulate the environmental bloom of the hatch cabinet must be established.

Literature Review

Formaldehyde

Formaldehyde is a colorless, flammable gas that is known for its pungent smell and distinct odor. It is known to cause a burning sensation to the eyes, nose, and lungs at high concentrations. It is classified as a volatile organic compound meaning it is a gas at room temperature. Formaldehyde can be manufactured as a solid, known as paraformaldehyde, or liquid, known as formalin (U.S. consumer Product Safety Commission, 2016). Formaldehyde in all states has uses in industrial and laboratorial processes. It is often utilized in resins for wood products, antiseptics and cleaning agents, carpets, cosmetics, and insulation as well as the production of polyoxymethylene plastics (P. Avo, 2011). Formaldehyde air concentrations as low as 0.1-0.5 ppm can result in nasal and eye irritation as well as decreased performance on short-term memory test and an increased risk for asthma and allergies in humans. The Occupational Safety and Health Administration has set the permissible exposure limit of 0.75 ppm formaldehyde in air averaged over an 8-hour workday (Agency for Toxic Substances and Disease Registry, 2008). The Environmental Protection Agency issued formaldehyde regulation CASRM-50-00-0 along with multiple publications indicating the carcinogenicity of formaldehyde and its associates with respiratory damage to organism inhaling it (CASRM-50-00-0).

The aqueous solution of formaldehyde, known as formalin, is 37-40% formaldehyde by weight. Formalin is utilized in the laboratory for tissue fixation (Dimensteirn, 2009). This process includes the penetration of cells that stops autolysis, covalent bonding, and cross linking, allowing for tissue samples to be held for long periods of time (Buesa, 2008). In laboratory settings, formaldehyde can also be utilized to inactive viruses, bacteria, and bacterial toxins for vaccines (FDA, 2019). The reaction of formaldehyde with aqueous solutions of crystalline amino acids has been found to result in a compound described as adduct, that exhibits antimicrobial activity against some Enterobacteriaceae (Bland and Richardson, 1999). Formaldehyde targets the cell walls of bacteria, amino groups of fungi, and spore cores of bacterial spores, all common microorganisms in poultry feed and hatcheries (Ricke et al., 2019).

Formaldehyde in Animal Agriculture

Feed Sanitation

In food producing animals, specifically poultry and swine, formaldehyde treatment of the feed is common practice to prevent the transmission of enteric pathogens like *Salmonella* and *Escherichia coli*. Formaldehyde was first utilized in the treatment of feed as a mold inhibitor and was later found to reduce *Salmonella* and to improve overall feed hygiene (Spratt, 1985; Wales et al., 2010). Salmonella was first isolated from poultry feed in 1948 and was later found that the pathogen could be spread to broiler chicks if ingested by the hen, further leading to possible zoonosis (Ellis, 1969; Shapcott 1984). Of 12 *Salmonella* isolates found in a 1969 processing plant, 6 were isolated from feed (Morris et al, 1969). Researchers later reported that the serotypes of *Salmonella* in feed directly correlated to those of infected table eggs. Moreover, these serotypes could potentially infect consumers (Shirota et al, 2000). While formaldehyde has

proven to be a strong disinfectant for poultry feed, its use in commercial feed mills still poses a threat to human health.

Hatchery Sanitation

Commercial hatcheries provide the ideal environment for bacterial proliferation. During the hatching process, eggshell and feather dander spread throughout the hatch cabinet resulting in the horizontal transmission of both pathogenic and apathogenic microorganisms (Nichols and Leaver, 1967). Pathogens vertically transferred from the hen at oviposition then can spread horizontally from one infected chick to others near. (Lock et al., 1992; Cox and Pavic, 2010). A single egg has the potential to house as much as 500 CFU at time of lay and 80,000 by the time of hatch (Mauldin, 1999). This number can include Enterobacteriaceae like *Salmonella* spp., *Escherichia* spp., *Staphylococcus* spp., *Pseudomonas* spp., and *Aspergillus* spp. (Mayes and Takeballi 1983; Bruce and Johnson, 1978; Depner et al., 2016). Dust generated from the contaminated eggs can then spread to other areas of the hatchery, continuing to spread potential pathogens (Bailey et al., 1998; Mitchell et al., 2002). If not diminished, these microorganisms have the potential to decrease hatchability, chick quality, performance, increase condemnations in the plant, and possible zoonosis. Moreover, these microorganisms cause considerable economic losses for the poultry industry (Scott and Swetnam 1993; Reid et al., 1961; Kabir, 2010).

The use of formaldehyde fumigation as a disinfectant for eggs was first investigated in 1908 (Pernot, 1908). In the modern hatchery, embryos are fumigated with formaldehyde when they enter the incubator and once again when the embryos are transferred to the hatch cabinet at day 18 of embryogenesis (Zulkifili et al, 1999). Once in the hatch cabinet, formaldehyde fumigation can minimize the number of pathogenic microorganisms.

While formaldehyde has proven to be an effective antimicrobial agent, it poses health concerns to the neonatal chick as well as humans exposed in the workplace. Formaldehyde fumigation damages the cuticle of the egg and recommended avoidance of fumigation as the cuticle functions as a barrier against microorganisms (Baker and Balch, 1962). During hatch, controlling temperature and humidity is critical to chick quality. Moreover, conventional disinfectants have the potential to increase humidity and therefore negatively impact chick quality. A gas is an ideal disinfectant as it does not impact humidity and can be applied during the duration of the microbial bloom (Cadirci, 2008). However, the use of formaldehyde is also associated with the degeneration of the epithelial linings of the respiratory tract of chicks, predisposing them to increased susceptibility to respiratory disease in early days of life (Furuta et al., 1989). Once the chick pips, it begins breathing the air in the environment instead of that via air exchange within the egg shell, breathing in the formaldehyde. Because formaldehyde acts on proteins and nucleic acids, it can be hypothesized that formaldehyde gas diffused within the egg prior to hatching could result in embryonic death or damage the airways and lungs of the hatching chick (Cadirci, 2008).

In more recent years, Johnson et al., (2018) found that chicks treated with formaldehyde during the hatching process had significantly reduced performance from days 0-7 of life than those that were heat stressed and the negative control. By d10, both the formaldehyde treated and heat stressed groups had significantly ($P<0.05$) reduced performance in comparison to the negative control indicating that in the absence of high microbial blooms, formaldehyde significantly hinders performance of broiler chickens from day of hatch to d10 (Johnson et al., 2018). These data, along with the threats to human and animal health indicate a need for alternatives to formaldehyde fumigation for hatchery sanitation.

Alternatives to Formaldehyde Fumigation

While formaldehyde is an effective disinfectant in the hatchery setting, the threat it poses to human health and animal welfare have pushed researchers to find new alternatives. Researchers had identified hydrogen peroxide and probiotic fumigation as potential alternatives to formaldehyde fumigation.

Sheldon and Brake (1991) investigated the use of hydrogen peroxide as an egg disinfectant and its efficacy in comparison to formaldehyde. The pair found that 5% hydrogen peroxide significantly ($P<0.05$) reduced the bacterial load on the egg in comparison to no treatment as well as reducing early embryonic death. When comparing to formaldehyde, they observed a significant increase in hatchability as well. They concluded that hydrogen peroxide was favorable when comparing it to formaldehyde. In later experiments, Padron dipped *Salmonella* Typhimurium contaminated eggs twice in 6% hydrogen peroxide reduced the bacterial load by 95% on the surface of the shell and reduced the total number of contaminated eggs by 55% (Padron et al., 1996). It was later observed that the utilization of a 3% hydrogen peroxide fog significantly reduced the bacterial load in comparison to fogging water into the hatching environment (Sander and Wilson, 1998). When compared to ozone or UV light, 2.5 % hydrogen peroxide was found the be the most effective disinfectant of hatching eggs. Only the 2.5% hydrogen peroxide solution was able to significantly ($P<0.05$) reduce *Salmonella* load on the eggshell as well as reducing colonization on the ceca of chicks. Investigators also found that the hydrogen peroxide did not have a negative impact on hatchability (Bailey et al., 1996). It has been shown that the combination of UV light and hydrogen peroxide can result in the production of hydroxyl radicals, which act as a microbicide (Rodriquez-Romo and Yousef, 2005). The combination of 2.5% hydrogen peroxide and 8 minutes of UV light exposure resulted in a 3-log

decrease in bacterial counts in comparison to a 2-log decrease when each treatment was utilized independently (Wells et al., 2010). These data indicate that the use of hydrogen peroxide, or the pairing of hydrogen peroxide with another treatment could be an effective alternative to formaldehyde fumigation.

In more recent years, Graham et al., (2018) evaluated the use of a spray probiotic to competitively exclude potential pathogens in the hatch cabinet and as pioneer colonizers of neonatal poultry. In modern poultry production, chicks are hatched away from the hen eliminating the potential for natural microbiota providers (Kogut, 2018). Most pioneer colonizers in commercial poultry are obtained through horizontal transmission via the environment or food (Smith and Rehberger, 2018). A probiotic would be an ideal candidate for a formaldehyde alternative because beneficial microbes could potentially exclude pathogens commonly associated with the hatching environment and pose no threat to animal welfare or human health. The experimental probiotic was sprayed into the cabinet four times from transfer to hatch. Over the course of three trials, Graham et al., found that the experimental probiotic significantly ($P<0.05$) reduced Gram-negative colonization on the gastrointestinal tract on day of hatch consistently as well as 24 hours post hatch. The probiotic did not reduce total Gram-negative bacteria within the hatching environment, but it did allow for a successful colonization of the gastrointestinal tract, allowing for beneficial microbes to serve as pioneer colonizers instead of potential pathogens.

Conclusion

Formaldehyde has proven to be a useful chemical in industrial, agricultural, and laboratory settings. In animal agriculture, specifically poultry production, formaldehyde is utilized to disinfect feed as well as hatching eggs to ensure that pathogens are not transmitted to the

animals. Bacteria like *Escherichia* and *Salmonella* can be found in commercial hatcheries and pose serious threats to the health of the animal causing decreased chick quality and increased mortality resulting in economic losses for the poultry industry. The prevention of colonization is imperative to producing quality animal protein products. For many years, formaldehyde fumigation in the hatchery has been the key to reducing pathogen loads within the hatch cabinet. Formaldehyde gas sprayed into the hatch cabinet acts as an extremely effective disinfectant without increasing humidity in the hatch cabinet. However, it also poses threats to animal and human health (Fischer, 1905). Formaldehyde has been identified as a carcinogen and therefore human exposure must be limited. Formaldehyde has been shown to damage the cuticle of the egg, cause embryonic death, damage the epithelial lining of otherwise healthy chicks, predisposing them for respiratory distress, and reduce performance post hatch.

Researchers have been working to find safe, reliable alternatives for formaldehyde for many years. The utilization of hydrogen peroxide has been thoroughly investigated. Researchers found it effectively reduced bacterial loads without damaging the cuticle of the egg or embryo (Sheldon and Brake, 1998). The utilization of a spray probiotic has also been investigated showing promising results (Graham et al., 2018). The experimental probiotic was found to successfully serve as a pioneer colonizer and out compete potential pathogens in the gastrointestinal tract of day of hatch chicks as well as 24 hours post hatch. The utilization of a competitive exclusion products could effectively modulate the early microbiota of the chick, resulting in increased performance and reduced early mortality (Schneitz, 2005).

In order to evaluate formaldehyde alternatives under laboratory models, consistent challenge models must be developed to evaluate their efficacy. While some of these alternatives have been evaluated in a laboratory setting, the dipping of an egg in to a single pathogen (Padron et al.,

1995) does not simulate the environmental bloom of a hatch cabinet. It is well known that a poultry hatch cabinet houses a multitude of microorganisms including but not limited to *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., *Enterococcus* spp., *Pseudomonas* spp., and *Aspergillus* spp. (Buchanan and Gibbins, 1974; Soucy et al., 1983). Reliable laboratory challenge models must be developed that simulate both the horizontal transmission of pathogens and the microbial bloom within the hatch cabinets of commercial poultry.

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Chapter 2

Application of an *Escherichia coli* spray challenge model for neonatal broiler chickens

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Abstract

Avian pathogenic *Escherichia coli* (*E. coli*) is an opportunistic pathogen often introduced to neonatal chicks during the hatching process. This commensal bacterium, particularly as a pioneer colonizer of the gastrointestinal tract, can have substantial implications in the rearing of poultry due to reduced flock performance. In order to mimic the effects of the natural bacterial bloom present during the hatch, a seeder challenge model was developed to expose neonatal chicks to virulent *E. coli*. On day 20 of embryogenesis, selected early hatched chicks (n=18/hatcher) were briefly removed and sprayed challenged with saline (vehicle) or *E. coli* at $1x10^7$ CFU/chick (Exp 1) and $2.5x10^7$ CFU/chick (Exp 2). These challenged chicks were returned to the hatcher to serve as seeders to transmit the pathogen to the indirect challenged, or contact chicks (n=195/hatcher). For two 7-day experiments, the efficacy of transmission was evaluated via enteric bacterial recovery, body weight gain (BWG), and mortality. For Exp 1 and Exp 2, significantly ($P<0.0001$) more Gram-negative bacteria were recovered from the seeder and contact gastrointestinal samples compared to the negative control samples on day-of-hatch (DOH). Additionally, there was a reduction ($P<0.05$) in 7-day BWG and significantly ($P<0.0001$) higher mortality in the contact-challenged chicks compared to the negative control chicks in both Exp 1 and Exp 2. These data suggest that this challenge model could be utilized to evaluate different methods of controlling the bacterial bloom that occurs in the hatching environment.

Introduction

During the hatching process, humidity and temperature increase, yielding the ideal environment for bacterial and fungal growth. Microorganisms, both pathogenic and apathogenic, are horizontally transmitted throughout the hatching cabinet (Heyndrickx et al., 2002). Pathogens can be vertically transmitted from an infected hen at oviposition and then later transferred horizontally during hatch (Berchieri et al., 2001). Bacteria present at incubation can penetrate the eggshell (Lock et al., 1992; Berrang et al.,1999), resulting in the colonization and horizontal transfer of microorganisms. During hatch, chicks may be exposed to hours of heat stress, increasing the possibility of being colonized by a pathogen (Lara and Rostagno, 2013). While there are many microorganisms present in hatch cabinets, *Escherichia coli* (*E. coli*) is one of the most prevalent (Graham et al., 2018). *E. coli* is a Gram-negative, opportunistic pathogen that serves as a pioneer colonizer of the gastrointestinal tract of chicks (Lu et al., 2003). It is often observed under stress or co-infection in chickens; therefore, playing a significant role in chick quality and health (Reid et al., 1960). The infection of an avian pathogenic *E. coli* (APEC) can result in septicemia, omphalitis, and high mortality in commercial broiler houses (Kendler et al., 1967). Pathogens with tropisms for the gastrointestinal tract have also been found to be transmitted via the respiratory route (Kullapura et al., 2014). Due to APEC having a tropism for both the respiratory tract and the gastrointestinal tract (Barnes and Gross, 1997), respiratory transmission during hatch is a concern. Moreover, APEC isolates have been described as resulting in substantial economic losses for the industry (Kabir et al., 2010).

Seeder challenge models have previously been used in food-producing animals to evaluate the horizontal transmission of pathogens (Lechtenberg et al., 1994; Michiels et al., 2012; Graham et al., 2019). A seeder model may be utilized to simulate commercial hatching conditions where the

entire hatch cabinet may become contaminated by a very low number of initial infected eggs or chicks (Gross, 1997). As chicks hatch, the high temperature and increased humidity serve as ideal environment conditions to promote the natural amplification of microbes, also known as the "bloom", and these pathogens horizontally spread throughout the hatching cabinet. The purpose of the presented study was to evaluate the effect of a virulent *E. coli* spray challenge seeder model on early performance parameters.

Experimental Design

E. coli culture and challenge

A virulent, non-lactose fermenting serotype O2 *E. coli*, previously associated with colisepticemia and mortality in both chickens and turkeys, was selected for these experiments (Huff et al., 2002; 2003). In these studies, 500µL of *E. coli* was removed from a frozen aliquot and added to 50mL of tryptic soy broth (tryptic soy broth, cat. no. 90000-378, VWR, Suwanee, GA 30024). The culture was incubated at 37°C for 18 h. Post-incubation, bacterial cells were washed with sterile 0.9% saline by centrifugation at 1,800 × *g* for 15 min and resuspended in saline. The wash procedure was completed three times. *E. coli* colony-forming units (CFU) enumeration was determined by the shake plate method on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024) to determine the estimate stock concentration and then cells were held overnight for approximately 16 h at 4°C (Sanders, 2012). The culture was then diluted to the desired CFU concentration for spray challenge (d 20 of embryogenesis, n=18 selected from early hatched chicks at 20% pip). *E. coli* challenge dose (CFU/mL) was confirmed as described above and reported in each experiment. Each seeder chick was removed from the hatching environment, spayed on its chest and back using a calibrated hand pump sprayer to

deliver approximately 0.5 ml inoculum at each location, and then immediately returned to the hatching environment to potentially horizontally transmit the pathogen.

Enumeration of bacteria

For both experiments, whole gut samples (ventriculus to cecum) were aseptically removed and collected into sterile tissue collection bags. Samples were weighed, homogenized, and 1:4 wt/vol dilutions were made using sterile 0.9% saline. Ten-fold serial dilutions of each sample, n=12 samples from each group, were made in sterile 96-well Bacti-flat bottom plates and the serially diluted samples were plated on culture media. Evaluation of the total number of presumptive lactic acid bacteria (LAB) was completed on De Man, Rogosa, and Sharpe agar (Difco Lactobacilli MRS Agar, cat. no. 90004–084, VWR, Suwanee, GA 30024), as well as enumeration of presumptive Gram-negative bacteria, specifically with colonies with lactose-negative morphology (challenge strain is non-lactose fermenting), on MacConkey agar (MacConkey Agar, cat. no. 89429–342, VWR, Suwanee, GA 30024). All plates were incubated at 37°C for 18 h and bacterial counts were expressed as Log_{10} CFU/g of sample.

Development of an Escherichia coli spray challenge model for neonatal broiler chickens

The objective of both experiments 1 and 2 was to evaluate the horizontal transmission of the pathogen by measuring the bacterial colonization in the gastrointestinal tract (GIT) at DOH in both seeder and contact chicks of both treatments by measuring bacterial colonization at d 7 and evaluating the challenge's impact on performance. Mortality was recorded throughout the 7-day trial period in each experiment. In each trial, embryonated Ross 308 broiler hatching eggs were candled at d 18 of incubation and placed into separate hatchers (G.Q.F. Manufacturing 1602N Hova-Bator Incubator with a circulating air fan kit) at random. Hatcher units were housed in separate facilities to prevent possible contamination between treatments during the hatch. On d

20 of embryogenesis, at 20% pip, seeder chicks (n=18 seeders/hatcher or 8.45%) were inoculated with 1mL of E. coli or 1mL 0.9% sterile saline (vehicle) per chick via spray. On d 21, dry chicks were removed from the hatchers, hatchability was recorded, and select chicks (n=12 per group) were euthanized to evaluate presumptive LAB and Gram-negative bacteria as previously described. The confirmed seeder challenge dose was $1x10^7$ CFU/mL/chick for Exp 1 and $2.5x10^7$ CFU/mL/chick for Exp 2. In both experiments, negative control chicks were weighed and allocated into eight pens (n=20/pen) and the contact-challenged chicks were weighed and allocated into 16 pens (n=20/pen). Weight allocation on DOH was performed to normalize BW and prevent initial treatment effects on BW. Pen BW was determined at placement and on d 7 to determine BWG. Mortality was recorded for the duration of each 7-day trial period. Chicks were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean meal diet meeting the nutritional requirements for broilers recommended by Aviagen (Aviagen, 2019). All experiments and animal handling procedures complied with the University of Arkansas Institutional Animal Care and Use Committee under permit #18079.

Statistical Analysis

All data were subjected to one-way analysis of variance at 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 means were further separated using Tukey's multiple range test for presumptive LAB and Gram-negative bacterial recovery. The pen was the experimental unit for the BW data and means were separated using Student's t-test on DOH and day 7. Mortality was compared using the chi-square test of independence to determine the significance threshold ($P<0.01$) for these studies (Zar, 1984).

Results and Discussion

Under commercial conditions, chicks may be exposed to APEC isolates in the hatching cabinet which cause colisepticemia, airsacculitis, and increased early chick mortality resulting in significant economic losses for the poultry industry (Kendler and Harry, 1967). Thus, a laboratory model could be utilized to evaluate the effects of exposure to APEC isolates during the hatching phase. At d 20 of embryogenesis, seeder chicks were inoculated via a spray with a virulent *E. coli* or saline vehicle and then placed back into the hatching cabinet to horizontally transmit the pathogen.

On DOH, there was a significant increase ($P<0.0001$) in presumptive Gram-negative recovery from GIT samples from the seeders and contact chicks. Results were consistent in both Exp 1 and 2, indicating that spraying the inoculum on seeder chicks horizontally transmitted the pathogen during hatch (Table 1). Significant differences ($P<0.0001$) in LAB were also observed between the challenged groups and negative control (Table 1), indicating that Gram-negative bacteria may contribute to colonization by LAB (Wilson et al., 2020). LAB are naturally found in the gastrointestinal tract of animals (Rine et al., 2019). Some researchers believe LAB plays a role in restoring the natural microflora after an infection (Higgins et al, 2009). This could potentially contribute to the amplification of LAB post-challenge reported in the present experiments. No significant differences were observed between the challenge and negative control groups on d 7 for presumptive Gram-negative recovery (data not shown).

The *E. coli* isolate used in these experiments was chosen based on negative impacts on both performance and mortality in previous experiments (Huff et al., 2002; 2003). In Exp 1 and 2, differences ($P<0.02$) in 7-day BWG were observed, indicating that the challenge had a negative

impact on performance (Table 2). The 7-day mortality was significantly ($P<0.001$) higher in the challenge group than the negative control group (Table 2).

Spraying select early hatching chicks, also known as seeder chicks, at d 20 of embryogenesis, effectively transmitted the pathogen throughout the hatching cabinet resulting in an increase in Gram-negative recovery at DOH, presumptive LAB at DOH, 7-day mortality, and a negative impact on 7-day BWG. In a commercial hatchery, chicks are exposed to Gram-negative bacteria that serve as pioneer colonizers of the GIT (Graham et al., 2018). Further research is being conducted to evaluate potential alternatives of pathogen control within the hatch cabinets employing seeder challenge models to mimic commercial conditions.

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Tables and Figures

Table 1. Presumptive Gram-negative and lactic acid bacteria (LAB) recovered from gastrointestinal tract at day-of-hatch (Exp 1 & Exp 2)

Treatment	Gram-negative bacteria[1] (Log_{10})		LAB (Log_{10})	
	Exp 1	Exp 2	Exp 1	Exp 2
Negative Control	0.00±0.00 [c]	0.00±0.00 [c]	0.00±0.00 [b]	0.25±0.25 [b]
Spray Challenged Seeder Chicks	8.30±0.11 [a]	7.78±0.45 [a]	6.53±0.92 [a]	4.74±0.82 [a]
Spray Challenged Contact Chicks	4.09±0.71 [b]	5.19±0.79 [b]	4.17±0.85 [a]	3.87±0.95 [a]

[a,b, c] Indicates significant differences between treatments per column (P<0.05).
[1]Data are expressed as mean log_{10} CFU/g ± SE.

Table 2. Effect of virulent *E. coli* horizontal transmission on average BWG and 7-day mortality in neonatal broiler chickens (Exp 1 & Exp 2)

Treatment	7-day BWG (g)[1]		Mortality (%)[2]	
	Exp 1	Exp 2	Exp 1	Exp 2
Negative Control	129.52±3.55 [a]	133.02±3.43 [a]	0 [y]	0 [y]
Spray Challenge Contact	115.89±3.07 [b]	119.05±3.06 [b]	10.31[z]	15.62 [z]

[1] Data are expressed as mean ±SE .
[a,b] Indicates significant differences between treatments (*P*<0.05).
[2] Data are expressed as number of deaths / total (%).
[y, z] Indicates significant differences between treatments (*P*<0.001).

Chapter 3

Evaluation of a multi-pathogen challenge models' effects on early performance of broiler chickens

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Abstract

Hatchery sanitation and hygiene are imperative to the production of healthy broilers and turkeys. Commercial hatch cabinets are known to harbor a variety of pathogenic and apathogenic microorganism including bacteria and fungi. These microorganisms can serve as pioneer colonizers of the gastrointestinal tract of poultry. Some of these pioneer colonizers, such as *Escherichia coli* and *Enterococcus* spp., are opportunistic enteric pathogens that lead to reduced performance at the broiler or breeder farm. Formaldehyde fumigation has been traditionally used to reduce the pathogen load in commercial hatch cabinets. To investigate potential alternatives, effective challenge models under laboratory conditions must be developed. In a companion paper, a multi-pathogen challenge model (PM) was developed and evaluated for its ability to simulate the microbial bloom that occurs in commercial hatch cabinets. The purpose of this manuscript is to evaluate the impact of PM challenge with and without formaldehyde fumigation on early performance in broiler chick. Three experiments were conducted to evaluate microbial contamination in the hatch cabinet environment (air samples, fluff samples), enteric colonization at day-of-hatch, and 7-day performance. In all experiments, there was significantly more ($P<0.05$) Gram-negative bacteria recovered from PM challenge control group compared to the non-challenged control (NC) and formaldehyde treated group (PM + F) from the GIT on DOH. There were no statistical differences in 7-day body weight gain or feed conversion ratio between the PM challenge group and NC or PM + F groups. These data suggest this model could be utilized to evaluate alternatives to formaldehyde fumigation in a laboratory setting. Seven-day performance parameters may be dependent upon the pathogenicity of the inoculum and are not consistently affected in the present experiment.

Introduction

Commercial poultry hatch cabinets harbor a plethora of apathogenic and pathogenic microbes. These potential pathogens can serve as pioneer colonizers to hatching chicks, negatively impacting chick quality, performance, and leading to possible zoonosis (Rehkopf et al., 2017). While mammalian species initial microbiota from their mother, this does not occur in commercial poultry (Kogut, 2018). Instead, pioneer colonizers come from their initial environment, specifically the hatching environment, broiler facility, or feed (Kogut, 2019). The hatching environment is warm and humid, creating favorable conditions for microbial blooms (Heyndrickx et al., 2002). After collection from the breeder flock, embryos are fumigated with formaldehyde to minimize the bacterial load within the hatch cabinet (Cidirci, 2008). Formaldehyde has been utilized to control the microbial bloom in the hatch cabinet for over 100 years (Pernot, 1908). While this is an effective disinfectant, formaldehyde fumigation is known it cause embryonic death and damage the egg protective cuticle (Whistler and Sheldon, 1989). Post hatch, formaldehyde has also been shown to damage the epithelial lining of the respiratory tract, predisposing the chicks for respiratory illness and decrease performance (Sanders et al., 1995). Johnson et al. (2018) showed that exposure to formaldehyde in the hatch cabinet significantly reduced performance in 7- and 10-day old broiler chickens compared the non-treated group.

Embryos are candled at transfer to prevent non-viable embryonated chicks from entering the hatch cabinet. In the warm hatching environment, these non-viable embryonated chicks, also known as potential “exploder eggs”, have the potential to explode due to bacterial overgrowth and gas production (Smith et al., 2005). These bacteria then spread throughout the hatch cabinet on to the surface of other viable embryos (Liu and Ngadi, 2013). If the cuticle has been damaged by formaldehyde fumigation, the potential for the neonate to be colonized by a pathogen could

increase as the cuticle has known antimicrobial properties (Wellmen-Labadie et al., 2008). During the hatching phase, microbes circulate throughout the hatch cabinet and serve as pioneer colonizers of the gastrointestinal tract (Mitchell and Waltman, 2003). If colonized by opportunistic pathogens obtained from the hatchery, flock performance could be severely impacted (Kabir, 2010). The poultry industry previously utilized growth promoting antibiotics in starter diets to reduce the severity of infections from opportunistic pathogens. In recent years, this practice has been limited to prevent antimicrobial resistance which has been associated with an increase in early mortality and reduced performance (Casewell et al., 2003; Gayatri et al., 2018).

Probiotic application in the hatch cabinet has been investigated with promising results. In a commercial trial, Graham et al., found a significant reduction of Gram-negative colonization within the gastrointestinal tract of day-of-hatch chicks as well as 24 hours post hatch. A probiotic is an ideal alternative to formaldehyde because it could competitively exclude the pathogenic microorganisms as pioneer colonizers, positively influencing early mortality and broiler performance (Graham et al., 2018).

To accurately compare formaldehyde to alternative disinfectants or competitive exclusion products, an effective challenge model simulating commercial conditions in laboratory settings must be developed. Our lab has previously developed horizontal transmission models within the hatch cabinet (Graham et al., 2019; Selby et al., 2021). These models utilized wild type or virulent *Escherichia coli* isolates in a seeder challenge model. By using a seeder challenge model, only selected embryos or chicks were challenged with the isolate and horizontally transmitted the pathogen throughout the cabinet. The *in ovo* administration of a virulent *E. coli* along with tetracycline to seeder embryos on d19 of embryogenesis increased Gram-negative

recovery from the GIT of contact chicks. Additionally, there was a significant (P<0.05) increase in mortality and reduced body weight gain from d0-7 for contact chicks in two of the three trials compared to the non-challenged control (Graham et al., 2021). In another study, at 20% hatch at DOE20, a subset of the initial chicks that hatched were sprayed with $1x10^7$ CFU/ml of virulent *E. coli* and placed back into the hatch cabinet to horizontally spread the pathogen. At DOH, Gram-negative bacterial recovery of the GIT was significantly (P<0.05) increased and early performance was significant affected for contact chicks (Selby et al., 2021).

Although challenge models that have used a singular challenge organism have been effective (Graham et al., 2021; Selby et al., 2021), a more representative challenge model to mimic commercial hatchery conditions should include multiple pathogens. As previously discussed, there are a multitude of pathogenic and apathogenic bacteria and fungi that are ubiquitous in commercial hatch cabinets. *E. coli*, *Staphylococcus* spp., *Enterococcus* spp., and *Aspergillus* spp. have all been isolated from commercial hatcheries and infected chicks (Wright et al., 1960; Whistler and Sheldon, 1989; Rodgers et al., 1999; Kense and Landman, 2011). Our lab has developed an innovative multi-pathogen challenge model to simulate the microbial contamination in commercial hatcheries under laboratory conditions (Graham, 2021). This challenge model involved the application of two wild type *E. coli* isolates, *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Enterococcus faecalis*, and *Aspergillus fumigatus* on the surface of the eggshell. At day 19 of embryogenesis (DOE), 100 µl of the pathogen mix (PM) was spread on a 28 mm area utilizing a sterile disposable loop, simulating an "exploder" egg. Extensive environmental sampling was used to enumerate selected pathogen circulation within the hatch cabinet, along with fluff sampling, chick rinses, and gastrointestinal tract sampling in order to compare our pathogen mix to a homogenate of material recovered from non-viable

embryonated eggs (EH) combined with a vehicle and was applied the same way as the PM. We found the PM to be a more suitable challenge than the EH, due to its consistent increase of Gram-negative bacteria in the fluff sampling, chick rinse, and gastrointestinal tract. The PM challenge also consistently elevated *S. aureus*, *Enterococcus* spp., and *A. fumigatus*, proving to be a consistent challenge method in comparison to the EH.

The purpose of the present work was to evaluate the PM challenge model on early performance and mortality of broiler chickens as well as the effects of formaldehyde fumigation post challenge.

Materials and Methods

Three experiments were conducted (Exp 1-3) to compare a non-challenged control to the PM challenge control, and PM challenge with formaldehyde fumigation. For each experiment, 1,701 fertile eggs (n=189 per hatcher x 3 hatchers per treatment x 3 treatments) were placed into separately assigned hatch cabinets to prevent cross contamination. Eggs for each hatcher were placed in a block random fashion to minimize potential source flock or incubator bias. All eggs were candled to ensure non-viable embryonated eggs were removed. As previously described, 100 µl of the PM challenge with 0.01% xanthan gum was applied on the morning of d19 of embryogenesis. Utilizing a disposable, sterile loop, the PM was spread on 28 mm of the blunt end of the eggshell (Graham, 2021). Post challenge, embryos were placed back into the hatch cabinet. In the PM challenge with formaldehyde group, the hatch cabinet was fumigated via drip application of 6 mL of formalin every 3 hours post challenge (Graham et al., 2021). Formaldehyde fumigation ceased 12 hours before hatch pull, approximately 7 pm DOE 20. On d21 of embryogenesis, dry chicks were pulled from hatch cabinet. Selected chicks (n=15/treatment) were immediately euthanized and utilized for gastrointestinal tract sampling

(GIT) for enumeration of relevant enteric pathogens. Each sample was homogenized with sterile saline, 10-fold diluted, and plated onto selective agar plates to enumerate various pathogens. After each experiment, hatch cabinets were disinfected, dried, and fumigated with formaldehyde to prevent contamination between experiments. In all three experiments, remaining chicks were weighed and allocated into 16 pens per treatment (n=20/pen). Weight allocation on DOH was performed to normalize and prevent treatment effects on BW. Pen BW was determined on DOH and day 7 to evaluate BWG. Feed consumption was also measured to evaluate FCR. Mortality was recorded throughout the duration of the trial. Chicks were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean meal diet meeting nutritional requirements for broilers recommended by Aviagen (Aviagen, 2019).

Animal Source

For all experiments, 18-day old Ross 308 embryos were candled, randomly allocated, and placed into separate hatchers based upon treatment group. All experiments and animal handling procedures complied with the University of Arkansas Institutional Animal Care and Use Committee guidelines permit #20017.

Challenge preparation

Bacterial and Fungal Isolates

The preparation of the PM challenge for each experiment involved 1 ml of each *E. coli*, *S. aureus*, *S. chromogens*, or *E. faecalis* was removed from a frozen aliquot and added to 100 ml of tryptic soy broth (TSB). *Staphylococcus* spp. cultures were incubated on an orbital shaker, while others were not. The cultures were incubated at 37C for 18 hrs. Post incubation, each culture was washed three times with 0.09% sterile saline by centrifugation at 1,800 x *g* for 15 minutes. Colony-forming units (CFU) was determined by serial dilution and plating on respective agar to

determine stock concentration. Stock was held overnight at 4 °C until stock concentration was determined. At DOE19 (day of challenge), each presumptive pathogen stock was concentrated by centrifugation based upon desired CFU concentration for challenge. An aliquot of *A. fumigates* was thawed and sterilely swabbed onto Sabouraud dextrose agar (SDA) supplemented with chloramphenicol 50g/L. This method was adopted by Graham et al., during the initial development of this model based upon Sala et al. (1972) and NIH model for invasive Aspergillosis. Challenge was confirmed using a hemocytometer and spread plating on SDA supplemented with chloramphenicol 50g/L as described by companion paper.

The concentrated bacterial cells and *A. fumigatus* spores were combined and resuspended in 2X TSB vehicle along with 1% xanthan gum. (Graham, 2021). The PM was then plated on selected media to confirm challenge. Confirmed challenge doses are reported in Table 1.

Enumeration of Bacteria and Fungi

Environmental sampling

The open-agar plate method was utilized to enumerate selected pathogens circulating within the hatch cabinet (Kim et al., 2010; Graham et al., 2018). For each selective media, three agar plates were placed open side up on top tray of hatchers (G.Q.F. 1550 Digital Cabinet Egg Incubator) using a modified sample port (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 *A. fumigatus* presence in the hatching environment. The open agar plates were placed in the hatch cabinet for 1 or 5 minutes depending on media type. MacConkey agar and SDA were both placed in hatching environment for five minutes (Graham et al., 2021; Graham, 2021). All other selective

media were placed in hatch cabinet for 1 minute. Environmental sampling took place at 4 time points over the hatching period specific to percent hatch. Samples were taken at 20% hatch or 8:00 am DOE 20, 50% hatch or 2:00 pm DOE 20, 80% hatch or 5:00 pm, DOE 20, and 100% hatch or 7:00 am DOE 21/DOH. Post sampling, agar plates were incubated at 37 °C for 18 hours. In order to enumerate *Staphylococcus* spp. and *A. fumigatus*, selected agar plates were incubated for 48 hours.

Gastrointestinal Tract

In each experiment, the GIT samples (n=5 chicks/hatcher, n=15 per treatment) from ventriculus to cecum, were aseptically removed and collected into sterile bags. They were then weighed and homogenized in a 1:4 wt/vol dilutions using 0.9% sterile saline. Ten-fold dilutions of each sample were made in a 96 well bacti-flat bottom plates and diluted plates were plated to evaluate presumptive Gram-negative bacteria, *Staphylococcus* spp., and *Enterococcus* spp on selective media. All plates were incubated aerobically at 37 °C. MacConkey and CO agar plates were incubated for 18 hours while MRS and MSA plates were incubated for 48 hrs. Bacterial counts are expressed as Log_{10} CFU/g of sample.

Fluff Sampling

At hatch, ~1 g of fluff was collected from each cabinet (n=3 samples/treatment). Gloves were changed between hatch cabinets to prevent cross contamination and eggshells were avoided. Fluff samples were weighed, diluted with sterile 0.9% saline at a 1:50 w/v dilution, and homogenized prior to drop plate samples on MacConkey agar, tryptic soy agar (TSA) agar, CO agar, MSA agar, and SDA plates. All plates were incubated aerobically at 37 °C for 18 hours.

Statistical Analysis

All data was subjected to a one-way analysis of variance at a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). The pen is the experimental unit for BW and FCR data. Means were separated using Tukey's multiple range test for all data. Mortality was compared using the chi-square test of independence to determine the significant threshold (P<0.01) (Zar, 1984).

Results

Bacterial Recovery from Hatching Environment (DOE 20-DOH)

In Exp 1-3, environmental sampling took place at four timepoints: 1) DOE 20 8:00am (20% hatch), 2) DOE 20 2:00pm (50% hatch), 3) DOE 20 5:00pm (80% hatch), and 4) DOE 21/DOH 7:00am (100% hatch, DOH). These timepoints were consistent across all experiments. Select bacterial and fungal recovery from each timepoint and experiment is shown in Table 3. In Exp 1 contamination associated with embryo source was apparent as the NC treatment had the highest Gram-negative recovery at 80% hatch (>100 CFU). Low level contamination (<100 CFU) was observed in each experiment. The PM challenged group had increased bacterial recovery in comparison to the Negative Control and PM + Formaldehyde treatments in all both Exp 2 and 3. Formaldehyde reduced the microbial load within the hatching environment across experiments.

GIT samples at hatch

Tables 3-5 represent the mean bacterial recovery from the GIT on DOH. Gram-negative recovery was significantly (P<0.05) higher in the PM challenged group in comparison to the negative control and challenge + formaldehyde group in all three experiments. In Exp 1 and 3, *Enterococcus* spp. recovery was significantly (P<0.05) higher in the PM challenged group in

comparison to the other groups as well. No differences in presumptive *S. aureus* recovery were observed in Exp 1-3.

Fluff samples at hatch

Tables 3-5 represent mean bacterial recovery from fluff samples taken on DOH. In Exp 1-3, the PM Control group had markedly ($P<0.05$) higher *Enterococcus* spp. and presumptive *S. aureus* recovery when compared to the negative control and PM + formaldehyde groups. Non-mannitol fermenting presumptive *Staphyloccus* spp were not detected from any of the samples collected in three samples. Gram-negative recovery was significantly ($P=0.0017$) higher than both the negative control and PM + formaldehyde groups in Exp 3. In Exp 2, the PM control was significantly higher than the PM + formaldehyde group, but not the negative control when evaluating Gram-negative recovery. In Exp 1, *A. fumigatus* recovery was highest in the PM + formaldehyde treatment group, indicating that formaldehyde, as used in the present studies, might not always be effective against *A. fumigatus.* Only in Exp 2 was *A. fumigatus* recovery significantly ($P<0.001$) higher in the PM group in comparison to the other treatment group. In Exp 3, no differences for *A. fumigatus* recovery were observed.

Performance Parameters

Body weight gain and feed conversion means are reported in Table 6 and percent mortality from d0-7 is reported in Table 7. Only in Exp 1 were significant differences observed during the trial period when evaluating performance parameters. In Exp 1, the PM + formaldehyde group had a higher ($P=0.0331$) body weight gain than the negative control. No other differences were observed in BWG, FCR, or mortality throughout experiments.

Discussion

Effective sanitation and egg handling are important for production of healthy chicks. Moreover, hatchery conditions in commercial poultry are known to have an effect on overall performance. (Lazarov et al., 2018). Embryos can enter the hatch cabinet colonized with pathogens transferred from the hen at oviposition, on the surface of the egg, or non-viable eggs can explode, resulting in pathogenic microorganisms being further spread throughout the hatch cabinet. As chicks are hatching, they begin to interact with the environment around them, up taking microorganisms that could potentially serve as pioneer colonizers of the gastrointestinal tract. It is well understood that bacterial contamination increases toward the hatching process (Magwood, 1964; Kim, 2010). Chick fluff is known to harbor and spread enteric pathogens specific to poultry (Warren et al., 2016). Investigators have found that chick fluff is one of the best representative samples of microorganisms within the hatch cabinet and can be utilized to evaluate hatchery sanitation (Chen 2002; Gehan, 2009). These pathogens are then given the chance to horizontally spread within the hatch cabinet and later at the farm (Lazaroy et al., 2018). Enterobacteriaceae isolated from chicks with omphalitis have been isolated from the air in originating hatcheries (Chute and Gershman, 1978).

Formaldehyde has proven to be an effective sanitation method for commercial poultry as it is effective against a multitude of microorganisms and inexpensive (Sheldon and Brake, 1991). However, in the United States it has limited exposure limits due to it being a known carcinogen (Wilson and Mauldin, 1989). Practical and effective alternatives for formaldehyde fumigation are needed but remain elusive. An ideal alternative would not increase humidity within the cabinet, damage the egg cuticle, or pose a threat to animal welfare or human health. Testing potential products under commercial conditions can be difficult and expensive. Moreover,

challenge models under laboratory conditions must be developed to accurately simulate the microbial bloom in commercial hatch cabinets. Single pathogen models have been utilized that involve the dipping of eggs into a selected pathogen as well as the *E. coli* seeder models developed in our lab (Padron, 1996; Graham et al., 2021; Selby et al., 2021). The use of PM model has proven to effectively simulate the microbial bloom in commercial hatch cabinets (Graham, 2021). By evaluating the environment, chick fluff samples, chick rinsing, and gastrointestinal tract sampling, our lab has shown that the mix *E. coli*, *Staphylococcus*, *Enterococcus*, and *A. fumigatus* effectively colonizes the gastrointestinal tract of chicks and simulates the microbial bloom in that cabinet.

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Tables and Figures

Table 1. Challenge dose (CFU/100µL/egg) for each presumptive pathogen in PM Exp 1-3

Isolate in PM	Exp 1	Exp 2	Exp 3
Gram-negative bacteria	$1.07x10^8$	$3.33x10^8$	$8.61x10^7$
Enterococcus spp.	$4.67x10^7$	$4.0x10^7$	$5.33x10^7$
Presumptive *S. aureus*	$7.67x10^7$	$4.0x10^7$	$1.63x10^8$
Total Aerobic Bacteria	$3.33x10^8$	$9.67x10^7$	$2.67x10^8$
A. fumigatus	$1.0x10^5$	$1.0x10^5$	$1.0x10^5$

Challenge dose (CFU/100µl/egg) reported as an average of 3 replicates

Table 2. Selected bacterial recovery (CFU/plate) from the hatching environment at DOE 20 (20%, 50%, or 80% hatch) or at DOH prior to hatch pull for Exp 1-3

Exp	Trt	Gram-negative bacteria				Presumptive *S. aureus*				*Enterococcus* spp.				*A. fumigatus*			
		~20%	~50%	~80%	DOH	~20%	~50%	~80%	DOH	~20%	~50%	~80%	DOH	~20%	~50%	~80%	DOH
Exp 1[1]	NC	0.00	3.33	142.78	44.44	0.22	1.11	37.11	7.78	0.00	17.56	53.89	141.22	0.11	0.11	0.00	0.00
	PM	4.56	20.7	41.50	48.22	11.00	15.67	17.89	15.00	23.22	51.78	71.89	274.67	1.89	17.89	29.78	6.22
	PM + Form	0.11	0.11	13.44	51.00	1.00	1.33	0.11	1.33	0.78	4.89	11.89	53.78	0.56	4.50	9.67	2.44
Exp 2	NC	0.00	3.33	4.56	7.89	0.67	0.33	0.22	1.11	0.00	0.00	0.00	18.00	0.11	0.00	0.00	0.00
	PM	0.00	4.33	32.78	9.00	0.11	1.67	1.44	5.89	0.00	6.44	4.78	17.67	0.00	5.56	5.56	8.11
	PM + Form	0.00	0.22	2.78	2.11	0.00	0.11	0.00	1.22	0.00	0.56	0.33	0.67	0.11	8.11	1.22	3.00
Exp 3	NC	0.00	0.00	10.89	0.00	0.22	0.00	0.00	0.00	0.00	1.78	0.00	0.00	0.00	0.00	0.00	0.00
	PM	9.89	9.33	20.67	15.22	5.00	8.78	8.22	7.33	13.78	20.22	41.00	90.67	7.00	17.44	12.44	5.11
	PM + Form	0.00	1.00	0.22	2.56	0.22	0.00	0.78	0.00	0.00	0.00	0.00	2.00	1.00	0.44	0.11	1.44

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

Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)

n=3/treatment placed in hatching environment for 1 or 5 minutes based upon selective media

Table 3. Selected bacterial or fungal recovery from GIT and chick fluff samples on DOH in Exp 1

GIT (Log_{10} CFU/g) [1]	**NC**	**PM**	**PM +Form**	***P*-value**
Gram-negative bacteria	4.05±0.71 [b]	6.97±0.49 [a]	4.52±0.75 [b]	0.0071
Presumptive *S. aureus*	0.00±0.00	0.24±0.92	0.00±0.00	0.3765
Enterococcus spp.	2.54±0.71 [b]	6.14±0.62 [a]	0.97±0.46 [b]	<0.001
Fluff (Log_{10} CFU/g)	**NC**	**PM**	**PM+Form**	***P*-value**
Gram-negative bacteria	4.83±0.32	4.59±0.16	4.54±0.16	0.6904
Enterococcus spp.	0.74±0.74 [b]	5.21±0.23 [a]	5.14±0.13 [a]	0.001
Presumptive *S. aureus*	2.00±1.00 [a]	0.00±0.00 [b]	0.00±0.00 [b]	0.0304
A. fumigatus	0.00±0.00 [b]	1.23±0.62[ab]	2.05±0.65 [a]	0.0317

[1]Log_{10} CFU/g reported as the mean of 15 replicates for GIT and 9 samples for fluff
Non-mannitol fermenting *Staphylococcus spp* were not detected in GIT or fluff samples
Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)
Data are expressed as mean log_{10} CFU/g ± SE.
[a,b,] Indicates significant differences between treatments per column ($P<0.05$)

Table 4. Selected bacterial or fungal recovery from GIT and chick fluff samples on DOH in Exp 2

GIT (Log_{10} CFU/g) [1]	**NC**	**PM**	**PM +Form**	***P*-value**
Gram-negative bacteria	0.00±0.00 [b]	5.50±0.51 [a]	0.58±0.58 [b]	<0.001
Presumptive *S. aureus*	0.00±00	0.20±0.20	0.00±0.00	0.3765
Enterococcus spp.	0.00±0.00	0.38±0.26	0.90±0.51	0.1679
Fluff (Log_{10} CFU/g)	**NC**	**PM**	**PM+Form**	***P*-value**
Gram-negative bacteria	3.86±0.98 [ab]	4.56±0.64 [a]	1.45±0.75 [b]	0.0289
Enterococcus spp.	2.34±1.17 [b]	5.29±0.14 [a]	1.92±0.41 [b]	0.0137
Presumptive *S. aureus*	1.45±0.73 [b]	4.11±0.53 [a]	0.41±0.41 [b]	0.0004
A. fumigatus	0.00±0.00 [b]	3.51±0.46 [a]	1.23 ±0.62 [b]	<0.001

[1]Log_{10} CFU/g reported as the mean of 15 replicates for GIT and 9 samples for fluff
Non-mannitol fermenting *Staphylococcus spp* were not detected in GIT or fluff samples
Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)
Data are expressed as mean log_{10} CFU/g ± SE.
[a,b,] Indicates significant differences between treatments per column (P<0.05)

Table 5. Selected bacterial or fungal recovery from GIT and chick fluff samples on DOH in Exp 3

GIT (Log_{10} CFU/g)	**NC**[1]	**PM**	**PM +Form**	***P*-value**
Gram-negative bacteria	0.00±0.00 [b]	4.78±0.53 [a]	02.40±0.76 [b]	<0.001
Presumptive *S. aureus*	0.00±0.00	0.00±0.00	0.00±0.00	1
Enterococcus spp.	0.00±0.00 [b]	5.46±0.49 [a]	0.00±0.00 [b]	<0.001
Fluff (Log_{10} CFU/g)	**NC**	**PM**	**PM+Form**	***P*-value**
Gram-negative bacteria	2.14±1.07 [b]	5.15±0.38 [a]	0.93±0.62 [b]	0.0017
Enterococcus spp.	0.00±0.00 [b]	6.11±0.39 [a]	0.89±0.59 [b]	<0.001
Presumptive *S. aureus*	0.00±0.00 [b]	4.56±0.18 [a]	0.00±0.00 [b]	<0.001
A. fumigatus	0.00±0.00	1.23±0.62	0.41±0.00	0.1379

[1]Log_{10} CFU/g reported as the mean of 15 replicates for GIT and 9 samples for fluff
Non-mannitol fermenting *Staphylococcus spp* were not detected in GIT or fluff samples
Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)
Data are expressed as mean log_{10} CFU/g ± SE.
[a,b,] Indicates significant differences between treatments per column ($P<0.05$)

Table 6. Effect of PM challenge on average BWG and FCR (Exp 1-3)

Treatment	**BWG d0-7**[1]			**FCR d0-7**		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
NC	111.66±2.31 [b]	103.64±2.21	99.34±0.99	1.33±0.05	1.36±0.05	1.41±0.22
PM	117.98±2.04 [ab]	102.21±1.84	100.37±1.57	1.30±0.03	1.39±0.04	1.44±0.04
PM + Form	119.87±2.00 [a]	102.57±2.16	96.96±1.55	1.29±0.10	1.32±0.04	1.40±0.04
P-value	0.0331	0.8800	0.2204	0.7138	0.5246	0.7760

[1] data are expressed as mean body weight gain in grams ± SE
Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)
[a,b,] Indicates significant differences between treatments within columns ($P<0.05$)

Table 7. Effect of PM challenge on mortality by percentage from d0-7

Treatment	**Mortality %**[1]		
	Exp 1	Exp 2	Exp 3
Negative control	0.938	0.89	0.00
PM control	1.250	1.19	0.00
PM + Formaldehyde	0.625	0.60	0.595

[1] Data are expressed as number of deaths / total (%).
Negative Control (NC); Pathogen Mix (PM); and Pathogen Mix+ Formaldehyde (PM + Form)

Overall Conclusion

In Chapter 2, the spray challenge model proved to be an effective model to evaluate horizontal transmission within the hatch cabinet. The present manuscript was intended to evaluate the PM models' effects on performance parameters from d0-7 post hatch. In Exp 1, the Negative Control had high contamination from embryo source. High levels of Gram-negative bacteria were observed within the hatching environment as well as GIT samples, possibly impacting performance of the Negative Control. The colonization upon arrival of these pathogens shows how "real world" this challenge model is. The isolates utilized in the present study were chosen because of their prevalence in the hatching environment, not necessarily for virulence or effects on performance. While significant differences in performance were only observed in Exp 1, it can be observed that formaldehyde mitigated the microbiol blooms effect on bacterial circulation within hatching environment, GIT colonization, and fluff samples in Exp 1-3. Moreover, these results indicate that this model could be utilized to evaluate formaldehyde alternatives in comparison to formaldehyde and their effects on early performance parameters.

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

18079

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

UNIVERSITY OF ARKANSAS

Office of Research Compliance

June 14, 2019

MEMORANDUM

TO:	Dr. Billy Hargis
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	New Protocol
PROTOCOL #:	19032
PROTOCOL TITLE:	Experimental horizontal transmission model to evaluate probiotic efficacy
APPROVED PROJECT PERIOD:	**Start Date** June 13, 2019 **Expiration Date** June 12, 2022

The Institutional Biosafety Committee (IBC) has approved Protocol 19032, "Experimental horizontal transmission model to evaluate probiotic efficacy". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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