Chapter

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

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Abstract

Pioneer colonization by beneficial microorganisms 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 microbes. 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 microbes. 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.

Keywords: hatchery, microbial bloom, pioneer colonization, model

1. 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 of 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 microbes on the surface of the eggshell [1]. Fecal material on the surface of the eggshell may harbor potential pathogenic microbes capable of penetrating the eggshell and membranous layers during incubation [1]. Eggshell contamination has been shown to negatively impact hatchability and hinder early performance [2]. Additionally, these contaminated embryos serve as reservoirs that horizontally transmit pathogens during the hatching phase [3]. As chicks hatch, the humidity in the hatching environment promotes replication of both apathogenic and pathogenic microbes. The composition of the microbial bloom during the hatching phase influences pioneer colonization of the neonatal gastrointestinal tract [4]. As such, cross-contamination of primary poultry pathogens readily occurs in commercial hatcheries [5]. 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 [2, 6, 7].

For over a century, formaldehyde fumigation has been utilized to control the dissemination of pathogens in some commercial hatcheries [8, 9]. Although formaldehyde eliminates microbes in the hatching environment, it has been associated with tracheal epithelial damage and mucosal sloughing in neonatal chicks [10–12]. As a biocide, formaldehyde effectively kills resistant forms of bacteria, fungi, and viruses [13], and likely eliminates airborne apathogenic and potentially beneficial microbes. Cost-effective and sustainable alternatives to formaldehyde fumigation to reduce 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 microbes to improve poultry health.

2. A brief overview from lay to artificial incubation of hatching eggs

In commercial broiler breeder facilities, eggs are removed from the hen and transported to a commercial hatchery for artificial incubation. Hens lay their eggs in clean (or dirty) nest boxes or may lay their eggs in a contaminated environment, such as the floor. Factors including facility design and the lighting program can affect the onset and location of lay. Since floor eggs have been shown to harbor more microbiological contamination than nest eggs [14], care should be taken to avoid disrupting the hen's laying process.

The egg collection procedure, and egg handling and storage, have been reviewed [15]. Conveyor belts or mechanical apparatuses transport the eggs post-lay to a common area for collection in modern breeder facilities. Prompt collection of eggs is ideal to avoid an increased risk of damage, contamination, and reduced hatchability [16]. The egg temperature declines post-lay and should not increase until the time of preheating before placement in the incubator. Fertile eggs are regularly stored in coolers (15–20C) to optimize survival until artificial incubation. Demand for broiler chicks will dictate how quickly the incubation process will begin for fresh or stored fertile eggs. Single-stage and multi-stage incubators have been used in commercial broiler hatcheries, although multi-stage incubators tend to be the most common. During multi-stage incubation, different embryonic stages are co-incubated to equilibrate the temperature. The multi-stage incubators can be more economically feasible, but regular sanitation is difficult. Single-stage incubators are becoming more popular.

Although temperature management can be more tedious for multi-stage incubators, the single-stage incubators can be sanitized after each 18-day embryonic cycle. At DOE18, embryonated eggs are transferred to hatch cabinets. Disinfectants are applied during the hatching phase to reduce the microbial load in the hatch cabinet. Hatchery sanitation practices, and the impact of hatchery contamination, will be discussed below.

3. Pioneer colonization of the gastrointestinal tract (GIT): critical timepoints during the neonatal period

Pioneer or initial colonizers of the neonatal GIT influence the diversity of the post-hatch intestinal microbiome [17, 18], promote functional development of the immune system [19], and inhibit colonization by enteropathogenic bacteria [20]. Once established, the commensal microbiota inhibits pathogen invasion and colonization by forming a microbial barrier and by competing for nutrients and attachment sites [21]. The commensal microbiota also modulates host immune development and maturation of the GIT [19]. The intestinal immune repertoire evolves to tolerate the resident microbes in the lumen of the GIT, which is critical for homeostasis [22]. 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 [23]. For avian species, transfer of the maternal microbiota occurs during oviposition [24] 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 [25, 26]. 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 [27–29] 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 [30, 31].

During incubation of eggs by hens, it has been shown that the number of pathogenic microbes on the eggshell decline during incubation, and resident microbes on the eggshell inhibit trans-shell invasion by pathogens [32, 33]. However, in commercial poultry operations, embryonated eggs immediately removed from the hen may be exposed to fecal or environmental microbes that adhere to and potentially penetrate the eggshell [1, 34]. 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. [14] 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 microbes during late embryonic development improved growth performance and immune system development [35, 36]. 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 [37, 38]. Moreover, the energetic costs

related to the activation of inflammatory pathways by opportunistic pathogens have been shown to cause protein catabolism [39]. Thus, it is important to mitigate exposure to and transmission of pathogenic microbes 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.

3.1 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 [40]. Chemical defenses within the developing embryo include antimicrobial properties of the albumen, alkaline pH, lysozyme, and conalbumin/ovotransferrin [40]. Potential contamination of the egg occurs both before oviposition (trans-ovarian route) or after oviposition (trans-shell route; [41]). Environmental temperature and humidity are also known to impact the rate of microbial penetration of eggshells [42]. High relative humidity is considered essential for trans-shell transmission of microbes because it promotes survival, growth and transport through eggshell pores [43]. As the egg cools after lay, a relative vacuum is generated and the negative pressure facilitates microbial penetration of the eggshell [41]. Additionally, the quality and thickness of the eggshell impact a microbe's ability to penetrate the eggshell [44]. Comprehensive reviews describing microbial contamination of the egg and penetration of the eggshell have been published [5, 40, 41].

The composition of the neonate's GIT microflora is thought to be predominantly influenced by fecal and environmental contaminants on the eggshell [45], but the composition may also be affected by microbes vertically transmitted from hen to offspring at oviposition. 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 [46]. 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 [2, 6, 7]. 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 ~1×10^3 colony forming units (CFU) per egg [47]. 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. [48] 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 hatching eggs have been shown to harbor extensive numbers of microbes [49]. Additionally, sanitization of both clean and dirty hatching eggs increased total aerobic bacterial recovery from eggshells at the time of transfer (day 18 of embryogenesis) from incubator to hatch cabinet. However, nest-clean eggs that were not sanitized had lower total aerobic

bacterial recovery at transfer compared to the time of collection. Handling after the sanitization process should be limited to prevent contamination or recontamination of the surface of the eggshell. Potential for eggshell surface contamination occurs during egg collection, transport, artificial incubation, and hatching. It is important to limit the risk of contamination at each point throughout the egg collection and artificial hatching process.

Although there are physical and chemical defense mechanisms to prohibit microbial penetration of the eggshell and endogenous replication during embryogenesis, certain microbes 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 [50]. At the time of lay, the eggshell may become contaminated with *Salmonella* by brief contact with contaminated nest box shavings [51]. Contamination of the eggshell surface with fecal material, nest box shavings, or egg-derived debris increased cultivable aerobic bacteria compared to clean eggs [52]. Using 16S RNA amplicon sequencing, Olsen et al. [52] 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 [52]. 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 [53]. In another study, 16S sequencing was used to compare the breeder hen's fecal microbiota to the eggshell microbiome in two independent flocks [54]. Of the eggshells that were sampled, Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes contributed to 90% of the overall microbiota [54]. Transfer of potentially pathogenic bacteria and those associated with spoilage from breeder hens to the eggshell surface, included *Salmonella, Escherichia coli,* and *Staphylococcus* spp. [54]. Maki et al. [55] showed that source or exposure to only eggshell-derived microbes, environment-derived microbes, or to both eggshell and environment-derived microbes modulate the composition of intestinal tract microbiota and fecal microbiota post-hatch. The eggs that were only subjected to the environment-derived microbes were sterilized prior to incubation which could have negatively affected the eggshell cuticle integrity. Also, any maternal microbes transferred during oviposition or that penetrated the eggshell may have confounded the results. Regardless, results published by Maki et al. [55] 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 microbes has been used to inhibit colonization of pathogenic microbes by competitive exclusion [56–58]. In addition to competitive exclusion and performance benefits, beneficial bacteria may also have immunomodulatory effects on the host [35, 36, 59]. 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 [60]. Early application by *in ovo* injection at DOE18 promotes uptake of the material (vaccine, probiotic, etc.) by the chick during the pipping process [61]. Teague et al. [62] 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 [62]. Thus, *in ovo* administration could be utilized to promote early colonization by beneficial microbes 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 [63]. Additionally, there was an increase in spleen weight at hatch related to *in ovo* administration into the amnion [63]. 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 [63]. 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 [64–66]. A direct correlation between immunocompetence and the weight of the spleen has been described [67]. Although probiotics have been shown to stimulate immune development [35, 36, 59] and suppress pathogen colonization or invasion when administered by *in ovo* application [36, 62], certain microbes 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 [68]. 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* [68]. Alternatively, *in ovo* administration of Norum TM, a mixed *Bacillus* spp. culture containing vegetative cells of two *Bacillus 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 [69]. *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 [18, 70]. 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 [18], and antioxidant effects were upregulated and inflammation was reduced in the GIT of chicks that received the LAB at day 18 of embryogenesis [70]. Though, *in ovo* administration with one strain of *Citrobacter* spp., but not both, increased oxidative stress and proinflammatory responses in the GIT at DOH [70]. Rodrigues et al. [17, 71] 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 [17] and was associated with impaired intestinal immune function [71]. 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.

3.2 Postnatal or post-hatch period

The GIT is rapidly colonized by microbes present in the environment shortly after hatch and readily established 72 h post-hatch [72]. The composition of the microbiota is impacted by the individual host and age of the host [73]. 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 [74]. However, Stanley et al. [75] 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 [75]. 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 [76]. 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 [76]. However, administration of the cecal microbiota as a therapeutic treatment after oral challenge treatment with SE was not protective [76]. To investigate the rate of natural transfer of the maternal microbiota from hen to progeny, chicks were placed in contact with hens for 24 h post-hatch [77]. It was shown that exposure and transfer of the maternal microflora influenced the chick's cecal microbiota [77].

Administration of beneficial bacteria has been shown to inhibit pathogen colonization and reduce horizontal transmission of pathogenic bacteria [78, 79]. Early establishment of beneficial pioneer colonizers is critical for pathogen exclusion since the GIT is rapidly colonized the initial microbes 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, microbes 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 microbes 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.

4. 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 microbes 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 [80]. Moreover, at DOE21, bacteria recovered from non-viable embryonated eggs was ~2.4 logs higher than the chicks that successfully hatch [81]. In a more recent study, *Enterococcus 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* [82]. Additionally, Karunarathna et al. [83] 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 [83]. 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 [84]. Recovery from the yolk sac suggests that the navel is a critical portal of entry for *E. faecalis* during the neonatal period [84]. Reynolds and Loy [85] 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 [85]. 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 [86]. Moreover, antimicrobial-resistant *E. faecalis* strains have been isolated from broiler breeder hens [87]. 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 [88]. Portals of entry of APEC include the respiratory tract or translocation from the intestinal tract during stress [89]. 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 [90] by evading host immune defenses [91]. 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. [92] 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 [92]. APEC infection has also been associated with increased 7-day mortality related to airsacculitis and colisepticemia [93]. Horizontal transmission of APEC during late embryogenesis has been replicated in small-scale hatch cabinets [94, 95]. 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 [96]. 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 [97]. In 2009, *S. aureus* isolates recovered from poultry were predominantly related to a clonal complex relevant to humans [97]. 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 [97]. 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 [98]. 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 [99].

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 [51]. Successful eggshell penetration by *Salmonella* does not necessarily have to occur during embryogenesis. For example, Cason et al. [100] demonstrated that initial *Salmonella* recovery from yolk sacs, GIT, and chick rinses remained low until the onset of pipping [100]. 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* [101, 102]. This is critical because bioaerosols are generated throughout production in commercial poultry operations. Cason et al. [1] 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 [1]. 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 [103]. 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 [104]. 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 [105]. 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 [106–108]. *Aspergillus fumigatus* is the most common cause of aspergillosis in poultry [109]. A single *Aspergillus fumigatus* hyphae produces thousands of hydrophobic conidia (spores) that are readily dispersed into the environment [109]. Inhalation of *Aspergillus fumigatus* spores has been associated with respiratory mycosis, or brooder pneumonia [6, 110]. These fungi degrade the cuticle of the eggshell and increase the likelihood of invasion during embryogenesis [43, 111]. Application of *Aspergillus fumigatus* spores in a wet suspension or dry suspension increased embryo contamination and incidence of aspergillosis [112]. Huhtanen and Pensack [113] 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 [114]. The egg yolk in non-viable embryonated eggs also serves as a nutritive source for *Aspergillus fumigatus* [114].

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 microbes and improve poultry health.

5. Formaldehyde fumigation

Formaldehyde is a byproduct of cellular metabolism and detoxification has been shown to be important for metabolic processes [115]. 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 [13]. The first published report of formaldehyde application in commercial hatcheries was in 1908 [9]. For decades, formaldehyde fumigation of hatching eggs has been recommended to control the microbial load in hatching environments [116].

Formaldehyde fumigation has been shown to reduce the bacterial load on the surface of eggshells by 99% [117] 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 [6]. 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 [118]. Steinlage et al. [118] evaluated the application of 37% formalin applied as a constant rate infusion (CRI, 1 mL/hour over 12 h period) as compared to the traditional method of a single dose application of formaldehyde (12 mL administered at one time point every 12 h). The maximum concentration of formaldehyde in the environment was lower with CRI at 20 ppm versus 102 ppm 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 [118]. Although contamination increased because of *in ovo* injection in this study, formaldehyde fumigation reduced the microbial load in the hatching environment and potentially eliminated microbes 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 et al. [118], formaldehyde applied by CRI in commercial hatch cabinets reduced circulating aerobic bacteria 4 h before hatch pull at DOE21 more readily than a single administration of 37% formalin at transfer from incubator to hatch cabinet [119].

Formaldehyde fumigation reduced circulating coliforms in the hatching environment, which reduced horizontal transmission and enteric colonization at hatch [120, 121]. However, formaldehyde fumigation has been associated with tracheal epithelial damage and mucosal sloughing in neonatal chicks [10–12, 122]. 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 [123]. The avian respiratory tract has been suspected to be a portal of entry for enteric pathogens, including *S. enterica* [101, 102]. 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 [12, 122, 124]. Although the application of formaldehyde during the hatching period effectively reduced aerobic bacterial contamination in commercial hatch cabinets [119, 121], it has been shown that the efficacy of formaldehyde fumigation decreases as contamination increases [125]. Additionally, formaldehyde is not selective and eliminates both beneficial and pathogenic organisms. During late embryogenesis, the fumigant has a limited effect on endogenous microbes inside the egg [117, 120]. The impact of formaldehyde fumigation during late embryogenesis on performance has also been investigated. Zulkifli et al. [122] demonstrated that feed conversion was negatively affected due to formaldehyde exposure. Alternatively, CRI of formaldehyde or a single administration of formaldehyde every 12 h marginally improved feed conversion ratio (FCR) but did not significantly affect body weight gain (BWG) from DOH to day 14 [118]. Mahajan et al. [11] 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 [124].

Although formaldehyde effectively controls the circulating microbes 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 microbes and control the microbial bloom in the hatching environment without the use of carcinogenic formaldehyde will be essential.

6. 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 [126]. During the hatching phase, bioaerosols and dust are generated and dispersed by the ventilation system in the hatch cabinet [127]. 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 [128]. 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 [81]. 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 [128]. Magwood [129] 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 [129]. The rating system developed by Wright [128] to assess hatching sanitation practices has been utilized in other investigations [129, 130]. Other investigators also confirmed that fumigation of hatching eggs reduced microbial recovery from fluff collected from the hatch cabinet [131].

The open-agar plate method [119, 121, 132] as well as air sampling machines [133] 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 [134, 135]. Gentry [135] sampled various locations in a commercial hatchery using the open-agar plate method and the Anderson air sampler [133] 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 [135]. 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 Marr [136] 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 [136]. 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 [125]. 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, [125]. 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. [132] 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 [132]. 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 [31]. 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 [100]. 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 [137], but the increased percentage of *Salmonella*-positive chicks at hatch suggest moderate replication and dispersion of the pathogen within the hatch cabinet environment. Bailey et al. [138] 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 [103].

The incidence of *Salmonella* in commercial hatcheries for other gallinaceous species, including geese, has been documented. Chao et al. [139] 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* [139]. 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. [140] isolated *E. coli* from 47 fluff samples collected from commercial hatcheries that contained less virulence-associated genes than the 20 APEC isolates evaluated [140]. 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.

7. Alternatives to formaldehyde fumigation

Research efforts to identify alternatives to formaldehyde to mitigate pathogen transmission of pathogens in poultry hatcheries have been reviewed [141]. 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 microbes 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 [142] 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 [2, 143]. Bailey et al. [144] 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 [145]. 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 [146]. 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 [147, 148]. The combined treatment only reduced the incidence of *Salmonella* on the surface of the eggshell, and did not prevent bacterial penetration of the eggshell [147]. The incidence of *Salmonella* in the GIT of chicks and early performance were not reported in this study. However, Rehkopf et al. [149] 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 [150] 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 [150]. However, both UV and peracetic acid treatment actually increased total aerobic bacteria and Enterobacteriaceae recovered from yolk samples 24 h post-hatch as compared to non-treated controls and formaldehyde treated group [150]. 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 [11]. 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 [151]. 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 [151, 152]. Pyrazines are naturally-occurring organic nitrogen-containing ring structures which can be chemically synthesized or obtained by microbial fermentation [153]. Alkyl pyrazines are typically used as flavoring agents or as fragrances) and have been shown to have antimicrobial activity [154].

Application of a volatile organic compound, an alkylated pyrazine (5-isobutyl-2,3-dimethylpyrazine), reduced viable microbes on the surface of the eggshell [155]. 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 [121]. Compared to formaldehyde fumigation, probiotic-application would not be expected to inhibit the microbial bloom in the hatching environment, but the beneficial microbes could perhaps displace the opportunistic pathogens in the hatching environment thereby promoting colonization by beneficial microbes.

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 microbes, artificial introduction of probiotic candidates during the hatching phase may be a promising method to enhance enteric colonization by beneficial microbes.

8. 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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