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Broiler Industry

Edited by Guillermo Tellez-Isaias, Juan D. Latorre and Yordan Martínez-Aguilar

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Meet the editors

Guillermo Tellez-Isaias received his DVM and MS in Veterinary Sciences from the National Autonomous University of Mexico (UNAM), and his Ph.D. from Texas A&M University, USA. He worked as a professor at UNAM for 16 years, eight of which he served as head of the Avian Medicine Department, College of Veterinary Medicine. Dr. Tellez was president of the National Poultry Science Association of Mexico and is a member of the Mexican Veterinary Academy and the Mexican National Research System. Currently, he is a research professor at the Center of Excellence in Poultry Science, University of Arkansas, USA. His research is focused on poultry gastrointestinal models to evaluate the beneficial effects of functional foods to enhance intestinal health and disease resistance.

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Contents

Preface

In 1945, the American grocer Atlantic & Pacific Tea Company (A&P) organized the first of its "Chicken of Tomorrow" contests with the national finals held in 1948. For the finals, breeders submitted a case of 30 dozen hatching eggs to an Eastern Shore hatchery, where the eggs were hatched and the offspring fed until they reached the market weight and were then slaughtered. Broilers were judged on several factors, including growth rate, feed conversion efficiency, and the amount of meat on breast and drumsticks. Because the original purpose of most birds was to grow large quickly, breeders used weight as the primary selection criterion. Genetic line companies found that weight was moderately heritable, with 20 to 40 percent of the trait genetically controlled. Though simple, this selection process helped improve broiler breeder performance across generations. Market forces have changed selection criteria over the years. A more integrated and consolidated industry learned that weight and growth rates alone could not be the only selection criteria considered. This was particularly true as feed costs increased. Today, feed accounts for between 65 and 70 percent of the input cost for a broiler. Newborn chicks grow 31% (55 g/bird) on day one, and 5,902% (2,521 g/bird) on day 35. This astonishing performance of the modern chicken comes from: (1) intensive selection for growth rate, (2) meticulous attention to health and husbandry, and (3) advances in feed formulation, matching the nutrient contents of the feed with the nutrient requirements of the bird. That is why gut health and feed efficiency are so crucial for broiler chickens since feed efficiency is considered "the money saver." In today's broiler industry, subclinical forms of coccidiosis or necrotic enteritis are often financially more devastating than acute, short-term infections. Likewise, dietary factors that modulate the immune system and gut microbiota should be considered when formulating diets and managing feeding practices. As the growth period is progressively shortened and feed efficiency continuously improved, the health care and nutrition of the bird are becoming more demanding. This makes it more important to pay attention to the minute changes that occur in the gut, which are often overlooked because the damage is subtle and usually characterized by microscopic changes in the mucosal layer. This book presents updated information on this fascinating industry in the areas of management, nutrition, health, diseases, and hatchery and incubation.

The editors express their sincere appreciation to all of the authors who contributed to this book for their hard work and dedication, as well as to the IntechOpen editorial team for allowing us to complete this project.

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Chapter 1

The Impact of Heavy Metals on the Chicken Gut Microbiota and Their Health and Diseases

Selina Acheampong

Abstract

It is important to consider the health and well-being of birds in various production methods. The microbial makeup and function of a bird's gastrointestinal (GIT) system may vary based on the bird's food, breed, age, and other environmental conditions. Gut flora play a critical role in maintaining intestinal homeostasis. Environmental exposure to contaminants such as heavy metals (HMs) has been linked to a wide range of disorders, including the development of dysbiosis in the gut, according to many studies. Changes in the gut microbiota caused by HMs are a major factor in the onset and progression of these illnesses. The microbiota in the gut is thought to be the first line of defense against HMs. Thus, HMs exposure modifies the gut microbiota composition and metabolic profile, affecting HMs uptake and metabolism by altering pH, oxidative balance, and concentrations of detoxifying enzymes or proteins involved in HM metabolism. This chapter will focus on the exposure of chicken to HMs from their feed or water and how these HMs affect the immune system resulting in various diseases.

Keywords: poultry, chicken, broiler, GIT, heavy metals, microbiota, diseases

1. Introduction

Among the most popular types of poultry raised for human consumption are domestic chickens. At 35–40 days of age, a typical broiler chicken will weigh around two kilograms [1]. During this period, they require approximately 3–4 kilograms of feed per day because of their rapid growth. While raising chickens in close proximity is necessary to meet the demand for chicken meat, this practice puts the birds at greater risk of infection and speeds disease transmission [2].

There is a wide range of microorganisms that colonize an animal's digestive system as soon as it is born or hatches, and these microorganisms change over time [3]. The gut microbiota of an animal, a human of the same species, and the location of the host's body all differ [4]. In the gut microbiota, which is a complex, interconnected community of organisms, the actions of all microbial components have a direct effect on its functions [5]. When the host and microbes interact in a way that benefits both of them, an ecological system is created [6]. As with humans, animals' gut microbiome serves many of the same functions: scavenging energy from undigested feed

components through fermentation, creating an immune barrier to keep harmful bacteria out of the digestive tract, and aiding in the absorption of vitamins and amino acids by animals [5]. This is largely true for both species. Farm animals must fulfill environmental and dietary responsibilities, as well as economic ones, in order to be productive [7]. The GIT microbiota has a significant impact on animal performance, particularly in young animals who are exposed to a wide range of stressful situations [8]. Dietary fiber, vitamins, and minerals are all provided by the microbiota that inhabit the GIT. The GIT microbiota may also play an important role in hen health and immunity, according to some evidence [9].

Data shows that heavy metal (HM) exposure may play a role in the etiology of metabolic disease by altering the GIT microbiota [10]. It's important to remember that the gut microbiota protects the body from harmful microbes. Furthermore, HM exposure alters the composition and metabolic profile of the gut microbiota, which in turn affects the uptake and metabolism of these HMs by altering pH, oxygenation, and the concentrations of enzymes or proteins that are involved in the detoxification process [11]. As the intestinal barrier is influenced by gut flora, HM absorption can also be affected.

2. The avian GIT microbiota

There are many different types of microorganisms in the animal microbiota, including those that are beneficial and those that are harmful [12]. The term "microbiota" is used to describe this microbial community. It includes commensal, symbiotic, and pathogenic microorganisms, as well as those that are beneficial or harmful to the host [13]. The microbiome refers to all of these symbionts' genetic material as a whole [14]. When an organism consists of both host and microbial components, it is referred to as "supraorganisms" because of the important role it plays in the health and development of the host [5].

In the chicken intestinal tract, there is a diverse and ever-changing community of microorganisms [15]. When the gut microbiota is first established, it's mostly anaerobic bacteria that take over [16]. The intestinal microbiota of newly hatched chicks is heavily influenced by the surrounding environment, and this is especially true for chicks that have only a small number of bacteria in their system [17]. As animals older, the GIT microbiota evolves from simple to complex and obligate anaerobes, reaching a relatively stable dynamic equilibrium [18]. A variety of functions and microbial compositions are found throughout the chicken GIT, which is divided into numerous sections [19].

The digestion and absorption of nutrients are dependent on the proper functioning of each section of the digestive tract. In chickens, there are two paired ceca, both of which are home to a similar bacterial community [20].

According to Wei *et al.* [21], a total of 915 operational taxonomic units (OTUs), or species (defined as having a phylogenetic distance of 3%), were found in 13 phyla, with *Firmicutes* (70 percent), *Bacteroidetes* (11.3%), and *Proteobacteria* (9.3%) accounting for more than 90% of all sequences analyzed. *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* dominated the 117 genera described in total. *Ethanoligenes* (*Firmicutes*), a genus of bacteria that produces ethanol, was found to be prevalent. The most common *Proteobacterium* was *Desulfohalobium.* The genus *Bifidobacterium* was found in only 1% of the *Actinobacteria* sequences. *Cyanobacteria*, *Spirochaetes, Synergisteles, Fusobacteria, Tenericutes,* and *Verrucomicrobia* were among

the lesser-known phyla. The phylum *Euryarchaeota* was the only Archaea phylum to be found in the chicken GIT, with only 11 out of a total of 3184 sequences. This supports the lack of methanogens in the chicken GIT [22]. There are fewer species of bacteria in chicken GIT than in the GIT of other animals, which may be due to the rapid transit and short retention times of food in the digestive system [5]. For example, a 29-day-old broiler chicken's typical retention time is between 4 and 5 hours, compared to humans' average of 20 hours [23]. *Firmicutes*, *Bacteroidetes, Proteobacteria,* and *Actinobacteria* are said to be the most common phyla found in the ceca [24]. The presence of Firmicutes and Bacteroidetes in the ceca suggests that the microbiota present is important in the production of essential amino acids, the digestion of non-starch polysaccharides, which stimulates the production of short-chain fatty acids (SCFAs), and nitrogen recycling using uric acid [5]. *Bacteroides* are the most common species in the Bacteroidetes phylum (40%). Others in this family include *Prevotella* (the genus), *Tannerella* (the genus), and Ri*emerella*, *Desulfohalobium*, *Escherichia*, *Shigella*, and *Neisseria* are the most common genera of *Proteobacteria* [21].

Clostridium, Bacteroides, and Ruminococcus are among the obligate anaerobes found in the cecum [25]. The small intestine, which includes the duodenum, jejunum, and ileum and is where nutrients are primarily digested and absorbed, has fewer microorganisms and is primarily colonized by acid-tolerant and facultative anaerobes such as Lactobacillus, Enterococcus, and Streptococcus [17]. The feacal microbiota composition varies greatly depending on the contributions of microbiota from different gut segments [12].

3. Importance of GIT microbiota to broilers

Microorganisms are primarily found in the gastrointestinal tract. Broilers and their intestinal microbiota interact in a variety of ways, with an emphasis on nutrient exchange, immune modulation, digestive system physiology, and pathogen exclusion being the most important [5, 26]. These functions are summarized in the following sections.

3.1 Nutrient exchange

Chickens benefit from the nutrients provided by commensal bacteria in their digestive systems, both directly and indirectly [5, 27]. SCFAs, ammonium, amino acids, and vitamins [12, 15] are all included in this category. Polysaccharides, oligosaccharides, and disaccharides can all be hydrolyzed to primary sugars by the majority of intestinal bacteria [28]. SCFAs such as acetate, propionate, and butyrate are produced by the fermentation of these sugars by intestinal bacteria [12, 15]. Passive diffusion in the ceca allows SCFAs to be absorbed and enter a number of metabolic pathways [29]. SCFAs are a carbon and energy source for chickens [15]. To further enhance their ability to modulate intestinal immune response, they regulate blood flow and stimulate the proliferation of enterocytes [29].

Nitrogen metabolism is also aided by bacteria in the intestines [30]. When uric acid is broken down into ammonium by bacteria in the urinary tract, it can travel from the cloaca to the cecum, affecting the metabolism in the latter and allowing ammonium to be absorbed by the host [29, 31]. This allows the host to use ammonium to synthesize amino acids. However, the same intestinal bacteria can also be a source

of amino acids and vitamins [29], Despite the fact that most of the proteins and vitamins produced by these bacteria are excreted, because most intestinal bacteria are found in the cecum, which cannot digest or absorb proteins [5]. Chickens, on the other hand, may be able to provide nutrients to the intestinal bacteria in a reciprocal manner.

3.2 Immunological modulation

Chickens' immune systems include both innate and acquired immune responses [32]. The microbiota plays an important role in modulating the regulation and activation of both elements [33]. In terms of the innate immune response, the intestinal mucosa is thought to be the first line of defense against infection and a barrier that prevents commensal bacteria from penetrating the intestinal epithelium [32]. The interior surface of the avian intestine is covered in a mucous layer composed of the glycoprotein mucin, which is secreted by calceiform epithelial cells [34]. Mucins containing sialic acid have been found to be more abundant in conventionally reared chickens than mucins containing sulfate, which are found in birds with low bacterial loads. These differences are visible as early as day four (4) after birth, implying that the intestinal microbiota is involved in regulating the establishment of the mucous layer [35]. The intestinal microbiota also regulates the production of antimicrobial peptides on the surface of the intestinal epithelium, which are capable of rapidly killing or suppressing the activity [36]. Some of these peptides are expressed naturally, while others are induced in host cells by bacteria.

Regarding the acquired immune system, it appears that commensal bacteria protect the mucosa membrane by modulating the immune response, controlling the amount of mediators secreted by acquired immune system cells, and stimulating helper T cells [37]. Using germ-free chickens, it was demonstrated that microbiota has a dramatic effect on the repertoire of intestinal T cells and their cytokine expression [38].

3.3 The physiology of the digestive system

After hatching from the egg, the chicks must transition from a yolk-based diet to one rich in carbohydrates and proteins, which is critical to their development and health [39]. So, in this stage of development, the digestive system's organs go through anatomical and physiological changes. An ideal environment for microorganisms to colonize is the rapidly developing digestive tract, and the microbiota also plays an important role in the development of this organ. Compared to conventionally reared chickens, germ-free chickens have smaller intestines and cecas that weigh less and have thinner wall thickness [38]. There is some evidence to suggest that SCFAs increase enterocyte proliferation and growth, which could explain some of the discrepancy [29]. Intestinal microbiota may also influence the enzyme activity in chicken intestines [5]. Compare germ-free and conventionally raised chicken alkaline phosphatase enzyme activity and you'll see that the latter has higher levels of activity [38]. Bifidobacterium and Lactobacillus, which increase the activity of proteases, trypsin and lipases, can be induced by diet as well [40]. Morphological changes can be caused by pathogenic bacteria as well [35]. Co-infection with Eimeria and Clostridium perfringens has been shown to reduce the length of the intestinal villi [41]. Chickens infected with Salmonella typhimurium were also shown to exhibit these symptoms [35].

3.4 Competitive exclusion

Ecologically speaking, two species that compete for the same resources cannot coexist indefinitely [42]. A single competitor will always win out, leading to an evolutionary change, shift to another niche or even the complete demise of the other [5]. To reduce pathogen adhesion and colonization, the intestinal microbiota competes with colonizing pathogenic bacteria [43]. There are a variety of mechanisms that could lead to this reduction, including the physical occupation of space, competition for resources in a specific niche, and even direct physical or chemical confrontation with a potential colonizer [5]. Bacteriocins, for example, have been linked to a reduction in the ability of pathogens to invade the body [44]. No mechanism has been discovered yet to explain the protective effects of the competitive exclusion process on Salmonella colonization in broiler chickens' intestinal tracts [5]. It has been shown that the pathogen can be controlled using a variety of products ranging from probiotics to inoculation of bedding with cultures drawn from the fecal matter produced in more productive sheds with better intestinal health [5, 17].

4. Factors affecting the GI tract microbiota

Intestinal microbiota, intestinal environment, and dietary compounds all work together to maintain a delicate equilibrium [45]. Disease can occur if this relationship is out of place [5]. Environmental factors, host age and health, and dietary habits all have the potential to influence microbial populations in either a positive or negative way [5, 45]. Aside from promoting growth and preventing the spread of endemic diseases, the use of low-dose antibiotics in livestock feed is a common practice in intensive farming [46]. Drug-resistant bacteria and public pressure to reduce the use of drugs in food-producing animals have created a need for 'natural' alternatives to boost performance and prevent disease spread [47]. However, these natural alternatives are not without their drawbacks. Intestinal microbiota can be influenced through the use of prebiotics and probiotics [48]. Specific changes in the composition and/or activity of the intestinal microflora, made possible by selective fermentation, that benefit the health and well-being of humans. "Live microorganisms that when administered in adequate amounts confer a health benefit on the host" is defined as [49]. Probiotics, prebiotics, or a combination of the two have been shown to improve the health of broilers in numerous studies [48, 49]. However promising probiotic supplements appear to be in the labs, their effects on commercial broilers vary widely [49]. There are many factors that can affect the intestinal microbiota's composition and must be taken into consideration when trying to manipulate the intestinal microbiota, including the complex relationship between the host and the microbiota [50].

Food is a major source of energy for intestinal bacteria, and as a result, diet has a significant impact on the population of bacteria in the digestive tract [29]. Since different bacterial species have different dietary requirements and preferred substrates, changing one's diet can have an impact on one's gut microbiome [51]. It has been found that when wheat was added to the diet of birds, it promotes the growth of bacteria with 50–55% Guanidine to Cytosine (GC) content and suppressed the growth of those bacteria with 60–79% content [52]. In contrast to diets based on maize, it has been revealed that populations of *lactobacilli* and *coliforms* increased in response to wheat and barley diets [53]. The *Lactobacilli* population and *C. perfringens* are the most affected by changes in the ileal microbiota due to dietary fat source [54].

When soy oil was substituted for tallow as a source of dietary fat, there was a rise in anaerobes in the intestinal microbiota and an increase in gut transit time [55]. This allows for the manipulation of chicken microbiota through dietary changes and the inclusion of specific components (essential oils, oligosaccharides, enzymes, and specific carbohydrate sources) aimed at enhancing growth and improving intestinal tract conditions for specific commensal bacterial groups [12].

Poultry living conditions and the management that go along with them have a significant impact on their intestinal microbiota as well [56]. As a result of poor hygiene, there will be an increase in food-borne illness and wet litter issues [57]. Since farm litter is a source of bacteria for the birds and a potential source of pathogenic bacteria, proper litter management is essential [56, 57].

Age has been shown to influence the composition of the intestinal microbiota, along with host genotype [58]. The diversity and complexity of the bacterial populations in the intestinal microbiota of older and younger birds are shown to increase as the birds age [59], according to culture-independent molecular profiling techniques [45]. According to Wickramasuriya *et al.* [60], broiler chickens' ileal and caecal microbiota had remarkably similar microbiotas at 3 days of age, but after 2 weeks, these subpopulations had diverged significantly. Many factors are likely to play a role in age-related GIT microbiota changes, including changes in diet, maturation of immune systems, changes in environmental influences, and increased interplay with other animals that expose individuals to a wider range of bacteria [61].

Birds raised in xenobiotic-rich environments are more likely to have a diverse and beneficial GUT microbiota [62]. Heavy metals, plastics, and agrochemicals are just a few of the potentially harmful substances on this list. HMs and the gut microbiota interact in a variety of ways. Exposure alters the normal gut microbiota's metabolism [62].

5. Heavy metals (HMs)

Finding a variety of toxic substances in animal feed or food additives, such as arsenic, lead, cadmium, mercury, and a host of other toxins is very common [63]. In general, it refers to a group of metals with high densities, atomic weights, or atomic numbers that are either not required or only required in trace amounts [64]. As a result of their widespread use in the manufacturing, medical, and agricultural sectors, these chemicals have begun to accumulate in the environment, raising questions about their potential dangers to both human and animal health as well as the environment [65]. Ingestion, inhalation, or dermal exposure to heavy metals can cause a wide range of health issues, including neurological and neurobehavioral disorders, abnormal blood chemistry, cancers, and cardiovascular disease in humans [62].

Poultry can be exposed to a variety of toxic metals from a variety of sources [66]. The application of sewage sludge, the disposal of industrial waste, the use of pesticides and fertilizers, and atmospheric deposition are all methods by which heavy metals can contaminate soil and water [67]. These heavy metals can be found in the air, water, and soil, it is difficult to remove them from animal feed and feed supplies [68]. Heavy metal bioaccumulation and indestructibility raise the possibility of these substances serving as toxins [69]. Metals cannot be catabolized, so chelation is an option for their removal [63].

5.1 Classification of heavy metal

Heavy metals can be classified into four major groups on their health importance.

Essential: Cu, Zn, CO, Cr, Mn and Fe. These metals also called micronutrients [70] and are toxic when taken in excess of requirements [69].

Non-essential: Ba, Al, Li and Zr.

Less toxic: Sn and Al.

Highly toxic: Hg, Cd and Cd.

Heavy metals are also called trace element due to their presence in trace (10 mg Kg^{-1}) or in ultra-trace (1 μg kg^{-1}) quantities in the environmental matrices [69, 70].

6. Channels of heavy metals exposure in broiler production

Poultry feed is a common source of heavy metal pollution, as are the majority of animal feeds [71]. Heavy metal contamination in poultry birds can occur from feed or water [66]. Bioaccumulation and the food chain can transfer heavy metals from the soil to plants, animals, and ultimately humans [62]. Due to the use of plants in poultry feeding, contamination of the plant is likely to be found in poultry feed [71]. Rice bran, rice polish, solvent extracted rice and wheat bran, and molasses are all common ingredients in poultry feeds [72]. Calcium, phosphorus, trace minerals (such as Fe, Zn, Mn, Cu, CO, and Me), and vitamins A, D3, E, K, and B complex are among the other minerals and vitamins that can be found [73].

6.1 Feed

Mineral nutrition is required by all animals and heavy metals have been shown to be essential nutrients [73]. It is essential to maintain animal health and productivity because of the numerous enzymes that coordinate many biological processes, such as Co, Cu, Fe, I, Mn, Mo, Se, Zn [74]. Catalysis and regulation are two other important functions that essential metals perform [75]. Minerals are frequently added to commercial feeds to promote optimal growth, functional bioactivity, and antimicrobial properties from the standpoint of mineral nutrition, as well as to prevent mineral deficiencies that could compromise production [73]. There are many factors to consider when it comes to the optimal concentration of essential metals in feed [76]: genetic influences, diet, interactions between nutrients, bioavailability, and subclinical toxic effects [74, 77]. Since soil and climate conditions around the world have a significant impact on farming practices, the levels of heavy metal contamination in feed can vary widely, making it difficult to generalize across locations and legal restrictions [74]. In order to accurately predict the risk of metal exposure, it is necessary to consider the production system [78]. The majority of chicken feed contains trace amounts of heavy metals.

6.2 Water

Water pollution is the term used to describe the process of polluting waterways (e.g. lakes, rivers, oceans and groundwater). This type of pollution happens when contaminants are not properly handled before returning to the environment via rivers [79]. Water pollution has a negative impact on all aquatic life, including individual

species and populations, as well as natural biological ecosystems [80]. "Heavy" or "toxic," when it comes to metals, is defined as having a density larger than five times the water density. It is important to note that these elements are stable (i.e., those that cannot be digested by the body) and bio-accumulative [63]. Among the heavy metals (the metallic form against the ionic form required by the human body) are mercury, nickel, lead, arsenic and cadmium, alluminum, platinum, and copper.

There are a lot of heavy metals in proteins that have a lot of sulfur in them. The heavy metal concentration in streams, lakes, and rivers is normally less than 0.1 ppm [81]. However, some water sources contained up to 80 ppm of heavy metals. A lack of research has been done on heavy metal concentrations in rainfall and snow [82]. Mono-methyl mercury salts and diethyl mercury salts are the most common water-soluble mercury compounds. Environmental contaminants such as heavy metals have been related to adverse effects on human and animal health [64]. When an animal consumes a large amount of an important metal, it becomes hazardous [66].

A decline in environmental quality can be brought on by the presence of heavy metals in water, soil, or the air [64, 68]. Pollution sources can be traced back to airborne particles. It can be brought to the ground by wind or by raindrops, for example [83]. Contamination of soil layers with Cd is one cause of toxic amounts of Cd in groundwater [83]. Cd will be more concentrated in the water in the pipe duct. Environmental damage occurs when heavy metals in groundwater influence organisms directly or indirectly through adverse effects on human and animal health [84].

HMS can have an impact on our gut microbiota.

In addition to morphological harm, long-term heavy metal ingestion can cause gut flora dysfunction and potentially lead to host metabolic disorders [85]. These germs can impose selection pressure on bacteria that cannot adhere to the mucosal surface [5] and hence affect gut health.

6.3 The impact of heavy metals on the makeup of the gut microbiota

HMs have been shown to limit bacterial growth in several studies [86]. When it comes to microorganisms, Cd has been proven to have harmful effects on growth and development, particularly through disrupting protein synthesis as well as numerous enzymatic processes [83]. Because HMs come into direct touch with the gut microbiota, they have a profoundly negative impact on its composition [85]. After exposure to HM, the majority of studies have shown a drop in Firmicutes and Proteobacteria abundance and a rise in Bacteroidetes abundance at the phylum level. Cd, Pb, Cu, and aluminum (Al) were shown to elicit metal-specific and time-dependent alterations in the gut microbiota of mice, and the quantity of Akkermansia reduced following exposure to these four HMs.

Antibiotics, like heavy metals, may be poisonous to microorganisms as well as dangerous to mammals [5]. As a result, antibacterial metals are being used more frequently in goods. If animals are exposed to heavy metals, their health can be affected both directly and indirectly through their toxicological effects on cells and systems as well as the impact on their animal microbiome [12]. Microbiota imbalance, or dysbiosis, has been associated to several chronic health consequences, including infection [5]. As the immune system matures, the microbiota plays an increasingly important role in ensuring that it stays in a state of homeostasis [13]. Mucus production, epithelial barrier function and inflammation are all affected by beneficial bacteria in the microbiota [27]. The microbiota and the immune system might both be weakened as a result of heavy metal exposure, raising the risk of infection. Furthermore, these

exposures might have a negative influence on health because of the rise in antibiotic-resistant bacteria [85]. Metal resistance, like antibiotic resistance, has been thoroughly documented across many different bacteria for many different metals, despite the fact that heavy metals may be hazardous to microorganisms [36]. Bacteria that are resistant to both metals and antibiotics are often found together. Co-selection of metal and antibiotic resistance genes in bacteria can be caused by a variety of methods. Antibiotic resistance and metal resistance are both coded by two different genes that microbes may have, with one stimulus triggering transcription of both genes either physically or transcriptionally coupled inside a genetic unit like a plasmid. It is also possible that bacteria may have just one gene that makes a protein set that is capable of resisting both metals and antibiotics. As a result of any of these scenarios, bacteria would be able to select for antibiotic resistance as well.

The health impacts of HMs after changes in gut microbiota caused by HMs.

Toxicity-induced gut microbiota alterations have been found to disrupt gut integrity and contribute to a number of downstream consequences [36].

Cucumber toxicity resulted in a deterioration of chicken cecum structure, with the mucosa falling off, vacuoles forming in the lamina propria, and an inflammatory response that was time-dependent. In addition to morphological harm, long-term heavy metal ingestion can cause gut flora dysfunction and possibly host metabolic disorders [11]. Another study found that alterations in the microbiota of the digestive tract have been linked to a number of ailments, including intestinal barrier permeability and inflammation [38]. It is believed that copper exposure might lead to an imbalance in the gut flora, which could have negative consequences for the health of chickens [21].

7. Conclusion

Heavy metals in the broiler chicken production environment affect the gut flora, which in turn affects the health of the animals. In order to minimize or eliminate any impact on the gut microbiota, proper rules for the use of heavy metals in feed and water should be put in place. This is critical for the consumer's health, as heavy metals may build up in the body over time and pose a health risk. Toxic heavy metals may lead to the growth of bacteria that are resistant to heavy metals and antimicrobial resistance at the same time. Regulators and testing should be put in place to limit the discharge and exposure of hazardous materials.

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Chapter 2

Low Pathogenic Avian Influenza: A Permanent Threat to Poultry Farming in Africa

Oumayma Arbani, Mariette Ducatez, Mohammed El Houadfi and Siham Fellahi

Abstract

Initially isolated from turkey flocks in Wisconsin in America in 1966, the avian influenza virus H9N2 has become a serious threat not only to the avian industry but also to human health. Since the 90s, the virus spread in chicken flocks in several countries, starting with China in 1992, then in many parts of Asia, the Middle East, and North Africa. Actually, the LPAI H9N2 subtype is believed to be one of the main causes of chicken respiratory diseases in Africa. Since the first introduction of AIV H9N2 in Morocco in 2016, the virus became enzootic and causes outbreaks in different parts of the country. The intensive uses of inactivated vaccines were insufficient to eradicate the disease, which affects intermittently poultry flocks in different parts of the country at different periods with different degrees of severities, depending on concomitant diseases, management, and other environmental factors. The objective of this chapter will be to explain the H9N2 infection with regard to both animal and human health in Africa and to highlight the assessment of African strategies for control of LPAI in poultry.

Keywords: low pathogenic avian influenza virus, H9N2, poultry, Eurasian lineage, Africa, Morocco

1. Introduction

The low pathogenicity avian influenza (LPAI) H9N2 virus is the most widespread subtype in poultry around the world, posing a concern for animal and public health [1]. Despite their low pathogenicity, H9N2 avian influenza viruses (AIV) are causing heavy economic losses, particularly during coinfection with other respiratory pathogens [2, 3]. Globally, the virus has become endemic in multiple regions of the world counting Asia, the Middle East, and Africa [4]. On the African continent, the first A(H9N2) outbreak was reported in Libya in 2006 [5, 6], then in Egypt in late 2010 [7]. Even though many studies later showed that the virus was present in the country earlier, cocirculating with highly pathogenic avian influenza viruses (HPAIV) of the

H5N1 and H5N8 subtypes have been associated with heavy economic losses in the poultry industry [2, 8–10]. Since then, many African countries started surveillance programs for influenza viruses in poultry and the emergence of G1 lineage H9N2 viruses has been documented. LPAI H9N2 viruses emerged in Tunisia in December 2009 leading to the circulation of the disease in many parts of the country [11]. Rapidly spread in the African continent, the disease has been declared in Morocco in early 2016 [12], then in Algeria in late 2017 showing more than 99% genetic and antigenic similarities with Moroccan strains [13]. Since then, the virus started to spread southward making its way to several Sub-Saharan countries: it was first detected in Burkina Faso in 2016 [14], in Ghana in 2018 [15], and it expanded to Togo, Benin, Uganda, Kenya, Nigeria between 2017 and 2020 and Senegal, with a human case reported in 2020 (**Figure 1**) [16–19].

LPAI H9N2 has not only been detected in poultry but also in some human cases, being a real threat to human health and a global concern for public health. Thus, different studies showed that circulating H9N2 strains acquired an affinity to mammalian like-receptors and gained high virulence and pathogenicity through amino acid substitutions in their viral proteins [11, 12]. Human infections with LPAIV H9N2 have so far been reported in just two African countries; Egypt with four cases, since 2015 [20] and recently Senegal with a case in a 16-month-old child [17]. To date no report of AIV H9N2 in poultry in Senegal is available. Finally, it has been reported that LPAIV H9N2 can easily undergo genetic reassortment and donate internal gene segments to HPAIV H5 and H7 [21, 22].

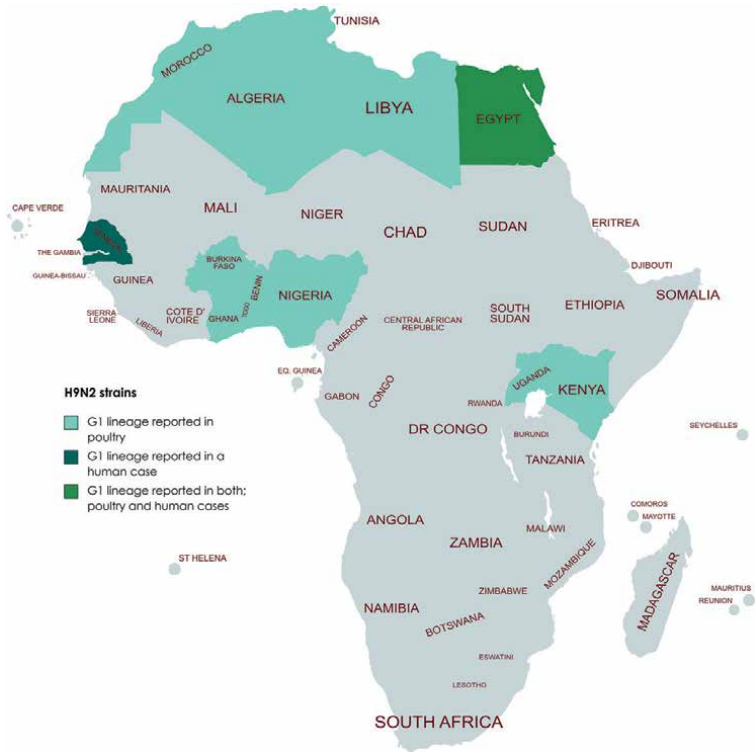

Figure 1.
Phylogenetic spectrum of H9N2 lineage in African countries. The emergence of G1-West lineage is shown in green reported in poultry and some humane cases (figure created with www.mapchart.net).

2. Low pathogenic avian influenza H9N2 subtype: a threat to both animal and human health worldwide

AIV belongs to the *Orthomyxoviridae* family, genus Alphainfluenzavirus (genus A) [23, 24]. These viruses are enveloped and contain negative-stranded RNA. AIV genome contains eight unique segments encoding no less than 10 core proteins including RNA polymerase subunits PA, PB1, and PB2, hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins 1 and 2 (M1 and M2), and nonstructural proteins 1 and 2 (NS1 and NS2) [24, 25]. Based on the genetic and antigenic variants of HA and NA surface glycoproteins, they are classified into 18 HA and 11 NA subtypes, of which 16 HA (from H1 to H16) and 9 NA (from N1 to N9) subtypes circulate in avian species. H17N10 and H18N11 influenza A subtypes were detected in bats in South America [26, 27]. Furthermore, based on molecular markers in the hemagglutinin (HA), AIVs can be broadly classified into two groups that affect their pathogenicity in chickens: HPAIV is highly pathogenic in chickens (high mortality rates in experimental chickens intravenously infected using the intravenous pathogenicity index; IVPI) and contain polybasic cleavage sites in HA; and LPAIV, characterized by low pathogenicity in chickens and mono- to tri-basic cleavage sites in HA. To date, only the H5 and H7 subtypes have shown HPAIV phenotypes in the field [28].

AIV H9N2 viruses are LPAIV and were first detected in turkeys in Wisconsin in America in 1966 [A/turkey/Wisconsin/1/1966(H9N2)] [29]. Since then, these viruses have been circulating worldwide and became the most prevalent AIV isolated from the poultry industry in the world. The subtype has even become endemic in a number of different countries in the Middle East, Asia, Africa, and Europe [30, 31]. Wild birds of the orders Anseriformes (like ducks, geese, and swans) and Charadriiformes (like gulls, waders, and terns) are the main natural reservoir of influenza A virus subtypes [24, 32]. However, there is no clear evidence for the international spread of H9N2 via migratory birds [33]. Instead, the trade and transportation of live poultry may contribute to the viral spread [34].

Phylogenetically, the HA gene of H9N2 viruses can be roughly divided into two main phylogeographic branches, Eurasian and American branches. Many clusters can be identified from these two major lineages. The American H9N2 viruses are mainly found in wild birds, especially sea birds, but they have been reported to infect farmed turkeys without the stable transmission in poultry [35]. However, during routine surveillance programs and at sporadic occurrences of other LPAIV in poultry, there have been no detections of the H9 avian influenza viruses in poultry in North America since 2001. In contrast, frequent isolations of the virus from wild birds have been detected [36].

Regarding the Eurasian H9N2 viruses branch, it is divided into four main lineages based on the hemagglutinin gene; G1 (A/Quail/HK/G1/97-like viruses), Y280 (also known as BJ94 or G9 lineage) (A/Duck/HK/Y280/97-like viruses), Korean-like or Y439 (A/Chicken/Korea/38349-p96323/96-like viruses) and European lineage primarily reported in turkeys. Lineages G1 and Y280 are most prevalent, and highly adapted to poultry [21, 35].

Genetic relatedness of H9N2 isolated in the Middle East and North Africa suggested the existence of two major lineages in the main G1 lineage: lineages A and B. Lineage A represents viruses detected in all countries of the Middle East and North Africa between 1998 and 2016, while lineage B represents viruses isolated in Saudi Arabia, Iran and Israel between 1998 and 2007 earlier [22]. Furthermore, lineage A contains the widespread H9N2 viruses (Panzootic-AIV H9N2) reported more recently in many of the Middle East and African countries.

H9N2 viruses are endemic in poultry populations. They are associated with mild to severe respiratory signs, among which are sneezing, coughing, rales and excessive lacrimation, and rattles [32, 37, 38]. Moreover, other clinical signs are reduction in egg production in breeder or layer flocks, reduced feed conversion with sometimes high rates of morbidity, and up to 20% mortality [31]. In commercial turkeys, H9N2 viruses mainly lead to acute respiratory syndromes and a drop in egg production. Vaccination programs are commonly undertaken in several Asian countries to reduce the economic impact of the H9N2 infection in poultry [28, 39, 40]. The virus also induces transient immunosuppression, which may exacerbate other concomitant or secondary infections. Thus, the severity of clinical signs and mortality rates in infected birds are often increased by co-infection with other avian pathogens, which can increase viral titers in oropharyngeal swabs and tissues [41, 42].

In recent years, A/H9N2 posed a global concern for animal and public health. It has been reported to infect humans by occasionally broadening its host range and crossing the mammalian species barrier. Since its first detection in humans, at least 59 cases have been reported so far and are often associated with mild flu-like symptoms [1]. However, studies in humans exposed to poultry in endemic countries showed that many people harbor H9N2 specific antibodies, demonstrating that subclinical infections are common in many countries, including China, Vietnam, Iran, Pakistan, Romania, and Hong Kong [31].

H9N2 viruses have been circulating among poultry and have acquired human-type receptor specificity, and thus recognize the pattern of sialic acids related to adjacent galactose in conformation α (2, 6) [9]. In addition, they are potentially posing a threat to public health because of their ability to contribute to the genetic diversity of AIVs with serious effects on humans. The internal gene segments of the AIVs responsible for fatal infections in humans (e.g., H5N1, H7N9, and more recently H5N6 and H10N8) are indeed derived from H9N2 viruses [1]. Moreover, A/H9N2 virus infection has been reported in pig farms in Hong Kong and China [21, 34], increasing the risk of zoonotic events. However, no evidence of human-to-human transmission of LPAI H9N2 viruses has yet been observed [9].

3. Assessment of national strategies for control of LPAI in poultry

Although all H9N2 are considered LPAIV based on the lack of mortality in the standardized *in vivo* pathotyping test in specific pathogen-free (SPF) chickens [14], their infections in poultry are quite different in the field compared to controlled experimental conditions. As mentioned earlier, birds show respiratory disease signs, decrease in egg production, and mortality is regularly observed [15]. The difference in the more severe clinical disease observed in the field is thought to be caused by co-infection with other pathogens including mycoplasma, Newcastle disease virus and infectious bronchitis virus (IBV), immunosuppressive infections with viruses like infectious bursal disease virus, and stressful environmental conditions including high temperature or high ammonia levels [43, 44]. Thus, in the last 20 years, the poultry-adapted H9N2 viruses have become a major concern not only for poultry health but also for human health as some of the H9N2 lineage viruses are zoonotic [45–47]. Moreover, one of the most outstanding characteristics of the H9N2 viruses from the G1 lineage is their ability to infect and efficiently spread in domestic bird species [48].

The use of vaccination of poultry likely provides the most practical control tool to reduce human exposure. Traditional vaccines for AIVs are made from influenza

isolates grown in embryonated chicken eggs (ECE), monovalent whole AIV H9N2 inactivated vaccine, or bivalent whole AIV H9N2 and Newcastle disease virus inactivated vaccine and delivered with mineral oil adjuvant [31]. Killed vaccines provide good protection in layer and breeder flocks, especially with multiple-dose vaccine regimens, where the birds usually receive up to three doses during the rearing period [49]. In broilers, vaccines are less effective and tend to generate modest hemagglutination inhibition (HI) titers compared to what is seen in layers and broiler breeders. This may be due to their shorter life span and the presence of maternal immunity when vaccinated early in life [39, 49–51]. However, production numbers are better in vaccinated broiler flocks compared to non-vaccinated flocks if infected [52]. Furthermore, the extensive use of vaccination in broilers and the continuous infection of vaccinated flocks in endemic countries may lead to the formation of escaped mutant viruses that are antigenically distinct from the vaccine [53]. In addition, we previously showed that LPAI H9N2 vaccination was more efficient on day 7 than on day 1 in reducing disease in a challenging experiment with both AIV H9N2 and IBV [54]. In Morocco, [55] showed a very high level of maternally derived antibodies against LPAIV H9N2 in day-old chicks. This was linked with vaccination or field infection of the parents. Indeed, maternally derived antibodies can interfere with vaccination, partially neutralizing vaccine antigens and they often last for 3–4 weeks in chickens [55].

In 2013, a new AIV H9N2 wild virus was isolated from vaccinated and infected broiler flocks in the Middle East. However, the high similarity of it's HA gene to the classical virus used for manufacturing the classical vaccines produced before 2004 was reported. A similar evolution of a new AIV H9N2 strain in vaccinated flocks in South Korea has been reported and the new strain provided better protection as a vaccine [53]. Hence, a new autogenous vaccine that can induce a higher antibody response in broiler chickens and reduce considerably viral shedding, was manufactured from this new field virus from the Middle East. That is why it is expected that the use of an autogenous vaccine will provide better protection for broiler chickens [52]. The use of high-quality, antigenically matched and properly applied vaccines can greatly reduce clinical disease in poultry and of equal importance can greatly reduce or eliminate virus shedding in birds that do get exposed to the virus.

H9N2 viruses hence continue to cause disease in vaccinated poultry. Sub-optimal vaccination may lead to antigenic drift and possibly clade replacement, with increased risk for zoonotic events [1, 11, 53]. Next-generation vaccines should then be developed with the aims of cost reduction, improved production capacities, increased efficacy, and broader protection against multiple H9N2 lineages.

In Ghana, once the virus was introduced for the first time in 2018, stamping out, which involves culling of potentially infected birds and birds presenting influenza-related morbidity has occasionally been used as the first line of defense against H9N2 [15]. But once the virus is endemic in a country, eradication becomes impractical and uneconomical, so vaccination is usually used after that. Eradication is more commonly used for HPAIV outbreaks as it is a reportable disease regardless of a country's outbreak/epidemic history [1].

4. Conclusion

Despite, the use of vaccination and all other tools as a control method for H9N2, many countries still see outbreaks resulting from H9N2 AIVs. For efficient control

of infection and transmission, the efficacy of vaccine and vaccination needs to be improved with a comprehensive control strategy, including enhanced biosecurity, education, surveillance, rapid diagnosis, culling of infected poultry, and proper management of concomitant viral and bacterial diseases. One health aspect would be particularly important to limit the spread of such AIV by elaborating preventive strategy, educating farmers on effective vaccination, and enhancing biosecurity measures to limit the co-circulation of zoonotic H5N1 and H9N2 viruses that has complicated the epidemiological situation in Africa.

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Chapter 3

Expression of Certain Cytokine Genes in Avian Cells Infected with Newcastle Disease Virus

Bindhu Jayaprakash and Divya Nair

Abstract

Newcastle disease virus (NDV) is an inescapable and financially significant microbe, which actually keeps on tormenting the Indian poultry industry. The illness has a wide variety in seriousness going from asymptomatic to 100% mortality. The causal specialist, NDV, is a negative-sense single-stranded RNA virus. Transmission happens by exposure to fecal and different discharges from tainted birds, and through contact with contaminated feed, water, devices, and apparel. In this study expression of cytokine genes in avian cells is identified as a basic proposal for researchers to tackle new castle disease.

Keywords: New castle disease, avian cells, cytokine genes

1. Introduction

The Indian economy is rural based and between 60% and 70% of Indian population depends on agriculture for their sustenance. The development of the Indian poultry industry over the most recent forty years from lawn side interest to the present coordinated scientific and vibrant industrial state is incredible. Subsequently, India has become one of the biggest manufacturers of eggs on the world and the development rate in the poultry industry is exceptional at 5–8% per annum. Besides the various issues that threaten the developments in poultry industry like feed cost, poor marketing and limited post harvest technology, incidence of infectious diseases like Newcastle disease (ND), avian infection bronchitis (IB), etc. also poses major hurdles. A large portion of the irresistible infection causes weighty mortality as well as leads to significant manufacturing misfortunes. The virus causing ND has been classified as the prototype of Avian Paramyxo virus (APMV). The eighth report of the International Committee on Taxonomy of viruses characterized Newcastle disease virus (NDV) under the order Mononegavirales in the family Paramyxoviridae, sub family Paramyxovirinae and the genus Avilavirus. Of the nine species in this genus (APMV 1 to APMV 9), APMV 1 APMV 1 with an intracerebral pathogenecity index (ICPI) value >0.7 has been reported to cause ND with respiratory distress and diarrhoea, with higher morbidity and death. The contamination has been accounted for to

be far and wide, in those 240 types of birds addressing 27 of the 50 sets of birds that were so far impacted by ND.

The genome of APMV 1 has been reported to contain approximately 15,186 nucleotides comprising of genes namely HN, NP, P, M, F and L. The HN protein has been shown to be multifunctional, playing a role in cell attachment and release as well as playing an important role in the infection process, notably in the globular head and stem regions of the HN gene, then the fusion protein (F) has been shown to facilitate the fusion of the viral envelope with the cell membrane, and NP has been shown to be highly immunogenic. Thus, it has been confirmed that the virulence of NDV is multigenic. NDV strains are classified as velogenic (highly virulent), mesogenic (mid virulence), or lentogenic (nonvirulent). Velogenic strains induce severe neurological and respiratory symptoms, spread rapidly, and cause up to 90% fatality. Mesogenic strains cause coughing, affect egg quality and yield, and cause up to 10% mortality. Lentogenic strains cause minor symptoms with trivial mortality.

Clinical indications vary greatly depending on viral strain, bird species and age, concomitant sickness, and pre-existing immunity. There are four broad clinical syndromes recognized.

Viscerotropic velogenic

- Sudden arrival
- Rapidly spreads
- Severe sadness and appetite loss
- Significant decrease in egg production
- Increased respiratory rate
- Extensive bright green diarrhoea
- Oedematous swellings of the head, comb cyanosis, and conjunctivitis
- Prostration, with many birds dying in a matter of days
- Nervous symptoms in those who survive the initial phase
- High mortality (>90% in vulnerable flocks)

Neurotropic velogenic

- Predominantly acute respiratory and neurological symptoms
- Sudden depression
- Appetite loss
- Decrease in egg output

- Chest discomfort and persistent coughing
- Torticollis, wing and leg paralysis, and gasping anxious symptoms
- Adult mortality rates range from 10% to 20%.
- Young chickens may have substantially greater levels.

Mesogenic

- A decrease in egg production and quality (lasting 1–3 weeks);
- Weight loss;
- Gasping nervous symptoms may appear late in the clinical phase of acute respiratory sickness
- Mortality rate is about 10%

Lentogenic
Commonly subclinical may be

- No mental symptoms
- Mild respiratory symptoms
- Temporary appetite loss
- Decline in egg production;
- Negligible mortality unless concomitant disease is present.

There are several gross lesions.
Young chicks and chickens dying suddenly sometimes don't have any lesions.
The following symptoms are present in the viscerotropic velogenic form:

- Hemorrhages and necrosis in the walls of the small intestinal tract, gizzard, and proventriculus. Other internal organs frequently get little hemorrhages.
- Thickened and clouded air sacs, acute laryngitis, and tracheitis, congestion, and catarrhal exudates are present in the neurotropic velogenic and mesogenic variants.
- Proventriculus hemorrhages occasionally, but infrequently elsewhere

2. The disease

An "infection of birds caused by Avian paramyxovirus 1 (APMV 1) with an ICPI value more than 0.7, possessing three arginine (R) or lysine (K) residues between

position 113 and 116 of the F gene, and possessing phenylalanine (F) at position 117" is what the Office Internationale des Epizooties (OIE) defines as ND. The OIE has reclassified the illness, which was formerly included as a list A infection and is now one of the notifiable avian diseases. There have also been reports of significant productivity and financial losses due to the sickness. The production and economic losses caused by ND were shown to be more significant and severe than its economic effects. The economic effects of ND on commercial poultry trade were found to more important and severe [1].

3. Historical perspectives

Between 1926 and 1930, reports of ND in hens were made in several nations, including Indonesia [2], Newcastle-Upon-Tyne, England [3] and Ranikhet Village, Chennai, India [4, 5]. ND has been reported in many countries, including the USA [6], Australia [7, 8], Malaysia [9], South Africa, and Mozambique [10]. The sudden emergence of ND in a virulent form during the beginning of twentieth century was attributed to number of reasons, which include a sudden change in host population, role of feral birds acting as natural reservoir, shift of virus from enzootic form to epizootic form or to the result of a major mutation at the genome level [1, 11]. These views were reinforced by the emergence of ND as panzootic and report of ND in caged birds and feral birds [12–15].

4. Impacts

Two hundred and forty one species of birds, or 27 of the 50 orders of that class, have been recorded to have ND, which predominantly affects chickens. House crows, pigeons, ducks and geese, emus, water fowl. Due to their apparent disease resistance, village chickens have been reported to be affected by the virus just as severely as commercial poultry.

5. Control

According to reports, NDV still poses a concern and continues to produce serious outbreaks even if control measures like good management practices and biosecurity standards are available at the farm level [8]. As a result, routine vaccination is the major goal of control measures. However, it has been noted that vaccination is not straightforward because it only prevents clinical sickness and mortality and does not stop virus multiplication, which makes the virulent become endemic [1].

Vaccination against ND in chickens has been reported to be carried out with live naturally occurring and artificially attenuated non pathogenic forms of the agent, inactivated viruses or their immunogenic determinants, subunit vaccines, live genetically modified vaccines, DNA vaccines, marker vaccines and edible vaccines. However, most of the currently available vaccines were not found to be able to provide desirable immunity even after using multiple doses [1], which has been justified by regular outbreaks of ND in vaccinated flocks.

While inactivated vaccines give primed birds a sustained high tire immune response, live vaccines were reported to stimulate the production of both humoral and cellular immune responses in addition to mucosal immune responses. Paranteral vaccination with inactivated virus often elicits serum neutralizing antibodies, and no local immune response, in contrast to attenuated NDV when used as live vaccines, which have been shown to have the ability to revert to virulent strains with transfer from bird to bird.

6. The virus

6.1 Classification

The International Committee on Taxonomy of viruses (ICTV) has been classified NDV under the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *paramyxovirinae*, and genus *Avulavirus.* The genus *Avalavirus* as on date has reported to have only one species, namely the *Avian Paramyxovirus* (APMV) that comprises of nine serotypes—APMV 1 to 9. Of the nine serotypes, APMV-1 has been identified to be responsible for clinical ND.

6.2 Morphology

The nucleocapsid of the NDV virus is reported to measure 1000 nm in length, 17–18 nm in width, and an envelope covered in spike glycoproteins measuring 8–12 nm in diameter. The NDV virus particles are described as being pleomorphic and varying in size from 150 to 400 nm. According to the "rule of six theory", which is unique to members of the family Paramyxoviridae, the genome was also found to be typical of Baltimore group v, single strand of negative sense RNA with a molecular weight of $5.2–5.7 \times 10^6$ Da and 15,186 nucleotides. Six significant proteins have also been identified to be encoded by the genomic RNA, especially the haemagglutinin neuraminidase protein (HN), phosphoprotein (P), matrix protein (M), F, and big protein (L). According to reports, Mrna editing at the P gene led to the formation of the two additional proteins, V and W.

6.3 Methodology

6.3.1 Sample preparation

6.3.1.1 Procedure

The experiment was conducted with infected (treatment) and mock infected (control).

1. The cells from chicken embryos (CEs) were grown in six-well tissue culture plates (Nunc, Denmark, Cat # 150229) for preparation of samples.

2. The CE cells from one well from each group were taken after confluent monolayer to serve as the sample for day 0. The remaining cell cultures in both

the treatment and control groups were infected with the seventh passaged NDV (D58) virus and MEM, respectively.

3. For the viral adsorption, the plates were incubated at 37°C for 1 hour.

4. The un-adsorbed virus was thoroughly washed off with MEM medium and the cell cultures were maintained in maintenance medium (2 ml/well).

5. The plates were incubated at 37°C and 5% CO_2 (CO_2 Incubator, Model 3131, Thermo Scientific, USA).

6. Throughout a 5-day time course as sample 1–5, the CE cells of one well from each group were collected every day at intervals of 24 hours.

6.3.2 Harvesting of CE cells

Prior to trypsinization using 250 μl of 0.1% trypsin 1:250 (Invitrogen, Canada, Cat # 27250-018) and 0.5 mM EDTA (Life-Technologies, USA, Cat # 15576-028) solution (made in sterile 1× PBS and sterilized by 0.22 m syringe filter) for 2–3 minutes, the cultures were immediately rinsed twice in pre-warmed PBS before being used to harvest the cells. To stop the trypsinization, 750 μl of growth media was added. Centrifugation at 500 g (∼2400 rpm) for 10 minutes at room temperature (25°C) was used to collect the cells. 750 μl of supernatant was discarded and remaining 250 μl along with cells pellet was preserved at −40°C for RNA isolation.

7. Reverse transcriptase polymerase chain reaction (RT-PCR)

7.1 Material for RT-PCR

7.1.1 RT-PCR kit

The iScript™ cDNA Synthesis kit (BIO-RAD, USA, Cat # 170 – 8891) was used to synthesize cDNA for cellular (CE-fibroblast) genes (β-actin, IFN-α, IFN-y, MHC-I and DDX1) and viral genes (M and F genes).

7.1.2 For RNA extraction required materials

Cellular and viral RNA was extracted from CEF samples using the following chemicals

1. TRIZOL® LS reagent (Invitrogen, USA, Cat # 10001 96-010)

2. Chloroform (Qualigens, India, Cat # 22465)

3. Isopropanol (Merck, India, Cat # 9634)

4. 75% ethanol (Changshu Yangyuan Chemical, China, Cat # XK-13-201-00185)

5. Protease, nuclease free water (GeNei™, Bangalore, Cat # 105437)

7.1.3 Other materials/reagents for PCR

Material/reagent	Supplier with Cat #	Purpose
PCR tubes	Axygen, USA, Cat # MCT-02-C	For cDNA synthesis and PCR
Microfuge tube 1.5 ml	Axygen, USA, Cat # MCT-150-C	For RNA isolation
Ampliqon (Taq DNA Pol 2.0× Master Mix Red)	Biomol, Denmark; Cat # AMP 180301	For amplification of DNA
DNA marker	100 bp (100–3000 bp) Cat # 1 kb (300–10,000 bp), Cat # Axygen, USA	In order to research the genes unique DNA migration pattern
Agarose	Genei™ India, Cat # 105193	For gel electrophoresis of PCR product
Ethidium bromide solution	Sigma-Aldrich, USA, Cat # 46067-50ML-F	Using a final concentration of 10 μg/ml for staining the gels

7.1.4 RNA extraction from cells and viruses

With a few minor adjustments during the RNA pellet washing stage, the TRIZOL® LS reagent was used to extract the RNA from preserved pelleted CE cells in accordance with the manufacturer's instructions.

1. The pelleted CE cells was suspended in supernatant and 250 μl of this suspension was taken into DNAse, RNase free microfuge tube.

2. After adding 750 μl of TRIZOL® LS reagent, the mixture was vortexed to combine the components.

3. For the complete dissociation of nucleoprotein complexes, this mixture was incubated for 5 minutes at room temperature (20°C).

4. Days after the initial incubation, 200 μl of chloroform was added, and the mixture was vortexed for 15 seconds and again incubated at room temperature for 10 minutes.

5. A refrigerated centrifuge machine (Eppendorf model # 5415 R) was used to centrifuge the contents at 12,000 g for 15 minutes at 4°C.

6. After separating the upper aqueous phase, RNA was precipitated by adding an equivalent volume of isopropanol.

7. To pellet the precipitated RNA, this mixture was centrifuged at 12,000 g for 10 minutes at 4°C while being held at 20°C for 20 minutes.

8. Then discarded the supernatant and the pellet was then centrifuged twice with 75% ethanol for 5 minutes at 7500 g before being air-dried for the remaining 5 minutes. The RNA pellet was once again dissolved in 20 μl of DNase RNase free water.

9. The concentration of RNA was determined to be 260/280 Å using a spectrophotometer (Bio-tek Instruments, Inc., μ Quant).

7.1.5 cDNA synthesis

Following the manufacturer's instructions, the iScript™ cDNA Synthesis kit (Bio-Rad, USA) was used to create cDNA synthesis.

1. The reaction mixture (20 μl) used to synthesize cDNA has the following ingredients

Component	Volume	Final concentration in the reaction
5× iScript reaction mix	4 μl	1×
iScript reverse transcriptase	1 μl	1×
Nuclease-free water	10 μl	1×
RNA template (100 fg–1 μg)	5 μl	1×
Total volume	20 μl	1×

2. The above reaction components were added and mixed properly by vortexing and spinned for few seconds to accumulate all of the components at the tube's bottom.

3. Reverse transcription was carried out in the tubes using a thermocycler (Applied Biosystem, Singapore, Model #2720) at 25°C for 5 minutes, 42°C for 30 minutes, and terminated at 85°C for 5 minutes.

4. Following cDNA synthesis, 10 μl of each sample's cDNA was pooled, serially diluted, and used as a standard curve in a relative quantitative PCR (qPCR) assay. The 10 μl of the cDNA was diluted ten times and kept at −40°C for a farther use.

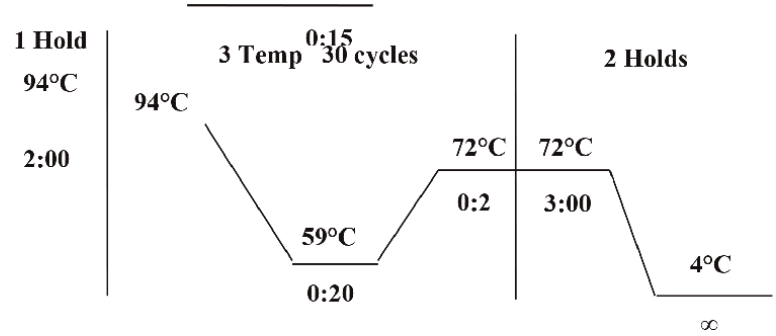
7.1.6 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using Ampliqon (Bio-Basic Inc.) following manufacturer's instructions for FPCS of F gene and M gene fragment. The primers designed for qPCR were used.

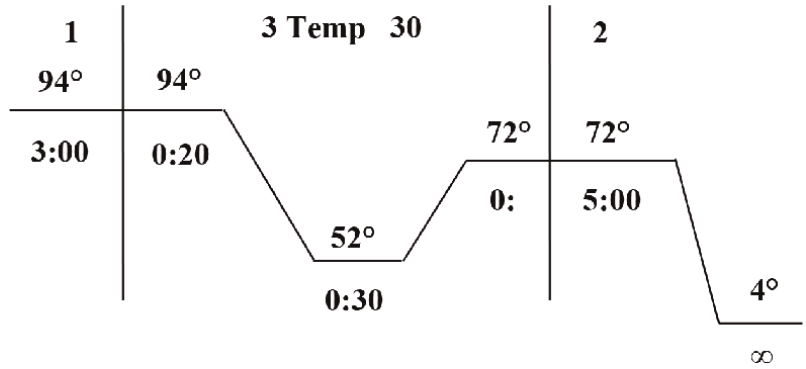
The reaction was set up as follow

Component	Volume	Final concentration in the reaction
Taq Master Mix Red	10 μl	1×
Forward Primer (10 pmol)	1 μl	0.5 pmol
Reverse Primer (10 pmol)	1 μl	0.5 pmol
Template cDNA	2 μl	—
Nuclease free water	6 μl	—
Total volume	20 μl	—

1. For amplification of M gene fragment, PCR was carried out as per cycle sequence provided below.



2. For the purpose of amplifying FPCS gene specific cDNA, PCR was performed using the cycle sequence shown below



3. The FPCS of the F gene and M gene fragment PCR products were electrophoresed at 150 V for 15 minutes in a 2.0% agarose gel and compared with a 100 bp DNA marker. The gel was then photographed and scanned using a gel doc system (Bio-Rad).

7.1.7 Other materials/reagents for qPCR

Sl. no.	Material/reagent	Supplier with Cat #	Purpose
1	Real time PCR tube (0.2 ml) strips and masterclear cap strips	Eppendorf, North America, Cat # 951022109	For qPCR
2	Micro tips (0.2–10 μl)	Tarsons, Kolkata; Cat # 52100	For dispensing the reagents
3	Real time PCR plates (96 wells/plate)	Applied Biosystem, USA, Cat #	For qPCR

7.1.8 qPCR

Using SYBR® Green JumpStart™ Taq Ready Mix™ (Sigma, USA, Cat # S4438) in accordance with the manufacturer's instructions, qPCR was performed. SYBR Green jumpstart Taq Ready Mix combines the convenience of an easy-to-use ready-Mix solution with the performance boost of jumpstart Taq antibody for hot start PCR with

SYBR Green I. It contains a fluorescent dye and the reagents for performing high-throughput qPCR and is provided in 2× concentration.

1. The composition of reaction mix (10 μl) used for real time PCR is as follow

Component	Volume	Final concentration in the reaction
2X JumpStart Taq Ready Mix	5 μl	Taq DNA Polymerase—1.25 units, Tris HCl 10 mM, KCl 50 mM, $MgCl_2$ 3.5 mM, dNTP 0.2 mM,stabilizers
Forward primer (10 μM)	0.4 μl	0.4 μM
Reverse primer (10 μM)	0.4 μl	0.4 μM
Template cDNA	___μl	2–20 ng
Nuclease free water	q.s. to 10 μl	
Total volume	10 μl	

2. The above components are combined and added to a 200 μl PCR tube with a thin wall and carefully combined by vortexing before being quickly centrifuged for a few seconds to let allow the contents settle at the bottom of the tube.

3. Real time PCR was performed in a real time thermocycler (Mastercycler® ep realplex, model # 22331, Eppendorf, Germany) for the amplification and relative quantification of cellular genes. The procedure started with a heat denaturing step at 94°C for 3 minutes, then the sequence cycle, final extension, and melting curve as follows

After each extension step, a single fluorescence `n end-point was measured. To establish the specificity of each amplification, the melting curves for PCR products were examined between 70°C and 95°C.

Conflict of interest

Authors express no conflict of interest.

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Chapter 4

Advances in the Nutrition of Functional Amino Acids in Healthy and Immunologically Challenged Birds

María de Lourdes Angeles and Sergio Gómez-Rosales

Abstract

The effects of some functional amino acids (AAs) such as arginine, threonine, and methionine on the development of immune and digestive capacities in poultry were reviewed. The information was examined analytically to identify the source of the AA requirements, growth potential, type of housing, and type and degree of immune challenge applied. Regardless of these factors, the level of functional AA required to stimulate the immune or digestive response was higher than that required to maximize the productive performance. The implications section describes the main obstacles to integrating and applying the concept of functional AA in conventional diet formulation, and its use in birds raised under commercial conditions experiencing different types and degrees of immune stressors. It is necessary to develop a profile of functional AAs and establish their strategic use during or after immunological challenge situations to aid in the recovery of productive parameters to prechallenge levels.

Keywords: functional amino acids, growth, immune response, digestive function

1. Introduction

The establishment and reevaluation of nutrient requirements in poultry is a dynamic process due to constant increases in feed consumption, growth rate, and the amount of dietary protein, and amino acid (AA) transformed into body protein in chicken meat over time, as a result of genetic selection programs [1–3]. The advancement of each of these factors necessitates the constant labor of AA to maintain high levels of productivity, especially when we consider that in modern animal production, new concepts such as sustainability in meat production are emerging, as a result of the constantly increasing societal demand for food production through environmentally friendly practices.

Decades ago, one of the responses to achieving high levels of efficiency in the transformation of feed into high-quality protein was the development of the concept of the ideal AA ratio, which seeks to maximize efficiency.

However, when comparing the AA profiles recommended by various sources, there is a significant difference between them. For example, among the main essential AA in poultry, arginine (Arg), methionine (Met), threonine (Thr), and tryptophan ratios of 94–120, 36–46, 58–73, and 14–20 have been proposed over time. One reason for these disparities is that these available AA ratios were proposed over 50 years, from 1965 to 2014 [3, 4]. During this time, significant advances in broiler growth have been made, as well as the development of other concepts such as the use of digestible AA ratios, in conjunction with a steady improvement in the nutritional quality of vegetable feedstuffs through selection, and an ever-increasing amount of new information on the composition and availability of nutrients in feed ingredients.

Another strategy, in addition to the use of ideal AA ratios, to improve broiler production efficiency is the use of low-CP diets supplemented with available feed-grade amino acids [3, 5, 6] to fulfill the AA requirements according to different recommendations [7, 8], ensuring the birds productivity at least at the same level shown by birds. Furthermore, using low-CP rations improves nitrogen efficiency by avoiding excess nitrogen excreted in the form of uric acid, reducing environmental pollution caused by nitrogen and ammonia emissions, and lowering the carbon footprint of feed manufacturing through changes in the type and amount of raw material included in the feeds.

While these feeding strategies are still being refined in terms of research and practical application, they must keep up with changes in broiler genetic potential. Furthermore, they must adjust to new nutritional concepts, such as the use of functional AA. Functional AAs are defined as AAs that participate and regulate key metabolic pathways that improve organism health, survival, growth, development, lactation, and reproduction [4, 9]. Functional AA's descriptive roles in nutrition and health, as well as the metabolic pathways involved, have been documented [4]. In recent years, there has been a large number of publications on this topic, as well as several outstanding reviews on the use of functional AA to improve the immune response and digestive capabilities in chickens, including the embryonic developmental stage and the growth stage on the farm [10–12]. According to a recent review [12], AA supplementation strategies can positively contribute to immune and gut health. In the present chapter, an attempt was made to analyze the available information on the use of Arg, Thr, and Met as functional AA aimed at establishing a pattern between the improvement in immune response and digestive physiology with improvements in broiler growth, estimate an AA requirement, and discuss the implications regarding the readjustment of feeds based on functional AA formulation and their application in broilers kept in commercial settings.

2. Arginine

Arg is known as an essential amino acid (AA) for birds due to its inability to synthesize Arg, so it must be supplemented in their diet [13]. Arg plays an important function in serving as the building block of proteins and polypeptides and fulfills several physiological roles through the regulation of key processes such as maintenance, growth, reproduction, and immunity. The recommended dietary Arg levels for optimum growth performance in broilers varies from 1.25 to 1.10% for starting and growing birds [7] and from 1.37–1.43 and 1.0–1.1 for starting and finishing birds, respectively [8]. There are also dietary Arg recommendations for different commercial strains of broilers. In addition to this, it has been documented that the addition of

Arg either in ovo or dietary Arg above the recommended level improves the digestive physiology and the cellular and humoral immune responses in nonchallenged and challenged birds; hence Arg has gained the distinction of belonging to the group of functional nutrients. The readers are referred to several comprehensive reviews about this topic [14–18]. In the next section, the recent findings on Arg feeding from in ovo to unchallenged and challenged broilers will be presented with emphasis on simultaneous effects on immunity/digestive physiology and productivity.

In ovo feeding (IOF) of Arg. IOF of Arg has been shown to boost glucose synthesis in the liver, which correlates with enhanced glucose 6-phosphatase activity at hatch [19, 20]. In this regard, it has been shown that IOF of Arg improved posthatch growth performance in chicks, and the effects of Arg have been linked to glucose synthesis and hormone production. Furthermore, supplementing with Arg has been proven to improve gut morphology (a sign of gut health), implying that it may affect the metabolism of this oxygen-demanding tissue [21]. IOF of Arg also stimulates the intestine mucin gene expression at 18th d of incubation and 14th d posthatch, as well as the IL-6 and IFN-γ humoral gene expression in 26-days-old birds [22]. In this section, the available literature was examined to find the best dosage of Arg that improves the immune response and gut morphology and that leads to enhanced growth performance in posthatch broilers.

In some experiments, the Arg IOF at day 14th of incubation has been evaluated. IOF of 35 mg Arg/egg depressed hatchability but increased the body weight of alive broilers at 11, 24, and 42 days of age; Arg inclusion increased the length of jejunum and ileum at 42 d of age and led to the greatest villus height and crypt depth in jejunum at 11 days of age [23]. In a similar experiment, Arg reduced hatchability again but increased the body weight in 42 days old broilers, and the relative weight of spleen and bursa of Fabricius at 11 days posthatch and antibody titer against SRBC at 30 days posthatch [24].

In several recent experiments, lower levels of IOF (0.6 mg Arg/egg) at 17.5 d of incubation have been tested. A summary of the results indicates that Arg IOF did not negatively affect the hatchability and improved the body weight at 7 and 21 d posthatch, and the ADWG from 1 to 21 d of age; Arg also increased the weights of digestive organs, the activities of digestive enzymes, alkaline phosphatase, maltase, and sucrase in the jejunum, the mRNA expressions of jejunal sensing receptors of taste and nutrient transporters of solute carriers [21]. Arg also increased the absolute weights of lymphoid organs, the activity and the mRNA expression and protein abundance of iNOS, the contents of IL-2, IL-4, and sIgA, the mRNA expressions of TLR-2 and TLR-4 in intestinal mucosa and serum; conversely, Arg decreased the iNOS promoter methylation percentage in jejunal mucosa [25].

In other reports, it was found that IOF of 0.6 mg Arg increased the weight of embryos at 19 days of incubation and the ADWG in chicks from 1 to 7 days posthatch [26], and the ADWG of broilers from 1 to 21 days and from 1 to 42 days of age [27]. Arg also increased the duodenum activities of alkaline phosphatase, maltase, sucrase, and inducible nitric oxide synthase of 7-days-old posthatch broilers, and the villus height and the ratio of villus height to crypt depth in duodenum of broiler embryos and posthatch birds and increased the density of goblet cells [26, 27]. The hatchability was high and similar to the control group in Arg supplemented eggs. Other benefits of Arg were the increased percentage of proliferating cell nuclear antigen positive cells of villus, and the mRNA expressions of mucin-2, claudin-1, and zonula occludens-1 and -2 in jejunal mucosa of 21-day-old broilers [27]. Furthermore, IOF of 0.6 mg Arg/egg increased the relative weight of breast muscle

at hatch and 7, 14, and 21 days posthatch, and increased the concentration of some essential AA in the breast muscle such as Thr, valine (Val), phenylalanine (Phe), lysine (Lys), and Arg at hatch and 21 days posthatch [20].

Some studies have also been published using higher levels of IOF Arg with positive results. The IOF of 2.5 mg Arg/egg at 18 days of incubation resulted in similar hatchability to the control group, and in higher chick weight at hatch and lower transit weight lost from the hatchery to the farm; Arg also improved the body weight and ADWG in broilers up to 21 days of age [28]. When using even higher levels of IOF Arg (11 and 22 mg Arg/egg) at 18 days of incubation, the hatchability was similar to the control group, and greater body weight in chicks at 7 days posthatch was reported with 11 mg Arg; improved development of duodenal villi in 7-days-old chicks and enhanced cell-mediated immune response after 24 and 48 h in 28-days-old broilers was observed with 11 and 22 mg Arg [29].

A summary of the results indicates that IOF of 0.6 mg Arg/egg at 17.5 days of incubation increased the growth performance of broilers up to 42 days of age, which could be explained by the enhanced immune humoral and cellular response and the early maturation of the digestive capabilities.

In several studies, the stimulatory properties of increasing levels of dietary Arg on the immune and digestive systems have been documented in broilers kept in nonchallenged and under-challenged conditions [30–33]. From this, a number of studies have been published that have evaluated the productive performance along with the immune and digestive responses to high levels of Arg supplementation. These studies, while few, may provide insight into whether improvements in the immune and digestive responses can be associated with increased productivity at the same level of Arg supplementation.

In nonchallenged, 1–28 days of age broilers kept in cages and fed 1.48% (considered a normal level in corn/soybean meal diets) and 1.58 dietary Arg (keeping an Arg:Lys ratio of 1.20), no differences in growth performance were found, whereas the addition of 1.58% Arg increased the percentage of mucosa T helper (CD4+TCRvβ1+) and T cytotoxic (CD8+CD28+) [34]. In nonchallenged chicks housed in floor pens from 1 to 21 days of age and fed increasing levels of Arg [1.00, 1.125, 1.250, 1.375, and 1.50% of NRC [7] recommendations for Arg requirements], the performance was improved at 1.25% Arg, while the relative weight of thymus increased in a nearly linear manner, and the cell-mediated immune response to phytohemagglutinin P and antibody titer against NDV increased linearly up to 1.375% Arg [35]. Similarly, using increased Arg levels (0.86, 1.31, 1.76, 2.21, and 2.66%, based on the recommended Arg requirement by NRC [7]), the ADWG and FCR were improved at 1.31% Arg, whereas serum total immunoglobulins and IgA increased up to 1.76 and 2.21% dietary Arg, respectively [36]. Furthermore, low growth potential chicks fed increasing total Arg levels (0.85, 0.97, 1.09, 1.21, and 1.33%, based on the nutritional requirements for Qingyuan partridge chickens) for 30 days showed maximum ADWG and FCR at 0.97% Arg, while mucosal jejunum IgG and ileum sIgA increased linearly up to 1.21% [37].

In challenged broilers vaccinated against *Salmonella enteritidis* at nine days of age and kept in cages from 1 to 28 days of age, similar performance was observed with 1.48 and 1.58% dietary Arg (keeping an Arg:Lys ratio of 1.20); however, increased suppressors monocytes (Kul+MHCII−) were found in birds supplemented with 1.58% Arg [34]. In broilers fed diets: deficient, normal, and excessive in Arg (1.05, 1.42, and 1.90% according to NRC [7]), kept in cages and challenged with an *Escherichia* (*E.*) *coli* lipopolysaccharide (LPS), showed higher ADWG and FCR at 1.42 and 1.90% Arg, respectively, after the challenge; depressed TLR4 and NF-kB in cecal tonsils relative

mRNA expression at 1.42% Arg and PPAR-γ in spleen and IL-1b in cecal tonsils relative mRNA expression at 1.90 Arg were observed after the challenge [38].

In several studies, the coccidiosis challenge has been used as a mean to demonstrate the functional benefits of Arg. A summary of some studies is given below:

Broilers from 1 to 26 days of age allocated in metabolic cages fed increasing levels of dietary Arg (1.04, 1.14. 1.24, 1.34, and 1.44%) and challenge with Eimeria sporulated oocysts at 12 days of age, showed better ADWG and FCR at 14 days postchallenge at 1.14% Arg. Higher levels of Arg (1.34%) improved the intestinal permeability at five d postchallenge and the tight junction proteins zonula occludens-1 and zonula occludens-2 at six d postvaccination, while the addition of 1.44% Arg increased the zonula occludens-1 and zonula occludens-2 at 14 days postvaccination [16]. In chicks also housed in cages from 1 to 21 days, added with 1.11, 1.33, and 2.01% dietary Arg and challenged with a coccidiosis vaccine at 14 d of age, showed similar ADWG regardless of the dietary Arg level and lower FCR at 1.33% Arg. Increased sucrase, sIgA, and relative IL-1RI mRNA expression and reduced abundance of TLR4 and MyD88 in jejunum at 7 days postchallenge were observed at 1.33% Arg, and increased mucosal density in the jejunum was observed at 2.01% Arg at 7v postchallenge [38].

In floor-pens reared broiler given 100, 105, and 110% of the standard recommended values of dietary Arg for Ross broilers, and challenged with a mixture of Eimeria species from 16 to 20 days of age, addition of 105 and 110% Arg, prevented depressed ADG in coccidia-infected broiler chickens during the finisher period. The FCR was improved at 110% Arg supplementation during the grower and finisher periods. Increased villi height to crypt depth ratio at 105% Arg and increased villi surface area at 110% Arg were found, as well as a linear decrease in fecal oocyst count [39]. Broiler chicks reared in pens fed 100, 125, and 150% Arg levels, according to Ross recommendations, and infected with Eimeria on day 21, showed better ADWG, FI, and FCR from 22 to 42 days of age at 125% dietary Arg (starter 1.71%, grower 1.54%, and finisher 1.375%); furthermore, at 125 and 150% dietary Arg, increased levels of serum NO and proinflammatory cytokine concentrations (IL-1β IL-2 IL-6 TNF-α IFN-γ) and reduced fecal oocysts were found [40].

In two additional studies with broilers subjected to viral challenges, it was observed that productivity and immune responses were improved with higher levels of dietary Arg than recommended. Broiler chickens fed diets exceeding by 2.5 times, the recommended NRC levels (starter 1.34 vs. 3.35, grower 1.13 vs. 2.8, and finisher 1.1 vs. 2.58), and challenged with an intermediate plus strain of IBD virus (10-fold greater than normal vaccination doses) at 28 days of age, showed enhanced body weight, ADWG, and FCR, as well as higher serum level of IFNα, IFNγ, immunoglobulin G, and lower lesion scores in the bursa and spleen compared to the control birds [41]. In the same way, broiler chicks fed 2% supplementary dietary Arg and vaccinated and challenged against hydropericardium syndrome virus showed higher body weight, lymphoproliferation, and cutaneous basophil hypersensitivity reactions, lymphoid organ weights, and highest survival rate compared to unvaccinated non-Arg supplemented chicks [42].

Results in four available studies, in which nonchallenged broilers were fed increasing dietary Arg concentration, indicate that the Arg needed to stimulate the immune system was higher than that needed to improve the growth performance. These results were irrespective of the basis of Arg formulation, the growth rate of the birds, and the type of housing (cages or floor pens).

The information also denotes that in four out of eight studies available, in which challenged broilers were fed increasing dietary Arg concentration, the Arg needed to

stimulate the immune system was also higher than that needed to improve the growth performance. These results were irrespective of the basis of Arg formulation, the growth rate of the birds, the type of housing (cages or floor pens), and the type and degree of challenge.

It was found that in three out of four studies, in which the growth performance and immune and digestive responses were enhanced at the same Arg levels, the Arg levels were higher than those recommended for optimum growth; it is noteworthy that in one of these studies, ADWG and FCR were improved in 1–49-days-old broilers with Arg levels 2.5 times higher than recommended.

3. Threonine

Thr is ranked as the third limiting AA [2, 4] and is very important for the synthesis and maintenance of proteins in the body. About 30–50% of Thr, as well as some other amino acids, is directly used by the small intestine and is not available for extra-intestinal tissues. Thr has special importance as an essential nutrient because, compared with other AA, it has the highest metabolism in the portal-drained viscera. One of the primary fates of absorbed Thr is the synthesis of intestinal proteins, which are mainly secreted into the lumen as mucus, whereby protecting the gut from pathogens and antinutritional factors. Mucins are particularly rich in Thr, proline, and serine, with Thr representing as much as 28 to 40% of its total AA profile [43].

The recommended dietary Thr levels for optimum growth performance in broilers varies from 0.80 to 0.68% for starting and growing birds [7] and from 0.85–0.89 and 0.65–0.68 for starting and finishing birds, respectively. Further to this, it has been demonstrated that supplementation of Thr either in ovo or dietary Thr above the recommended level improves the digestive physiology and the cellular and humoral immune responses in nonchallenged birds and those subjected to different immune challenges [43]. IOF of Thr has shown to increase the expression profile of growth factors and immunity-related genes, including higher mucin gene expression on incubation day 18, higher expression of mucin gene on day 14 postinoculation, higher humoral expression of IL-6 and TNF-α, and higher IL-12 cellular gene expression in 26-days-old broilers [22]. In the next section, the recent findings on Arg feeding from in ovo to unchallenged and challenged broilers will be presented with emphasis on simultaneous effects on immunity/digestive physiology and productivity.

In several experiments, the IOF of Thr at day 14th of incubation enhanced various immune and digestive responses. IOF of 20 or 30 mg Thr/egg improved the ADWG of broilers from 14 to 28 days of age and enhanced the humoral response to sheep red blood cells; there was a tendency for digestive enzyme activities in proventriculus, jejunum, and pancreas to be higher in Thr-injected chicks at 21 days of age [44]. IOF of 25 mg Thr/egg increased the body weight of broilers at 11, 24, and 42 d and the FI from 1 to 42 days of age; Thr also enhanced the ileum villus height in 11-days-old chicks and the relative weight of the jejunum and ileum and the length of the jejunum in 42-days-old broilers [23]. IOF of 25 mg Thr/egg also increased the ADWG and FI in broilers from 1 to 42 days of age and the antibody titer against sheep red blood cells in broilers at 30 days posthatch [24]. In both studies, hatchability was similar to the control group.

In few experiments, the IOF of Thr in the last days of incubation has been also evaluated. IOF of 10.5, 21.0, 31.5, and 42 mg Thr/egg on day 17.5 of incubation

improved the chick hatch weight and growth performance from 1 to 21 days of age; Thr increased the villus height, villus height: crypt depth ratio, and villus area at hatch and 21 days posthatch. At hatch, all Thr levels increased the expression of MUC2 and PepT1 compared to the control group [45]. IOF of 15, 30, and 45 mg Thr/egg at 18th embryonation d increased the ADWG in broilers up 21 days posthatch, and the FCR was improved at 45 mg Thr; Thr increased the thymus weight (d0), bursa weight (d3), spleen weight (d3 and d7), whereas quadratic effect was observed on weights of bursa, thymus, and spleen at d21. IOF of Thr also increased the weights of gizzard, intestine, and liver at hatch, proventriculus at d7, as well as intestine and liver at d21 [46].

A summary of the results indicates that IOF of Thr at 14 and 17.5–18 days of incubation increased the growth performance of broilers up to 42 days of age, which could be explained by improved immune responses, but especially by increasing the development of the digestive capabilities. The best dosage for IOF of Thr appears to be around 25 mg/egg. In all cases, a high hatchability is maintained.

During the growth out of broilers, there are several studies of Thr supplementation as functional AA in nonchallenged conditions. Ross male broilers fed diets containing 0.8% (NRC [7] requirement), 0.87% (average of NRC and Ross requirement), 0.94% (Ross requirement), and 1.01% (more than Ross requirement) Thr had improved growth responses as dietary Thr increased from 0.8% to 0.87%; similarly, the villi height, crypt depth, and villi surface increased as dietary Thr increased from 0.8% to 0.87% [47]. In broilers from 1 to 21 days fed increasing standardized ileal digestible Thr levels from 0.4 to 1.1%, it was reported that ADWG was higher at 0.84–0.89% Thr, while the villus height in duodenum, jejunum, and ileum were increased linearly up to 1.1% Thr [48]. In broilers from 1 to 21 days of age fed 0.79, 0.87, and 1.07% Thr showed no differences in growth performance due to the supplementation of Thr; opposite to this, Thr supplementation increased the relative weight of spleen and thymus. Thr supplementation linearly increased the intestinal villus height, the ratio of villus height to crypt depth, as well as the goblet cell density and the jejunal immunoglobulin G and M. At the highest Thr supplied, the ileal secretory immunoglobulin A content and mucin-2 mRNA expression were increased, while the mRNA abundances of interferon-γ and interleukin-1β in the ileum were downregulated [49].

In broilers reared in floor pens and fed increasing dietary Thr levels (starter from 0.69–1.21% and grower 0.62–1.12% Thr), which correspond to 85–150% of NRC [7] recommendations, the ADWG and FCR were improved at 100% Thr, whereas the villus height in duodenum and jejunum, crypt depth in duodenum, and villus height/crypt depth ratio in jejunum were increased a 150% Thr in 21-days-old broilers, and the villus height and villus height/crypt depth ratio in jejunum were increased a 125% Thr in 42-days-old broilers [50]. Floor pen reared broilers fed increasing levels of dietary Thr (starter from 0.94–1.22% and grower from 0.74–0.96% Thr), equivalent to 100–130% of Ross 308 recommendations, had higher growth performance at 110% Thr inclusion, but the antibody titers against NDV and SRBC increased up to 120% Thr supplementation [51]. In two floor pen experiments using slow-growing broilers and a basal feed formula that met the requirements mentioned by Rostagno et al. [8] and added with increasing levels of digestible Thr, it was estimated that the lowest FCR was reached at 0.762 and 0.767 for starter and grower broilers, respectively, while the production of intestinal mucin was highest at 0.697% Thr in the starter phase [52].

In another study, in which broilers from 1 to 21 days of age were fed diets to match the Thr supply to 100% NRC specification, and from 100 to 130% Thr of Vencobb-400 strain specification, the ADWG was highest at 100% Thr of Vencobb-400 strain specification (0.87% Thr); the villus height, crypt depth, villus surface area, goblet cell number/villus, villus width, and goblet cell density were higher at 120% Thr and the weight of bursa and thymus, the total immunoglobulins, titers against Newcastle disease virus, lymphocyte proliferation, and neutrophil phagocytic activity were increased linearly up to 130% Thr [53]. Broilers fed dietary Thr levels that matched 100, 110, and 120% of NRC recommendation and kept in floor pens from 1 to 35 days of age showed enhanced ADWG and FCR at 110% Thr as well as higher villus height, lower crypt depth, greater VCR, greater weight of thymus and bursa, and greater infectious bursal disease titer [54]. Similarly, 1–21 days of age broilers fed dietary Thr level of 100, 120, and 140% of the NRC recommendation had improved performance ADWG and FCR at 120% Thr; anti-SRBC titer were increased at 120% Thr, and the jejunal crypt depth increased and the jejunal and ileal crypt width decreased at 140% Thr [55].

Some experiments were carried out using increasing dietary Thr addition in broilers under bacterial and coccidial challenges. Broilers from 1 to 10 days of age fed two dietary Thr levels (0.857 and 0.956%) and challenged with *Salmonella Enteritidis* at 2 days of age showed no difference in performance, but the intestinal integrity was improved in chicks fed the higher Thr level, including higher villus height, villus:crypt ratio, and goblet cell counts in the jejunum and ileum [56]. In the same way, broilers from 1 to 10 days of age fed two dietary Thr levels (0.81 and 1.00%) and challenged with *Salmonella Enteritidis* at 2 days of age showed no difference in performance, but had increased villus height and villus:crypt ratio in the duodenum [57]. Broilers of 1–21 days age kept in cages and fed two levels of dietary Thr (0.784 and 1.084%), undergoing a challenge using *E. coli* LPS from 17 to 21 days had improved ADWG and FCR at the higher Thr level and reduced serum IL-1β, and TNF-α, IFN-γ in jejunal mucosa and L-1β in ileal mucosa [58]. In three floor-pen experiments, different Thr-to-Lys ratios (from 0.56 to 0.77) were evaluated (as standardized digestibility) in the diets of broilers subjected to a subclinical Clostridium infection at nine d of age; from 9 to 37 days of age, the ADWG in broilers fed the high dietary Thr was increased, but the intestinal damage (incidence and lesion severity) was not affected by Thr supplementation [59].

The results indicate that in eight out of 12 studies, in which nonchallenged and challenged broilers were fed increasing dietary Thr concentrations, the Thr needed to stimulate the immune and digestive system was higher than that needed to improve the growth performance. These results were irrespective of the basis of Thr formulation, the growth rate of the birds, the type of housing (cages or floor pens), and the type and degree of challenge.

4. Implications

According to the literature reviewed, the level of AA required to stimulate the immune and digestive systems in unchallenged and challenged chickens is higher than that required for optimum growth performance; however, from a practical standpoint and the formulation of commercial diets, there is not enough information to confirm any benefits of adding functional AA, especially when issues such as sustainable

poultry production, in which the economic return and environmental concerns are key components, come across.

The promotion of concepts such as phase feeding, an ideal AA profile, the addition of AA on a digestible basis, and the use of low-CP diets supplemented with crystalline AA in modern feed formulation aims to maintain high levels of productivity while having a low environmental impact. The recommendations on the required levels of AA in each specific situation have been established by taking into account the stages of development, environmental conditions, management, and degree of immunological challenge due mainly to the presence of infectious agents. All of this is done to ensure that the birds consume the amount and proportion of AA that best suits their maintenance and growth needs while avoiding any excess or deficiency of AA.

The incorporation of functional AA into practical formulation is comlex, owing to the fact that levels of AA above the established requirement for growth must be included. When this occurs, an AA imbalance may exist, affecting digestion, absorption, and metabolism of AA from the same group, as has been demonstrated with dibasic AA such as Arg and Lys. This could result in a deficiency of one or more AAs from the same group, resulting in the deamination of all AA not required in the various metabolic processes, in order to eliminate excess nitrogen in the form of uric acid, resulting in the excretion of excess nitrogen through urine. At the same time, significant amounts of energy associated with uric acid synthesis would be excreted. This problem has not been addressed in the literature.

Experiment models in animals subjected to various challenges attempt to simulate what happens in commercial farms, where animals are exposed to various sources of stress as well as viral, bacterial, and parasitic infectious agents. During the growthout process, the main factors that cause immune challenges (dietary components, management, environment, and infectious agents) can be present simultaneously and sequentially. If experimental and field challenges elicit the same level of immune stimulation and type of immune response, implying that the stimulatory effects of functional AA are similar in both scenarios, it is important to note that in AA nutrition, the ultimate response to AA additions is measured by the productive response. This implies that perhaps, with the information at hand, the use of higher levels of AA beyond the levels necessary to maximize growth and FCR is questioned. In other words, in challenged birds, the use of higher than recommended levels of AA to stimulate a greater immune and digestive response is not worthwhile if this is not reflected in increased growth.

This controversy could probably be explained by drawing on much of the information already known about the metabolic effects of immune challenges to redirect AA to protective functions involving various humoral and cellular mechanisms. To increase the supply of AA, body protein will be broken down and production performance will decrease. This is necessary since the entire immune response process requires amounts and proportions of AA that vary for each type of response. In contrast to this, in most of the reviewed studies, functional AAs have been evaluated individually, or adjusted to a profile to cover the growth recommendations, but in very narrow ranges. This is explained by the difficulty of adjusting the amount and profiles of AA when feeds are balanced with AA concentrations far above the normal requirement, especially in low-CP diets. In addition, the lack of information of a proper AA profile for immune-challenged situations makes this task more difficult.

It is noteworthy that several authors have hypothesized that the addition of synthetic AA would particularly improve the animals' immune response against intracellular pathogens. If this hypothesis is confirmed, the use of functional AA could play a critical role in pathogen reduction and, as a result, in the spread of antimicrobial resistance factors. These advantages should be confirmed in farm animals that are normally subjected to acute and chronic stressors, which may be concurrent and synergistic. It is critical at this point to determine whether episodes of immunosuppression caused by stress can be overcome by functional AA.

It is also unclear whether functional AA should be used continuously or only on a case-by-case basis, particularly when birds are stressed or when there are conditions that increase the risk of disease. If the application is strategic, it should be specified the best moment and the period they should be supplemented.

It is also possible that functional AA should be supplemented during or after an immunological challenge to aid in the recovery of affected individuals and to restore productive parameters to prechallenge levels.

5. Conclusions

The use of functional AA such as Arg, Thr, and Met to improve the health and productivity of birds exposed to immune challenges is promising. It has been proposed that functional AA can help the immune system fight intracellular pathogens. It is necessary to determine whether episodes of immunosuppression caused by stress can be overcome by functional AA in field-raised birds, as well as to define the strategic use to reduce disease risk. It is also possible that functional AA should be supplemented during or after an immunological challenge to help affected individuals recover and return productive parameters to prechallenge levels.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

An Overview of Poultry Meat Quality and Myopathies

Basheer Nusairat, Guillermo Tellez-Isaias and Rasha Qudsieh

Abstract

The increased demand for poultry meat and the shift toward portioned and further processed products has been accompanied by genetic improvement and progress in nutrition and management to increase growth rates and improve feed efficiency. Animal protein continues to be the most demanded and expensive protein source worldwide. Poultry is an animal protein commonly accepted among different faith groups and relatively more affordable than other animal protein sources. In addition, poultry meat has lower fat, cholesterol, and sodium content compared to red meat. This review aims at summarizing the available information about skeletal muscle structure, conversion of muscle to meat and how it affects poultry meat quality, the different myopathies historically been identified and other emerging myopathies, then discussing how meat quality affects consumer perception and consumption trends, and finally discussing few of the proposed solutions to overcome the issues of decreased meat quality, including nutritional strategies.

Keywords: meat quality, nutrition, muscle myopathies, color, poultry

1. Introduction

The poultry industry has witnessed significant improvements over the past several decades achieving higher market weight with improved feed efficiency, thus reducing production cost. During the past 60 years, the amount of time and quantity of feed per pound of meat required to reach broiler market weight had been reduced by 50% [1]; furthermore, according to the National Chicken Council [2], modern broiler chickens can achieve market weight 16 days earlier with 35% higher weight compared to the 1960s broiler chicken. These improvements have resulted from a combination of genetic improvement and progress in nutrition and poultry management.

The U.S. is considered the world's largest producer of poultry meat; the U.S. provides approximately 17% of the global poultry meat output, followed by Brazil and China, mainly dominated by broiler meat followed by turkey meat and a small fraction for other poultry meat. The production and consumption of poultry meat have increased rapidly worldwide and are expected to continue to grow [3] due to its relatively low price compared to other meats, the absence of cultural or religious obstacles, and its dietary and nutritional properties as it has lower fat, cholesterol, and sodium content [4] with an increased preference of white chicken meat [5, 6].

Additionally, consumers have shifted from the consumption of whole chicken toward portioned (especially breast fillets) and further processed products [7, 8]. These changes were driven by the need for convenience with meal preparation in a fast-paced industrialized era and meeting consumer preference of specific carcass parts. The poultry industry has responded to these changing demands by further enhancing genetic selection for increased breast yield, faster growth rate, and improved feed efficiency. Meanwhile, feed cost has increased, and ethanol production has forced producers to use alternative feed ingredients such as the distiller's dried grains with solubles (DDGS) produced as byproducts of ethanol production. However, since the selection of broiler chickens initially focused on increasing growth performance and improving body composition [9], this has led to indirect and often deleterious effects on meat quality traits, such as excessive deposition of abdominal fat, the formation of which represented the inefficient use of feed [10, 11]. Coincidently, several studies have shown an increased incidence of abnormalities, mainly in breast muscles [12, 13]. In the early 1980s, Wight and Siller [14] recognized an abnormal condition in the pectoralis minor, in which the muscle is basically "suffocated" leading to ischemic necrosis; this condition known as deep pectoral muscle myopathy is only the first in a list of fast-growth-related muscle abnormalities that eventually affect meat quality and its functional properties.

In poultry meat, appearance and texture have been considered the two most important attributes responsible for initial consumer meat evaluation and final product acceptance [15], so consumers are expected to reject meat with observed defects such as bruises and hemorrhages. Several appearance defects have been reported in the poultry industry, such as pinking of raw and cooked meat, bone darkening, red/bloody discoloration, white striping, wooden breast, spaghetti meat, and pale, soft, exudative appearance of breast meat. However, many of the underlying causes of appearance defects have not been fully explained. Understanding the structural organization of the muscle fibers and physiology can help in explaining some of these defects.

2. Overview of skeletal muscle structure

The basic structural unit of a muscle has been defined as the muscle fiber, which is constituted of several myofibrils (contractile units). Each muscle fiber is surrounded by a connective tissue called the endomysium; muscle fibers are then grouped into fascicles and surrounded by another layer of connective tissue called the perimysium. Then, the whole muscle is made up of a group of fascicles and surrounded by epimysium that connected the muscle to bones. Collagen is the major constituent of these connective tissues. These connective tissues influenced muscle development and subsequent meat quality.

Skeletal muscles growth was achieved by increasing the size of preexisting muscle fibers (hypertrophy). The number, size, and type of fibers vary with the function and anatomical location of the muscle. Meat quality is also affected by these factors. A muscle that contained high proportion of oxidative fibers tends to have red color due to a greater amount of myoglobin (e.g., thigh muscles) as compared to glycolytic fibers, which tended to appear white in color, which affected the appearance of muscle/meat (e.g., chicken breast muscle). Glycolytic fibers are larger and have lower rate of protein turnover. Therefore, the white muscles are larger and more efficient. In poultry, genetic selection for increased breast yield resulted in pale breast meat color in broilers [16], ducks [17], and turkeys [18], which could result in poor meat quality.

Collagen is the most abundant protein in the body and in connective tissues. The structure of collagen supports its function of providing strength to muscle and other tissues with more than 20 different types of collagen identified in vertebrates [19]. Glycine constitutes about one-third of all the amino acids found in collagen, while proline, which has been classified as an imino acid, and its analog hydroxyproline also constituted about one-third of all amino acids in collagen [20]. Lysine has been considered to be another constituent of collagen where both proline and lysine are covalently modified to hydroxyproline and hydroxylysine, respectively. A collagen molecule (tropocollagen) is composed of three left-handed polypeptide helices coiled around each other to form a right-handed supercoil where glycine is found at every third residue [19].

The strength of the collagen fibrils is due to the covalent bonds formed between and within tropocollagen triple helices, where collagen is cross-linked by lysine side chains that contribute to the strength of the collagen in meat, which has an essential role in the development of meat tenderness [21]. Furthermore, in a recent study, it has been shown that muscle with spaghetti meat abnormality had an altered immunoreactivity to specifically procollagen type III (precursor of collagen type III) suggesting a possible defect in the collagen turnover and synthesis process [22], while Sanden et al. [23] reported that spaghetti meat has poorly packed thin, loose, and immature collagen fiber bundles.

2.1 Conversion of muscle to meat

The process of converting muscle to meat in poultry starts immediately upon sacrificing the bird. Exsanguination results in blood/oxygen supply removal, during which the muscle tries to maintain its functions even after oxygen depletion through the anaerobic glycolysis of its glycogen reserves to produce adenosine triphosphate (ATP), but in the absence of blood supply to remove waste, the accumulated heat and lactic acid in the muscle decreases the pH. Owing to ATP depletion, the muscle remains contracted due to actin and myosin binding that leads to muscle stiffness (rigor mortis). This marks the onset of rigor mortis and the conversion of muscle to meat, where muscle proteins start to denature due to high temperature and low pH. Temperature and pH are the main postmortem factors influencing meat quality through affecting the onset and progression of rigor mortis and subsequent resolution [24–27]. During resolution, the proteolysis of Z-disk proteins takes place, and myofibrillar proteins degrade into myofibrillar fragments by proteolytic enzymes that affect meat tenderness. In chickens, the process of converting muscle to meat has been found to start immediately after slaughter and be resolved within 2–4 h. The extent of meat tenderization postmortem could be altered by the conditions under which the meat is processed. Factors include temperature and chilling duration, deboning time, postchill aging/holding duration, and marination.

2.2 Poultry meat quality

Meat quality is a collective term used to describe the indicators of a meat product wholesomeness and freshness, such as color, texture, flavor, pH, and juiciness. The two most important quality attributes for poultry meat are appearance and texture since they influence the initial consumer selection of a product as well as final satisfaction [15]. Appearance quality attributes include skin color, meat color, and appearance defects such as bruises and hemorrhages. Any deviation from a normal

appearance would result in meat product rejection, subsequently leading to consumer complaints. Despite the importance of these quality attributes, the poultry grading system used is still based on aesthetic attributes, such as conformation, presence or absence of carcass defects, bruises, missing parts, and skin tears, without taking into consideration the functional properties of meat [28], which have been important for the further processing industry that was mainly interested in the functional properties of meat; the importance of incorporating functional properties and quality indicators is becoming increasingly important as the recent muscle myopathies not only affect consumer acceptance based on appearance but also the quality of further processed meat manufactured using meat with such defects.

Many factors influence poultry meat quality, including sex, strain, age, environmental factors, exercise, diet, and processing practices mainly focused on chilling, deboning time, marination, and electrical stunning [29–32].

Another important quality attribute that influences customer perception is the tenderness of the meat. This attribute comes second after appearance; consumers usually correlate acceptable appearance with better quality and tenderness. Tenderness development is a function of myofibrillar protein denaturation, connective tissue content, and juiciness. Deboning time, age, and strain are some of the major factors that affect poultry meat tenderness [31, 33]. Lyon and Lyon [34] reported that as the time before deboning increased from 0 to 24 h postmortem, consumer acceptability of the meat texture increased, with fillets deboned at 0 and 2 h postmortem considered tough by a consumer panel, and samples deboned at 6 and 24 h postmortem considered slightly tender to moderately tender. Liu et al. [35] reported a decreased shear force of chicken breast as deboning time increased from 2 to 24 h postmortem. Similar results were also reported by Cavitt et al. [33].

Furthermore, Mehaffey et al. [8] reported that fillets deboned 2 or 4 h postmortem from broilers raised to 7 weeks were significantly tougher than those raised to 6 weeks, indicating that age affected tenderness when deboning was performed shortly after harvest. Northcutt et al. [31] reported that breast fillets harvested at less than 2 h postchill aging were tenderer when taken from broilers slaughtered at 42 or 44 days of age than those harvested from birds 49 or 51 days of age, irrespective of any sex effect. On the other hand, Young et al. [36] reported that females had greater fillet yields than males.

Connective tissue content has been reported to increase with age and is correlated with tenderness; as mentioned earlier, collagen is the most abundant protein in the body, making up the majority of the connective tissue proteins [37, 38]. In young broilers (6–8 weeks), it is expected that connective tissue would not affect tenderness since mature cross-links should have not yet formed between tropocollagen molecules, which are the structural units of the collagen fibril. On the other hand, the contraction of myofibrillar protein, which depends upon time and rate of rigor mortis development after the bird is sacrificed, is related to processing rather than intrinsic factors [15]. Furthermore, tenderness, indirectly associated with connective tissue, is one of the quality attributes that are negatively affected by the emerging muscle myopathies emphasizing the importance of further investigating and attempting to mitigate the negative impacts.

Another important meat quality attribute is meat juiciness, or water-holding capacity, which refers to the ability of raw meat to retain its inherent water during force application and/or processing [39]. Water in muscle has been divided into three general types: bound, immobilized, and free. Bound water is held tightly via myofibrillar protein charges and represents 4–5% of water in muscle [39, 40]; it is resistant

to freezing and could only be removed by severe drying processes, not including conventional cooking [41]. Immobilized water is found within the muscle ultrastructure (within the space between actin and myosin), but it is not bound to myofibrillar proteins as in the case of bound water. Immobilized water accounts for the largest portion of muscle-bound water (88–95%). Finally, free water is held within muscle by weak capillary forces [42].

2.3 Poultry meat color

Poultry has been determined to be the only species known to have muscles/parts with apparent differences in color, as meat from poultry has been classified as either white or dark. In chicken, fresh raw breast meat is expected to have a pale pink color, while the raw thigh and leg meat are darker and redder. Meat color plays a significant role in consumer purchase decisions [43–45]. Consumers tend to associate color with flavor, tenderness, safety, storage time, nutritional value, and satisfaction level [46], and as an indicator of freshness and wholesomeness.

Meat color is what the human eye sees as light is reflected from the meat surface. Poultry meat absorbs most blue and green color spectra and reflects most of the yellow, orange, and red color spectra, which is what the human eye perceives.

The most commonly used colorimetric scale is the CIE Lab [47], even though other color scales have been used, such as the Hunter L, a, b, and YXZ space. However, the accuracy of these instruments has depended upon thickness, background color, and illuminant wavelengths [48, 49].

The CIE Lab system components measures include L^* that refers to lightness and has a range from 0 to 100 (black to white), component a^* had a range from –60 to +60 (green if negative to red if positive), and b^* has the same range as a^* (blue if negative to yellow if positive) [50, 51]. Another more recent system used for color measurement is the computer vision system, which has been shown to give reproducible results with the ability to measure the color of the entire sample instead of specific spots, as has been the case with widely used colorimeters [52]; in fact, Tomasevic et al. [53] recommended using computer vision program as a superior approach for poultry color determination.

Meat color is mainly related to the myoglobin pigment present in the muscle fibers. Myoglobin consists of a protein (globin) and a nonprotein heme ring, which has an iron molecule in its center. Iron can bind one of several ligands (e.g., oxygen, carbon monoxide, and nitric oxide) on its sixth coordination site. The forms of myoglobin (deoxymyoglobin, oxymyoglobin, carboxymyoglobin, and metmyoglobin) differ depending upon the ligand bound to iron and on the redox state of the iron. Thus, myoglobin and iron states are the two main ways through which meat color changes.

Myoglobin (or deoxymyoglobin) has a red-purple color in its nature when not bound to any ligands; the state of myoglobin changes to oxymyoglobin when oxygen is present and to carboxymyoglobin when carbon dioxide is present. In both the forms, the color is bright red (bloom), and iron is in the reduced ferrous form (Fe^{++}). The oxidation of myoglobin changes the form to metmyoglobin and the iron to the oxidized ferric form (Fe^{+++}), which has a brown color. These myoglobin color changes are reversible; however, if heat-treated, metmyoglobin becomes denatured and color changes irreversibly to grayish-brown. Curing with nitrites/nitrates causes an irreversible color change to red color that, upon heating, converts to pink. The replacement of iron with zinc results in a stable red color of myoglobin due to the

formation of Zn-protoporphyrin IX (ZPP), which has been shown to give Parma ham its stable, bright red color [54, 55]. Within a chicken carcass, chicken breast muscles are mainly composed of white fibers (glycolytic) that have low myoglobin content. Thus, breast meat appears white, while thigh muscles are composed of red fiber (oxidative) and appears darker. Fleming et al. [56] reported a myoglobin concentration of 0.16 and 0.30 mg/g in broiler breast and thigh muscles, respectively. Furthermore, Miller [57] said a lower myoglobin content of 0.01 and 0.40 mg/g in white and dark meat of 8-week-old broilers, respectively.

Froning [58] classified the factors influencing meat color into three main categories (**Table 1**). Smith et al. [59] investigated the effect of age, diet (carbohydrate source), and feed withdrawal on broiler meat color by slaughtering birds each day from 42 to 45 and 49 to 52 days of age with a carbohydrate source that was either corn, milo, or wheat, with feed withdrawal times of either 0 or 8 h. Color was not affected by age. Still, feed withdrawal increased fillet lightness (L*) from an average of 46.1 to 48.9, decreased redness (a*) from 4.1 to 3.1, and increased yellowness (b*) from 2.8 to 3.7. Fillets from the birds fed the wheat diet were lighter than fillets from the corn or milo fed birds. The milo diet resulted in redder fillets than corn or wheat diets, while the corn diet produced more yellow fillets than milo or wheat diets.

In addition to meat color, skin color has been considered a critical quality attribute, mainly in a whole carcass and skin-on cuts sale. The color of poultry skin has varied from cream-colored to yellow. This variation is primarily the result of genetic variation and natural pigments in feed. Birds had differed in their ability to deposit the black melanin pigment in the epidermis and dermis layers of the skin and varied in their ability to deposit carotenoids from the feed as the combinations of different amounts of melanin and carotenoids produced different skin colors. However, in commercial strains, the ability to deposit melanin has been eliminated through genetic selection. Different skin colors as adopted from [60] are illustrated in **Table 2**.

Heme pigments	• Myoglobin, hemoglobin, cytochrome c, and their derivatives
	• Presence of ligands complexing with heme pigments
Preslaughter factors	• Genetics (fast growing strains)
	• Feed (e.g., moldy feed)
	• Feed withdrawal time
	• Hauling and handling stress
	• Heat and cold stress
	• Preslaughter gaseous environment of the bird
Slaughter, chilling, and further processing	• Stunning techniques
	• Presence of nitrates
	• Additives and pH (e.g., phosphates, salt)
	• End-point cooking temperature
	• Reducing conditions
	• Washing surimi-like processing of mechanically
	• deboned poultry meat (MDPM)
	• Irradiation

Table 1.
Factors influencing poultry meat color [58].

Skin color	Dermis	Epidermis
White	None	None
Black	Melanin	Melanin
Yellow	None	Xanthophyll
Green	Melanin	Xanthophyll
Blue (Slate)	Melanin	None

Table 2.
Combination of possible skin colors due to dietary xanthophyll deposition in epidermis or melanin production in either dermis or epidermis [60].

Defect	Description	Possible causes
Bruises and hemorrhages	Classic bruises, pin-point blood spots in meat, blood accumulation along bones and in joints	Physical trauma, nutrient deficiencies, mycotoxins, stunning
Overscalding	Incomplete removal of epidermis, cooked discoloration on surface of meat	Too high scalding temperature, too long in scalder
Surface drying	Mottled appearance of skin or meat due to surface dehydration	Incomplete removal of epidermis, exposed meat, poor packaging, freezer burn
Heme reactions	Normal color ranges from raw pink meat, tan to brown raw meat, grey to brown cooked meat, pink cooked meat, cured meat color	Oxidative or redox state of the myoglobin, myoglobin complexing with nitrates/nitrites or other compounds such as carbon monoxide
Dark meat	Darker than normal appearing meat, possible mottling	High muscle pH due to antemortem depletion of muscle glycogen
Light meat	Pale breast meat	Low muscle pH (PSE-like condition)
Dark bones	Dark brown to black bones	Freezing, blood accumulation around bone

Table 3.
Summary of poultry color defects [60].

However, considerable variation in color and discoloration of poultry meat has occurred and remains of great concern for the industry. Discoloration may occur in the entire muscle or only in a portion of a muscle due to bruising or broken blood vessels [58]. Possible poultry color defects are presented in **Table 3**.

2.4 Poultry meat color defects

2.4.1 Pink discoloration of cooked white meat

The pinking of cooked white meat has been an undesirable color defect found in poultry; its occurrence was noticed sporadically and has negatively influenced consumer purchasing decisions (Maga, I994). According to Maga [61], pink color might have resulted from the presence of high levels of myoglobin that were not completely denatured during heat processing, incidental nitrate/nitrite contamination either in feed or water or during processing. The presence of carbon monoxide and nitric oxide gases in oven gas while roasting has caused pink color on the surface of turkey

meat, with carcasses from younger turkeys more susceptible than older ones [62]. The proposed mechanism for pink color development of fully cooked is related to the ligands to which the denatured myoglobin was bound, such as amino acids, denatured proteins, and nitrogen-containing compounds that form denatured hemochromes globin. Therefore, depending on the ligand to which the denatured heme will bind, different pink shades would result.

Binding of nitric oxide to myoglobin from preslaughter contamination (feed and water and gases from the truck exhaust) or during/after processing (processing water, ice, spice mix, and oven gas) has formed the pink nitric oxide myoglobin that, upon cooking, was converted to pink nitrosohemochrome. Furthermore, carbon monoxide binding to myoglobin has led to pink carboxymyoglobin developing upon cooking in oven gases or during irradiation.

Cooking meat harvested from birds before rigor mortis resolution could also cause pink color when meat is cooked when pH was higher than 6.0. At this high pH, myoglobin is not denatured, and cytochrome C (electron transport protein), which is heat stable, increases and contributes to the delayed denaturation of myoglobin since cytochrome C is still able to deliver electrons to myoglobin. Ahn and Maurer [63] showed that a pH above 6.4 leads to binding of myoglobin and hemoglobin with most naturally present ligands, such as histidine, cysteine, methionine, nicotinamide, and solubilized proteins, which leads to pink color of the meat. At high pH, amino acids and protein ligands can donate electrons to Fe, resulting in stable pink ferrohemochrome. High pH also reduces the susceptibility of meat pigments and lipids to oxidation resulting in a cooked pink color [64].

2.4.2 Bone darkening or discoloration

Bone darkening has been described as a dark reddish brown or black discoloration on the surface of bone and muscle adjacent to the bone after cooking. The darkening was due to bone marrow passing from inside the bone onto the bone surface and adjacent tissue, usually after freezing the meat [65, 66] and after cooking of the frozen meat [67]. Lyon and Lyon [30] described the variation in bone discoloration due to different preparation methods (precook, freeze, and reheat). They found that freezing before cooking increased the severity of discoloration more than cooking followed by freezing and reheating. Lyon et al. [65] demonstrated that meat and bone darkening of thigh pieces was related to pigment migration from the femur to muscle tissue. The commercial further processing industry has reported that redness was usually accompanied by blood in bone-in chicken carcasses and parts, which consumers could reject as the product appears undercooked and unsafe for consumption [59].

The migration of pigments from the femur to muscle tissues has created darkening that was more prevalent in younger birds since their bones were less calcified, were more porous, and had more red marrow than older birds. The epiphysis of long bones in older birds is more calcified than young birds, so the pigment is more difficult to escape from bones onto surrounding tissue. However, bone darkening only affects the appearance and not the organoleptic properties of the meat product [67].

Smith and Northcutt [59] studied discoloration prevalence in commercially fully cooked breasts, thighs, and drumsticks from various market sources. They speculated that about 11% of products could face consumer complaints or rejection since they were severely discolored. Furthermore, cooking chicken breast samples with bone marrow collected from femurs increased the darkness and redness of both raw and cooked broiler meat [68].

2.4.3 Red discoloration of white meat

Red and/or bloody discoloration of poultry meat, raw or cooked, has been a chronic yet sporadic problem for the poultry industry. Raw breast meat with red discoloration is objectionable to many customers, and cooked white or dark meat with red defect is unacceptable to consumers due to the perception that it is undercooked. Red discoloration of white meat is closely related to bone darkening but with higher redness. Little research has been available concerning this red discoloration defect in poultry meat [59]. According to Smith and Northcutt [66], bone marrow is an effective inducer of red, bloody discoloration in breast meat samples. In a previous investigation conducted concerning the color of different parts of chicken, Lyon et al. [65] reported that the initial color of breast was lighter and less red than thighs because breasts had a lower proportion of total bone area to muscle mass, fewer large, calcified bones, a lower proportion of blood vessels per muscle mass (less hemoglobin), or lower myoglobin content than thighs or drums [66].

The bright red color development has been investigated in Parma ham, where this north Italian traditional dry-cured ham "Prosciutto di Parma (Parma ham)" has been made from only the legs of fattened pigs and was salted with sea salt, dried, and matured over 1 year [69]. It was initially postulated that sea salt used was contaminated with nitrate/nitrite. However, that was later investigated, and results showed that this pigment was also formed in a nitrate/nitrite-free environment and that endogenous enzymes as well as microorganisms were involved in this pigment formation [54, 55]. These results suggested that the bright red color in Parma ham is caused by Zn-protoporphyrin IX (ZPP), in which the iron in heme was substituted by zinc heme separated from the native heme protein. Investigations on this lipophylic myoglobin derivative showed that it was a stable red pigment that increased with aging [70]. This process has now been patented for producing red pigments for food use that were heat-stable [71]. The addition of salt accelerated the reaction and increased redness [72]. The process has also occurred in live animals, including humans, as lead poisoning and iron deficiency caused an increase of ZPP in blood as zinc replaced the iron in hemoglobin. The level of ZPP can be evaluated with a simple screening test using a hematofluorometer. The measurement of ZPP has been used with ducks to test for lead poisoning [73]. An increased ZPP/heme ratio indicates that Zn has replaced Fe in the heme, thus changing the color of hemoglobin and myoglobin. Based on findings in Parma ham, ZPP may be responsible for the red discoloration in poultry meat, which could be formed in myoglobin found in muscles or hemoglobin stored in bone marrow. Thus, ZPP leaking out of the bones could cause the increased stable redness observed in white meat.

2.4.4 Green discoloration

Green discoloration of live muscles, raw meat, and cooked deli products can be produced by various mechanisms that lead to condemnation by the industry and consumers. In live muscles, green muscle disease (deep muscle myopathy) is caused by the lack of blood supply to the deep pectoral muscle that results in the death of the muscle fibers, thus giving the muscle a green appearance. The bruising of live birds has caused a rupture of blood capillaries and blood accumulation under the skin or in the meat. The color of the bruise subsequently developed over time and turned either yellow or green depending upon heme degradation. Using lactic acid as a decontamination approach resulted in the greening of chicken skin color [74]. The irradiation

of fresh beef and pork meat has been thought to affect the stability of iron in the myoglobin and cause the breakdown of the porphyrin molecule and/or the formation of sulfmyoglobin that caused green pigments to appear [75].

In cooked meat, contamination with microorganisms such as Pseudomonas fluorescens has produced a shiny transparent greenish exudate on the meat surface due to microbial degradation of the heme pigment. In sausage-type products, the presence of green rings is an indicator of microbial contamination where the microorganisms oxidized the heme pigment before applying thermal treatment.

Iridescence, which is the appearance of a green-orange color on the surface of meat products such as deli meat, is mainly associated with the meat surface microstructure that could be interpreted as a color diffraction problem related to the ability of certain muscles to split the white light into its component. Thus, the reflection of the meat surface would appear in green-orange. If a sharp knife was used to cut the meat, the smooth surface resulting from the cut causes this color diffraction, but if a dull knife was used instead, this problem would be eliminated.

3. Existing and emerging muscle myopathies

3.1 Breast muscle myopathy

Deep pectoral muscle myopathy, also known as green muscle disease and Oregon disease, was first identified in turkeys [76] and later in broiler breeders [77] and 7-week-old broiler chickens [78]. This disease affected the wing elevating muscle (*M. supracoracoideus* or pectoralis minor) and was characterized by the death of the muscle (tenders) but did not cause the death of the bird. Dead muscle decay, while the bird was still alive, resulted in the appearance of a yellowish-green color due to the breakdown of hemoglobin and myoglobin to bile salts; muscle myopathy could affect just one (unilateral) or both (bilateral) pectoralis minor muscles. Since affected tenders were located deep in the breast, this defect resulted in consumer complaints when the carcasses were sold as a whole.

The pectoralis minor muscle is confined in a tight space between the sternum and the pectoralis major muscle (large breast fillet). It is also encased in a rigid fibrous sheath that restricts any increase in muscle volume in response to any physiological changes caused by muscle exercise such as wing-flapping [79] which requires increased blood flow to supply the oxygen and nutrients needed by the muscles. The incidence of green muscle disease has also been reported to be higher in high yielding crosses, especially males.

On the other hand, the incidence of focal pectoral myopathy has increased, and it has been associated with increased growth rate and muscle size [12, 80]. Further investigation is required to determine the causes of this muscular defect since focal myopathy has an even more detrimental effect on the poultry industry. It has affected the pectoralis major muscle leading to consumer complaints and industry economic loss.

3.2 Pale, soft, and exudative-like condition in poultry muscles

The incidence of pale, soft, and exudative (PSE) meat has been well-documented in swine, where meat has a very light gray color, soft texture, and cannot hold water

[81, 82]. This condition has been associated with heavy muscling [83]. In poultry, similar PSE characteristics have been reported in turkey meat [84, 85], chickens [86, 87], and ostriches [88]. However, it is more difficult to distinguish and identify these characteristics in poultry meat compared to pork. This condition has been referred to as PSE since characteristics were similar to PSE in pork, which is misleading since both conditions were not exactly the same. Poultry researchers have preferred to refer to the condition in poultry as "PSE-like" or "Pale poultry muscle syndrome" [86, 89]. The PSE and PSE-like conditions are detrimental to the industry profitability since it affects important meat quality attributes involved in the production of value-added products and further processed meat. Affected muscles have been reported to lose their rheological properties and become unable to hold water. For example, mortadella prepared with PSE-like chicken meat has reduced water-holding capacity, altered texture, diminished emulsion stability, and required additives to restore the functional properties of normal meat [90]. In addition, poultry processors have been concerned with the appearance of PSE-like meat in fresh tray packs. The pale color affected color uniformity within the package and, thus, consumer acceptance. The occurrence of PSE-like in poultry meat has been believed to be the result of accelerated postmortem glycolysis (rapid pH decline), while the carcass was still warm [91]. In poultry, normal pH values at 15 min postmortem (pH_{15}) are around 6.2–6.5 [92, 93], whereas normal ultimate pH (pH_u) values are approximately 5.8 [60, 88, 94]. If the pH_{15} value is low (below 6.0) when the muscle is still warm, the proteins are subject to denaturation, which leads to a decreased water-holding capacity and a lighter color of the meat.

The reasons for PSE-like condition have remained unclear, but up to 30% of broiler breast meat and up to 40% of turkey breast meat have shown this defect in commercial processing plants [95–97]. Furthermore, it has been reported that the occurrence of PSE-like meat in birds may be affected by alteration to the intracellular calcium homeostasis caused by a mutation in the ryanodine receptor gene, which is different from the ryanodine receptor gene in swine, and also depends upon the several aspects of preslaughter and postslaughter management practices [98, 99]. It is thought that the application of "snow chilling" with carbon dioxide intensified meat quality abnormalities [100]. In addition, other factors have been thought to contribute to this problem, such as heat stress during the finisher period or the preslaughter period [86], and stress and struggling before slaughter [101].

Differentiating PSE-like meat from normal meat has been based on the instrumental or visual assessment of color lightness (L^*). However, the cutoff value for classifying meat as PSE-like has differed among researchers. Petracci et al. [102] considered an L^* value of 56 as the cutoff, while Barbut [28, 103, 104] suggested classifying turkey breast meat as PSE-like when L^* values were greater than 52 at 24 h postmortem. Fraqueza et al. [105] classified breast meat as PSE-like when the L^* was greater than 50 and pH_u was less than 5.8, while Woelfel et al. [106] used L^* values greater than 54 in broilers as their standards.

Using L^* *per se* as an indicator of PSE-like condition has not been considered accurate and could be misleading because several factors influence poultry meat color. Feed ingredients used in poultry have been reported to change breast meat color (e.g., wheat-based versus corn-based diets). In addition, it has been shown that genetic selection for increased growth and breast meat yield resulted in a marked increase in muscle fiber size [107, 108] with a shift toward a greater proportion of white fibers (glycolytic) and reduced dark fibers (oxidative), which produced meat that appears

pale but still has a high pH_u. Muscle thickness [48, 49] and color measurement position on the fillet [109] also affects color measurement. Therefore, color, pH_u, and water-holding capacity should be considered when classifying poultry as PSE-like meat.

3.3 White striping, woody breast, and spaghetti meat

White striping, woody breast, and spaghetti meat can be collectively referred to as the myopathies of modern broiler. These nomenclatures were simply based on the appearance of the defective muscles. White striping is a condition described in broiler chickens and characterized by white striations parallel to the direction of muscle fibers on both breast fillets and thighs of broilers. White striping is considered to be an emerging issue by the poultry meat industry that could be associated with enhanced growth rate and heavier body weight in birds [110–112], especially in the age of 6–8 weeks [110], and higher fat content in broiler breast fillets [111]. The incidence of white striping was evaluated under commercial conditions, and the overall incidence in broiler breast meat was 12.0%, of which 3.1% had severe striping [113]. It is possible that the intense selection for rapid growth rate in birds could have accidentally been accompanied by the selection for inadequate capillary/fascial growth or muscle fiber defects leading to myopathic changes referred to as growth-induced myopathy [13], under which these three different myopathies can be classified.

The precise etiology of white striping has not been defined yet [114]; however, several speculations have been reported. In turkeys, Wilson et al. [80] reported that rapid growth rate may have led to the limited ability of muscle support systems leading to a condition called focal myopathy, which affected the major pectoral muscle.

Ischemia could also result from a rapid growth rate and lead to muscular damage in turkeys [115]. It is also possible that reduced oxygen supply to breast muscle resulted from lower capillary density in fast-growing chickens [116]. A higher growth rate could also lead to defective cation regulation in muscles leading to an increased sodium, potassium, magnesium, and calcium in muscle tissue [117]. An increased level of calcium in muscle tissue could initiate several tissue changes, including the activation of intracellular proteases or lipases resulting in myopathic changes [13, 118–120]. Kuttappan et al. [114] reported that breast fillets showing severe white striping had reduced protein content and myopathic lesions, while Petracci et al. [113] observed poor cohesion beneath the striation area.

Poultry producers started noticing and complaining about woody breast in the late 1990s [12, 121]. The woody breast muscle is usually characterized by increased firmness in all or parts of the pectoralis major muscle that can start in the live birds and can be detected by palpating the breast muscle. Sihvo et al. [121] reported that woody breast might result from fibrosis, which leads to an accumulation of interstitial connective tissue. This myopathy affects consumer acceptability and meat quality; even when trying to mitigate by diverting to further processed poultry products, woody breast meat is still required to be mixed with normal meat to maintain the quality of the further processed product [122, 123].

Spaghetti meat, or previously known as mushy breast, is the most recent emerging myopathy of breast meat in poultry. As the name implies, the breast muscle loses its structure and firmness. One distinct feature the spaghetti meat has that would differentiate it from white striping and woody breast is the loss of endomysial and perimysial connective tissue that compromises the fiber bundles cohesion, coupled with a loose connective tissue deposition [124] leading to the separation of the fascicles into "spaghetti" strings.

Sanden et al. [23] investigated the collagen of muscles with either woody breast or spaghetti meat abnormalities. They showed that collagen in woody breast muscle was a mix of thin and thick fibers, whereas spaghetti meat had thinner, fewer, and shorter. However, both myopathies generally resulted in a higher content in connective tissue (mainly in perimysium) compared to normal muscle.

Several researchers have investigated these myopathies to understand their etiology and effect on meat products quality [114, 121, 124, 125]. It is believed that cellular stress and hypoxia (ischemia) caused by muscle hypertrophy are the main triggering factors behind white striping and woody breast, in addition to being strapped within a relatively rigid connective tissue that limits the hypertrophy capabilities. However, what is interesting is that spaghetti meat, where the opposite issue is faced concerning connective tissue, started appearing. It is possible that geneticist, while trying to reduce the rigidness of the connective tissue, led to the emergence of the most recent abnormality of spaghetti meat, which is worth investigating in the future with poultry strain companies.

4. Nutrition and muscle myopathies

Researchers have investigated multiple factors that may have either contributed or helped in eliminating the emerging myopathies starting at different incubation conditions [126] all the way to management during growing [127, 128] and nutritional manipulations [129–133].

Several white muscle defects and myopathy have been reported. According to the literature, these problems spiked in the 1970s and 2000s concurrent with increased feed prices. It was suggested that producers were driven to use less expensive feed and use alternative feed ingredients (e.g., DDGS) to control costs. One significant consequence of feeding less expensive feed was that the essential amino acids (e.g., lysine and methionine) became a primary concern when formulating these diets, while the nonessential amino acids (e.g., arginine, glycine, and proline) were neglected despite their essential role in connective tissue formation, which may have contributed to the emerging of muscle defects as genetics for enhanced growth and muscle accretion were improved even further.

The spectacular advancements in genetics witnessed by the broiler industry have resulted in broilers with a higher growth rate, while the role of nutrition has become even more critical in supporting the increased growth demands of what may have become a relatively fragile animal. Profit-driven decisions about formulating feed in a least-cost manner while neglecting the essentiality of nonessential amino acids in nutrition would eventually be evidenced by increased condemnation at the processing plant and increased consumer complaints.

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Chapter 6

Broiler Amino Acid Research: Then and Now

Craig W. Maynard and Michael T. Kidd

Abstract

The interconnected nature of the amino acids in broiler nutrition pose an interesting quandary for poultry nutritionists. Two primary antagonisms exist in poultry, that among lysine and arginine and the branched-chain amino acids. Throughout the discovery and investigation into these amino acids, researchers have questioned the existence of these antagonisms as well as their impact on practical formulation. The lysine-arginine antagonism was the first discovered in poultry and was largely solved when protein formulation shifted from crude protein requirements to formulation based on amino acid levels. In contrast, while branched-chain amino acid antagonism was discovered over 50 years ago, increased refinement of dietary amino acid profiles has allowed for this antagonism to become a reemerging concern. These antagonisms and the interplay of amino acids on dietary requirements will continue to challenge researchers for years to come and innovative formulation strategies will need to be developed in order to optimize broiler diets and production.

Keywords: arginine, lysine, amino acid antagonism, branched-chain amino acids, leucine

1. Introduction

The first amino acid to be isolated is credited to L.N. Vauquelin and P.J. Robiquet, who discovered asparagine in 1806 [1]. Isolation of the amino acids continued throughout the nineteenth and early twentieth century, with the last significant discovery occurring in 1935 with the identification of threonine as both a new and essential amino acid [2, 3]. During the time of which threonine was discovered, W.C. Rose conducted a series of experiments identifying the amino acids required for growth. At the conclusion of these experiments, Rose reported that 10 amino acids were indispensable and must be included in diets in order to support growth [4]. The evolution of indispensable amino acids based on the experimentation of Rose is presented in **Table 1**.

Post World War II, interest in animal nutrition increased exponentially, reaching a peak in the 1970s, as more importance was placed on food production [7]. Early poultry amino acid nutrition research mirrored the amino acid studies of the time, with the determination of essential amino acids in poultry [8–12]. These original studies were conducted in commercial-type type diets, with the first reports of the use of

	1932	1938	1948
Alanine	Unknown	Dispensable	Dispensable
Arginine	Dispensable	Indispensable	Indispensable
Aspartic acid	Dispensable	Dispensable	Dispensable
Citrulline	Not listed	Dispensable	Dispensable
Cystine	Indispensable	Dispensable	Dispensable
Glutamic acid	Dispensable	Dispensable	Dispensable
Glycine	Dispensable	Dispensable	Dispensable
Histidine	Indispensable	Indispensable	Indispensable
Hydroxyglutamic acid	Dispensable	Dispensable	Not listed[2]
Hydroxyproline	Dispensable	Dispensable	Dispensable
Isoleucine	Unknown	Indispensable	Indispensable
Leucine	Unknown	Indispensable	Indispensable
Lysine	Indispensable	Indispensable	Indispensable
Methionine	Unknown	Indispensable	Indispensable
Norleucine	Unknown	Dispensable	Not listed[2]
Phenylalanine	Unknown	Indispensable	Indispensable
Proline	Dispensable	Dispensable	Dispensable
Serine	Unknown	Dispensable	Dispensable
Threonine	Not listed[3]	Indispensable	Indispensable
Tryptophan	Indispensable	Indispensable	Indispensable
Tyrosine	Dispensable	Dispensable	Dispensable
Valine	Unknown	Indispensable	Indispensable

[1]*Adapted from Rose [4, 5] and Rose et al. [6].*
[2]*Removed from list of dispensable amino acids.*
[3]*Prior to identification.*

Table 1.
Status of amino acids as indispensable or dispensable as reported by W.C. Rose[1].

purified-type diets, with crystalline amino acids serving as the only source of protein, being that of Hegsted in 1944 [13]. The use of purified-type diet laid the foundation for accurate determination of amino acid requirements, as they allowed for researchers to know the exact dietary amino acid contents.

In the early phase of purified diets, researchers attempted to create reference diets that could produce similar growth performance to chicks fed commercial-type diets. Extensive work was done at the University of Illinois to construct the aforementioned reference diet [14–18]. In 1965, Dean and Scott [19] published a report detailing a reexamination of the findings of their previous work at the University of Illinois. In this set of experiments, it was found that after refinements had been made to the amino acid levels in the reference diet, earlier determined amino acid requirements were over estimated and could be reduced without negatively affecting performance. These findings indicated the interplay among the amino acids when determining requirement estimates.

2. Identification of classic amino acid antagonisms in the chick

2.1 Lysine and Arginine

In early work concerning the arginine requirement for poultry, Almquist and Merritt [20] found that the arginine requirement increased at a constant rate as crude protein was increased, citing requirement values of 0.9, 1.2, and 1.8% of the diet for arginine at crude protein levels of 15, 20, and 25%, respectively. Taking this into account, Anderson and Dobson [21] noticed that the arginine requirement fluctuated in diets containing similar crude protein levels [22], and postulated that amino acid balance was the more important variable than crude protein in general at the conclusion of their own experimentation. Furthermore, Anderson and Dobson [21] indicated that a relationship between arginine and lysine might be expected due to similarities in their chemical structure and potentially their metabolism. Likewise, Fisher et al. [23] indicated that the amino acid content of casein, used in purified diets to determine amino acid requirements, likely increased the arginine requirement compared to diets containing soybean meal, which contains approximately a third the lysine of casein. This disparity between purified and commercial-type diets had been previously discovered by Krautmann et al. in 1957 [24], but Krautmann et al. [24] had failed to make the connection of amino acid content and instead proposed an "unidentified factor of plant origin" was to blame for the disparity in arginine requirements among diet types.

Due to the extreme variation of requirement estimates that had been published at the time, Lewis et al. [25] attempted to establish to an arginine requirement based on commercial-type diets used in the United Kingdom. Lewis et al. [25] not only investigated the effects of varying crude protein levels, but also examined the influence of an amino acid imbalance induced by excess lysine based on the previous work of Anderson and Dobson [21]. The results of these studies indicated that under practical conditions using commercial-type diets it was unlikely that an arginine deficiency would occur unless excess lysine was introduced into the diet [25]. Despite these findings, work continued using purified diets in order to understand the mechanisms behind the lysine-arginine antagonism.

Jones [26] studied the antagonism between lysine and arginine using diets containing casein and gelatin as the protein contributing ingredients. In this study, Jones [26] indicated that excess lysine depressed the utilization of arginine in purified diets containing these protein sources. Boorman and Fisher [27] then reported that the antagonism was not reciprocal, indicating that excess levels of arginine did not result in further growth depressions when lysine was deficient. Boorman and Fisher [27] went further to indicate that a lysine-arginine antagonism did not exist, but that the results of their experiment showed a response of a general amino acid toxicity.

A major step in the identification of a mechanism behind the lysine-arginine antagonism was reported by Jones et al. in 1967 [28]. First, Jones et al. [28] showed that both control and excess lysine fed chicks were able to effectively digest and absorb arginine, dispelling the theory that lysine reduces the utilization (i.e., digestion and absorption) of arginine. Secondly, Jones et al. [28] proposed three potential mechanisms behind the antagonism, of which the primary effect of lysine was indicated to be increased catabolism of arginine or a reduction in renal tubular resorption of arginine. The increase in dietary lysine was associated with an increase in kidney arginase activity, but as this was a delayed response, Jones et al. [28] did not believe it

to be the primary cause of the increased arginine catabolism. Boorman et al. [29] later showed that intravenous infusions of lysine resulted in increased plasma lysine levels and inhibited renal reabsorption of arginine in cockerels.

Nesheim [30] studied the influence of lysine on chickens selected for high and low arginine requirements. In these studies, Nesheim [30] found that excess lysine had a greater growth depressing effect on chickens selected for high arginine requirements compared with those selected for low requirements. Despite the larger effect observed in the high arginine birds, Nesheim [30] observed growth depressing effects of lysine on the low arginine requirement birds, seemingly independent of kidney arginase levels. Neisheim [31] also observed an increase in urinary arginine loss when high levels of lysine were fed. Conversely, Austic and Nesheim [32] observed two to four-fold increases in arginase activity when excess lysine, histidine, tyrosine, and isoleucine were fed with and without arginine. These responses were determined to occur in concert with the depressions in body weight gain through the implementation of time-course studies. Therefore, Austic and Nesheim [32] concluded that arginase activity was a major factor in the variation of the arginine requirement, in stark contrast to previous research.

In 1970, D'Mello and Lewis [33] published the first of their series of papers on amino acid interactions in chick nutrition, focusing on the lysine-arginine antagonism. As previous researchers had challenged the existence of a lysine-arginine antagonism [27], D'Mello and Lewis [33] utilized a basal diet limiting in methionine and only marginally adequate in arginine. When excess lysine was added to the diet, depressions in chick performance could not be corrected with additional methionine, but only when arginine was added. These responses suggested a restructuring of the order of limitation in the basal diet and confirmed a direct relationship between lysine and arginine. The third paper in the D'Mello and Lewis [34] series defined the arginine requirement when diets contained excess lysine. When arginine was titrated at four lysine levels, D'Mello and Lewis [34] reported a linear increase in the arginine requirement (**Figure 1**).

Allen et al. [35] titrated arginine in diets containing dietary lysine levels of 0.55, 0.95, 1.35, 1.95, and 2.55%. These titrations allowed for the comparison of growth curves to show the declining efficiency of arginine to promote weight gain. Arginine efficacy decreased linearly to 58.8% of control levels as lysine was increased to 1.84%.

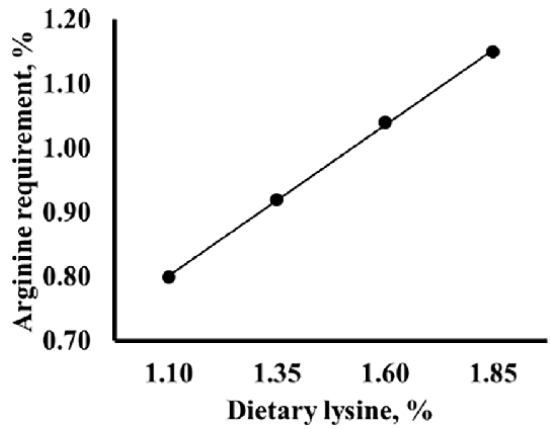

Figure 1.
Influence of dietary lysine level on the determined arginine requirement. Adapted from D'Mello and Lewis [34].

Further increases of dietary lysine had no effect on arginine efficiency. Based on these observations, Allen et al. [35] concluded that the lysine-arginine antagonism was based on lysine magnifying the effects of an arginine deficiency. Allen and Baker [36] then determined the arginine requirement when dietary lysine levels were 0.95 and 1.95%. The required arginine level increased by 52 and 37% for body weight gain and feed conversion, respectively (**Figure 2**).

Wang et al. [37] investigated the influence of excess dietary lysine and arginine on the enzyme activity of lysine-ketoglutarate reductase and arginase. Increased supplementation of L-lysine HCl, ranging from 0 to 1.0%, resulted in a in an approximate two and five-and-a-half-fold increase in lysine-ketoglutarate reductase and arginase, respectively. Conversely, supplementing L-arginine from 0 to 2.0% resulted in an approximate two-fold increase in kidney arginase activity, but arginine supplementation had no effect on lysine-ketoglutarate reductase activity.

Kadirvel and Kratzer [38] examined the intestinal uptake of L-arginine and L-lysine when excesses of lysine, leucine, and glycine in vitro. Focusing on arginine and lysine, it was discovered that arginine absorption was reduced when lysine was added to the solution, but progressive amounts of lysine had no further influence on arginine absorption, indicating that limited competition between lysine and arginine exists during absorption. Kadirvel and Kratzer [38] then displayed the effects of feeding the aforementioned amino acids to broilers and evaluated their effects on bird performance. During the in vivo study, only excess lysine resulted in the appearance of arginine deficiency symptoms, which Kadirvel and Kratzer [38] interpreted to indicate that lysine-arginine antagonism is mediated through a metabolic effect as opposed to competitive absorption. Robbins and Baker [39] revisited the influence of amino acid excess on kidney arginase activity. They found that not only did lysine, arginine, and histidine influenced arginase activity, in agreement with Austic and Nesheim [32], but also an effect of total nitrogen that exceeded that of individual amino acids. Robbins and Baker [39] concluded that total nitrogen level was equally important in the activity of arginase as dietary arginine and lysine.

Based on research evaluating lysine-arginine antagonism from its discovery until the early 1980s several conclusions can be drawn characterizing the antagonism. First, a specific antagonism exists among arginine and lysine that appears to be

Figure 2.
Influence of dietary lysine level on the arginine requirement for body weight gain (solid line) and feed conversion (dashed line). Adapted from Allen and Baker [35].

non-reciprocal, displaying only effects of lysine on arginine metabolism. Secondly, the reason for the discovery of said antagonism lies in the amino acid contents of specific protein sources that were used in the diets of the period used to characterize amino acid requirements, namely casein due to its low arginine content relative to lysine. Lastly, the mechanism behind the lysine-arginine antagonism has not been cleanly defined but it does appear to be linked to the reduced capacity for the renal tubes to reabsorb arginine. While the role of arginase, and lysine's effect on it, is still debated, the findings of Robbins and Baker [39] combined with the findings of Keene and Austic [40] twenty years later may potentially explain the conflicting reports on arginase activity. Keene and Austic [40] found that catabolic enzymes are stimulated more by dietary protein than by the single amino acid targeted by the enzyme. The response of arginase by multiple amino acids is likely the response of increased dietary nitrogen, or in the case of Robbins and Baker [39] a balanced amino acid mixture, as opposed to the individual amino acids.

2.2. Branched-chain amino acids

Knowledge of the branched-chain amino acids concerning poultry started in much the same way as arginine with the determination of essentiality for poultry in 1944 [12, 13], and the first data set outlining their requirements was published two years later in 1946 [41]. The original requirement values were established to be 0.5, 1.5, and 0.7% of the diet for isoleucine, leucine, and valine, respectively, and varied little during the years of experimentation contributing to crystalline amino acid diets [14, 16, 19, 42].

In 1960, Laksesvela [43] reported that that deletions of isoleucine resulted in a 27% reduction in the "combinative protein value" of herring solubles, whereas additions of leucine resulted in a 16% reduction in the aforementioned metric. The implications of this discovery would not be fully appreciated until 1968 when Mathieu and Scott [44] reported that feeding excess leucine in diets containing isoleucine and valine near adequacy resulted in depressions in body weight. This report started investigations into branched-chain amino acid antagonism, as the existence of amino acid antagonisms were known (i.e., the existing work on the lysine-arginine antagonism) as well as its previous discovery in rats [45].

The second interaction investigated in the early 1970s by D'Mello and Lewis in a series on amino acid interactions in chick was that among the branched-chain amino acids [46]. As with lysine and arginine, D'Mello and Lewis sought to confirm the existence of the former amino acid antagonism in chick nutrition. Through the course of five experiments, D'Mello and Lewis [46] isolated and definitively showed the existence of an antagonism between leucine and isoleucine and leucine and valine, but theorized that up to six antagonisms existed among the branched-chain amino acids. D'Mello and Lewis [46] further defined that this antagonism is most prevalent when valine and isoleucine are included in the diets at adequate levels but could present itself if valine was not the limiting amino acid in the basal diet, in the case of a leucine-valine antagonism.

In their next series of experiments, D'Mello and Lewis [34] determined the influence of excess leucine on the requirements of valine and isoleucine. Excess leucine shifted the requirements of both valine and isoleucine in order to obtain maximal body weight gain (**Figure 3**). Of particular interest, D'Mello and Lewis [46] had indicated that the leucine × valine interaction was probably of more practical importance to broiler production and later showed that adjusting valine to maximize growth at higher leucine levels improved average daily gain above control levels whereas

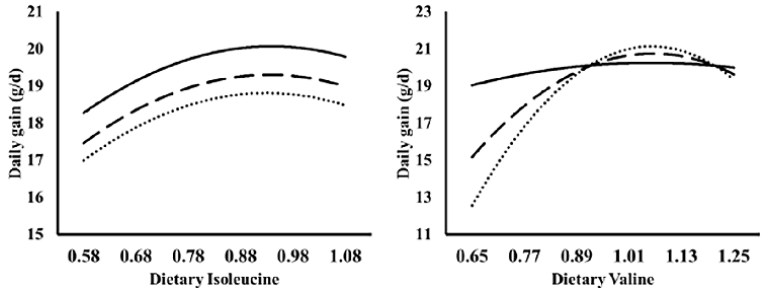

Figure 3.
Influence of titrating isoleucine and valine on average daily gain at low (solid line), medium (dashed line), and high leucine (dotted line). Adapted from D'Mello and Lewis [34].

increasing isoleucine resulted in a decrease in optimal average daily gain [34]. In their final study, D'Mello and Lewis [47] established the existence of a metabolic mechanism behind the branched-chain amino acid antagonism by pair feeding diets containing adequate and excessive leucine, as depressions in body weight gain were still observed in birds fed diets containing excess leucine compared with consuming an equal amount of a control diet.

The following year, Allen and Baker [48] conducted a series of experiments to determine the efficacy of isoleucine and valine when leucine was fed in excess. The ability of isoleucine to sustain body weight gain was linearly reduced to 80% of control levels when leucine was increased from 0 to 3% of the diets, conversely the ability of valine to support body weight gain was quadratically reduced to 74% of control levels as leucine supplementation was increased to 6%. Due to the difference in leucine excesses employed in the isoleucine and valine experiments, the minimal efficacy values do not allow for a direct comparison. Equalizing leucine inclusion levels to 3% displays an average efficacy of 81 and 79 for isoleucine and valine, respectively, agreeing with the postulations of D'Mello and Lewis [46] that the valine × leucine interaction would likely have more impact on poultry production.

Due to the similarities in chemical structure and common enzymes used in the transamination and decarboxylation steps of catabolism [49, 50], researchers believed that the antagonism among the branched-chain amino acids was linked to increased catabolism brought about by excessive leucine. Researchers at the University of Nottingham tested this hypothesis by monitoring the activity of aminotransferase for leucine and valine [51], as well as the catabolism of C14 labeled valine [52]. Both studies failed to observe any influence on the rate of catabolism of valine when leucine were fed in excess. Conversely, Smith and Austic [53] observed a small increase, approximately 2% of ingested levels, in the catabolism of C14 labeled valine and isoleucine when leucine reached 2.25% of the diet. Similarly, Calvert et al. [54] observed a 50 and 43% increase in isoleucine and valine, respectively, when leucine was fed at 5%. In addition, Calvert et al. [54] pair fed chicks diets containing 1.2 or 5.0% leucine to gauge the effect of reduced feed intake on branched-chain amino acid antagonism responses. Calvert et al. [54] found that growth depressions associated with excess leucine persisted when feed intake was equalized, agreeing with the previous findings of D'Mello and Lewis [47]. Based on their overall findings, Calvert et al. [54] proposed that 70% of the negative effects associated with branched-chain amino acid antagonism is linked to feed intake as opposed to a primary effect of metabolic changes.

Jackson and Potter [55] reported that the classic responses of branched-chain amino acid antagonism observed in poultry also occurred in turkeys. Branched-chain amino acid antagonism had previously observed in turkey poults [56, 57], but Jackson and Potter [55] also discovered that a reciprocal antagonism between isoleucine and valine that could result in depressions in body weight when either was fed at adequacy while the other was fed in excess. Mendonca and Jensen [58] latter confirmed the existence of isoleucine and valine antagonism in chickens, when it was found that supplementing isoleucine reduced performance, whereas a concomitant addition of isoleucine and valine had no effect.

Unlike with lysine-arginine antagonism, less is know about the mode of action behind the branched-chain amino acids with many theories lacking critical evidence to definitively prove. Despite this, cornerstone data were generated during the research conducted from the 1960s to 1980s. Firstly, the branched chain amino acid antagonism is a reciprocal antagonism, in that it can present itself by targeting both valine and isoleucine, and subsequent interactions between valine and isoleucine. Secondly, leucine appears to be the primary antagonist, but apparent performance gains can be made if proper supplementation of valine is made to account for the antagonism. Lastly, the largest piece of information that can be gleaned from classic research is that the antagonism is most apparent when isoleucine and valine are at adequacy levels, indicating that negative effects are likely to occur in reduced crude protein diets.

3. Evolution of poultry amino acid nutrition

In the 1990s, poultry amino acid nutrition reports placed more emphasis on practical aspects than those of previous decades. Large advancements in least-cost formulation strategies for broiler integrators, brought about by linear programing and personal computers that could conduct it, occurred in the mid 1980s, allowing nutritionists to rapidly produce mock formulas [59]. Lack of experience with this technology caused a distrust with feed-grade amino acids limiting their use, which would later be overcome with the widespread adoption of L-threonine in the 1990s [59]. This allowed for dietary crude protein to settle on the 4th limiting amino acid which varied depending on the ingredients included in broiler diets [60]. Therefore, research during the 1990s and early 2000s largely shifted to the determination of amino acid requirements, although some antagonism work remained.

By the 1990s research evaluating the lysine-arginine antagonism had largely come to an end. Mendes et al. [61] failed to observe any response to variations in dietary lysine or the arginine to lysine ratio when feeding broilers three to six weeks of age. The classic responses observed were largely the result of the ingredients used in non-practical diets (i.e., casein) and not something that would typically occur in poultry production. Similarly, studies determining the arginine requirement began to produce relatively consistent requirement estimates, likely resulting from the constraints placed on lysine during formulation (**Table 2**). In addition to arginine's role in animal growth, research into its influence on animal health gained popularity and was added to requirement parameters [67–70].

Conversely for the branched-chain amino acids, Farran and Thomas [71] implemented central-composite, rotatable design to model the branched-chain amino acids

Reference	Strain	Sex	Age, day	Requirement estimate[1]
Corzo and Kidd [62]	Ross × Ross 308	Male	0–18	101
Cuca and Jensen [63][2]	Peterson × Arbor Acres	Male	0–21	106
Chamruspollert et al. [64][3]	Ross × Ross 208	Mix	7–21	105
Mack et al. [65]	Ross	Male	20–40	112
Corzo and Kidd [62]	Ross × Ross 508	Female	21–35	ND
Mendes et al. [61]	Ross × Ross	Male	21–42	110
Corzo et al. [66]	Ross × Ross 308	Male	42–56	115

[1]*Ratio of arginine to lysine.*
[2]*Select trials used due to experimental design.*
[3]*Non-heat stressed.*

Table 2.
Estimations of the arginine requirement for broiler chickens of various age, strain, and sex.

and determine the requirements of the three simultaneously. Farran and Thomas [71] found significant interactions between valine and isoleucine, but were unable to identify any effect of leucine, differing from historic data. Due to the lack of effect of leucine, Farran and Thomas [71] eliminated leucine from their model, only determining requirements for valine and isoleucine, and began working with valine instead of continuing antagonism work [72, 73].

Also in the early 1990s, Burnham et al. [74] implemented a dilution technique in order to assess the effects of increasing isoleucine at different dietary valine and leucine levels. Burnham et al. [74] found that valine had no effect on the isoleucine requirement, and that leucine only depressed body weight when isoleucine was at the lowest tested levels. These findings resulted in Burnham et al. [74] postulating that the negative influences of leucine would not be an issue in practical diets if the ingredients used contained adequate amounts of isoleucine. Barbour and Latshaw [75] also evaluated the influence of valine and leucine on broiler isoleucine requirements but implemented practical type diets. No influence of valine nor leucine were observed on the isoleucine requirement. Barbour and Latshaw [75] indicated that the lack of a response was due to their experimental design in which not only were basal diets formulated with practical ingredients but adjustments in valine and leucine were brought about by practical ingredients available to the broiler industry. The final experiment of this era was conducted by Waldroup et al. [76]. Similar to the design of Barbour and Latshaw [75], Waldroup et al. [76] tested the effect of excess leucine by varying the amount of corn gluten meal in the diet. No negative effects were observed as a result of the excessive leucine levels, reaching over 3.5% of the diet. Waldroup et al. [76] indicated that the lack of response was driven by the increasing levels of isoleucine and valine that accompanied the excess leucine levels as a result of using intact protein sources to drive the leucine level. These universal excesses among the branched-chain amino acids allowed for the bird to account for potential losses of valine and isoleucine associated with the antagonism. Waldroup et al. [76] concluded their report theorizing that as more feed-grade amino acids entered poultry formulation, branched-chain amino acid antagonism may become a practical concern due to the elimination of excess valine and isoleucine in broiler diets.

4. The return of antagonism research

The doctoral work of I.C. Ospina-Rojas, resulted in three papers investigating interactions between valine and leucine [77–80]. To evaluate the relationship between valine and leucine and its influence on live performance and carcass traits, Ospina-Rojas [77] conducted two 5 × 5 factorials, after which results were displayed via response surface graphs to allow for visual observations of trends. During a 1–21 day starter phase, Ospina-Rojas et al. [80] observed valine × leucine interactions for fed intake and feed conversion. Ospina-Rojas et al. [80] was able to determine leucine and valine to lysine requirement values of 104 and 77 and 102 and 73 for feed intake and feed conversion, respectively. Feed intake was most severely impacted when valine levels were low and leucine levels were high, whereas feed conversion spiked when both amino acids were fed at low levels.

When varying valine and leucine levels were fed during a 21–42 day period, Ospina-Rojas [78] observed significant valine by leucine interactions for feed intake and body weight gain. Unlike with the previous growth phase, requirement values could not be estimated for maximal feed intake as a ridge occurred for feed intake between valine to lysine ratios of 82 and 91 for the entire range of leucine. Feed intake values remained relatively constant across leucine levels but it was again minimized when valine levels were low and leucine levels were high. For body weight gain, a requirement estimate was determined at a valine and leucine ratio to lysine of 111 and 83, respectively. As with feed intake, body weight gain was lowest when dietary valine was low and leucine was high.

Zeitz et al. [81] evaluated the influence of excess leucine on broiler performance and carcass traits when branched-chain amino acid levels were either fixed [82] or allowed to drop in relation to leucine level [81]. When branched-chain amino acid ratios were fixed, no differences were observed in growth performance over a 1–35 day period, but breast yields were decreased when leucine was increased by approximately 60%. However, no differences were observed for a 1–34 day period nor day 34 carcass traits when levels valine and isoleucine ratios in relation were allowed to drop when leucine increased.

Ospina-Rojas et al. [83] evaluated the influence of high leucine levels on the valine and isoleucine requirements for a starter (1–14 day), grower (14–28 day), and finisher periods (28–42 day) through the implementation of central-composite, rotatable design. Ospna-Rojas et al. [83] observed consistent influence of branched-chain amino acids on feed conversion across all three feeding phases, but body weight gain was not affected until the finisher phase. Unlike previous experiments, Ospina-Rojas et al. [83] did not generate response surface graphs, but did report regression equations. The lack of response surface graphs was due to the significant effect of three factors that cannot be displayed on a three-dimensional graph. Requirements estimates needed for optimal body weight gain reported by Ospina-Rojas et al. [83] generally showed that valine and isoleucine requirements decrease as the bird ages, but leucine needs increase.

A pair of studies published in 2021 implemented the use of Box-Behnken design to characterize the broilers response to various branched-chain amino acid levels [84, 85]. The studies were completed as part of a ring study and followed the same experimental design, with the only difference being the type of birds used (i.e., strain and sex). Maynard et al. [85] found significant interactions between valine and isoleucine for body weight gain, feed conversion, and breast meat yield when branched-chain amino acid levels were varied in diets fed to Cobb MV × 500 broilers. The effect of leucine was

limited to an interaction between leucine and valine on breast meat yield. Maynard et al. [85] came to a similar conclusion to that of Farran and Thomas [71] that leucine may not be a significant factor under practical conditions and eliminated it from their model, replacing it with glycine + serine due to the potential limitation of glycine or nonessential nitrogen in the reduced crude protein diets implemented. When leucine was removed from the model, the interactions between valine and isoleucine were virtually eliminated, indicating that the "real" effect of leucine may be a "shadow effect" that does not present as a traditional significant response Maynard et al. [85]. Kidd et al. [84] focused on the branched-chain amino acids in their study but conducted it in male and female Lohman Indian River broilers. Contrary to the findings of Maynard et al. [85], Kidd et al. [84] did observe significant influence of leucine, citing interactions between leucine and isoleucine for body weight gain and feed conversion and leucine × valine interactions for carcass and breast meat yield. Furthermore, Kidd et al. [84] found that female broilers were more responsive to branched-chain amino acid supplementation than males.

Maynard [86] followed up the findings from the Maynard et al. [85] studies through the implementation of factorial designs meant to confirm the modeling responses. The first factorial study conducted by Maynard et al. [86] sought to determine the shift in the valine requirement when high and low levels of isoleucine and leucine were fed in practical type diets. Interestingly, a three-way interaction was observed for feed conversion but two sub interactions, valine × leucine and valine × isoleucine, were observed for body weight gain. The body weight gain responses observed by Maynard [86] (**Figure 4**) closely resembled those observed by D'Mello and Lewis [34] 50 years ago, without using purified diets or large swings in dietary leucine. Maynard [86] then attempted to characterize the sub interactions, valine × leucine and valine × isoleucine, observed in the larger study but failed to get a response. This lack of response again highlighted and expanded upon the observations Maynard et al. [85], that investigations into the branched-chain amino acid antagonism require testing of all three due to the complex nature of the antagonism.

The most recent study evaluating the branched-chain amino acids was a central-composite, rotatable design presented by Corzo and Silva [87]. Corzo and Silva [87] observed significant three-way interactions for body weight gain, feed conversion, carcass yield, and breast meat yield. General trends showed that increased isoleucine and valine were needed when leucine was fed in excess, but potentially more important, that the negative effects of leucine could be overcome for all parameters, except

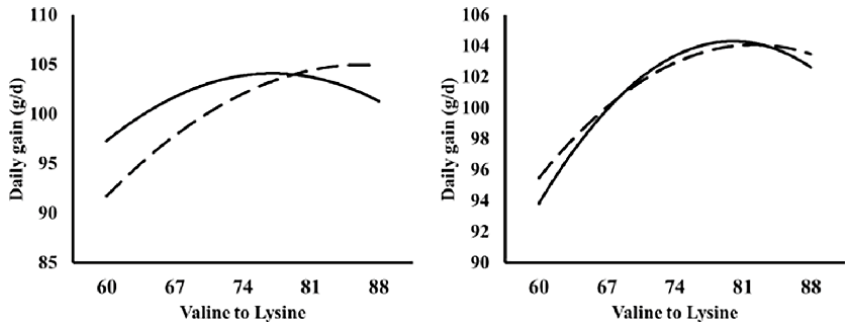

Figure 4.
Influence of titrating valine on average daily gain at high (solid line) and low (dashed line) leucine (left) and isoleucine (right). Adapted from Maynard [86].

carcass yield, when leucine levels continued to increase with proper isoleucine and valine supplementation.

These recent studies have shown that unlike the lysine-arginine antagonism, branched-chain amino acid antagonism presents in practical type diets and will continue to be an issue for practical broiler production as crude protein levels decrease. The work of Corzo and Silva [87] is promising as it appears the effects of this antagonism could be turned from a negative to a positive. Maynard et al. [88] indicated that in the future this phenomenon may be referred to as the branched-chain amino acid synergism based on a meta-analysis conducted on branched-chain amino acid research conducted from 2000 to 2021.

5. Future research

In the modern era, broiler amino acid research is centered around more complex problems as opposed to the simple strategies of the past. Titration studies used to determine amino acids requirements will remain the gold standard, as laid out by Lewis [89], but further refinement of these requirements will require researchers to consider test diet nutrient profiles compared to those observed in commercial practice. With the present known, and potentially unknown, antagonisms influencing amino acid requirements, generated values from test diets may not accurately represent those that produce optimal performance under commercial or practical conditions. Likewise, differentiated responses to branched-chain amino acid levels were observed when broiler strain or sex was changed under similar experimental conditions.

The double-edged sword of evaluating these complex interactions is the need for larger research facilities to achieve necessary experimental unit and replication. Another more manageable approach is the use of modeling. Previous researchers have shown that modeling research (i.e., Box-Behnken design) can be used in order to reduce the treatments necessary to characterize large scale interactions [90]. By effectively halving the number of treatments necessary to test a 3 × 3 × 3 interaction, the number of replicates can be doubled without increasing the number of necessary pens. This approach can be used over a broad range of inclusion levels in order to map general responses, then if a significant response is observed, treatment ranges can be reduced to reflect those observed in commercial practice to allow for a targeted approach. While Maynard [86] largely failed in the attempt to follow this strategy, the larger valine titration factorial was successful in observing a shift in valine requirements.

It is important to note that the collective work of Kidd et al. [84], Maynard [86], and Maynard et al. [85] used P-values $\geq$0.10 to identify significant interactions due to repeated observance of these levels. Originally, Kidd et al. [84] and Maynard et al. [85] set significance levels at $P \geq 0.10$ due to the modeling approach used in their studies, but subsequent work by Maynard [86] observed similar P-values in their factorial approaches. P-values for the three-way interaction observed by Maynard [86] for feed conversion were found to be between 0.05 and 0.10, but when the data was broken into the individual titrations, P-values were found to be highly significant (i.e., $P < 0.01$). Relative consistency in responses to the branched-chain amino acids have been historically observed and noted by previous researchers [91].

While the current body of literature does not allow for concrete formulation strategies, promising studies have been recently conducted and the prevalence of this

style of research is increasing. The original observations of these antagonisms were brought about through the use varying ingredients, which changed the amino acid profiles of the diets implemented. The implications of how these discoveries were made are still relevant today with the ability of nutritionists to simply monitor the levels of nutrients in diets through the addition of nutrients to formulation software. While requirement minimums or formulation constraints will not necessarily be added for these nutrients, their inclusion in matrices allow for monitoring that can be reevaluated if negative performance or responses are observed in the field.

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Chapter 7

Thermal Manipulation: Embryonic Development, Hatchability, and Hatching Quality of Broiler Chicks

Brian Tainika

Abstract

Here, PRISMA guidelines were utilized to systematically evaluate the publications reporting the effect of thermal manipulation during embryogenesis on incubation performance, hatchability, and hatching quality of broiler chicks. The search and selection of eligible publications was through databases web of science, PubMed, and Scopus. Publications written in English between 2015 and September 2021 were considered. It is evidenced that during TM, key considerations include duration and strength of TM besides stage of embryonic development. The moderate elevation in incubation temperature (38.5–39.5°C) intermittently (3–18 h/d) between E07 and E18 improves the chick's thermoregulation capacity and reduces any adverse effect of TM on hatchability, and chick quality (e.g., hatch weight and chick length) compared with continuous TM. In addition, high temperature TM (38.5–39.5°C) between E7 and E18 has no significant effect on embryo mortality, hatchability, and chick quality compared to standard incubation temperature (37.8°C). TM above 39.5°C significantly increases and decreases embryo mortality and hatchability, respectively compared with standard incubation temperature. In conclusion, the results of TM studies on embryogenesis, hatchability and hatching quality of broiler chicks are still contradicting, which is a possible limitation for its commercial use.

Keywords: broiler industry, chick quality, epigenetic adaptation, hatchery industry, incubation, thermotolerance acquisition

1. Introduction

In recent decades, the need to increase hatchery efficacy is increasing with demand for quality chicks. Therefore, during incubation, new techniques which are associated with embryo management are increasing with this demand. A possible reason is that the newly developed broiler genotypes have diverged considerably compared to traditional genotypes in terms of the biological, physiological, and biochemical requirements [1]. Thus, manipulation of different incubation conditions

to meet the requirements of modern broiler genotypes is under continuous investigation. Incubation conditions have a significant effect on hatchability, chick quality and post-hatch performance in chicken. In addition, while the first quarter of incubation is critical to chicken embryogenesis [2], the last quarter is very significant to hatch and post hatch performance [3].

Incubation temperature is the most significant incubation condition [4] and there has been an increase in studies regarding thermal manipulation (TM). TM (i.e., increasing or lowering incubation temperature) and broiler chicken embryogenesis has been deeply studied by Collin A, Tzschentke B, Piestun Y, Yahav S, and Halevy O, and the technique enhances chick quality through improved body weight gain, increased expression of genes in the breast muscle, and thermotolerance. Earlier studies laid the foundation for implementation of TM between different days of embryogenesis in addition to key factors such as duration and strength of TM to enhance the chick's ability to cope with environmental challenges of cold and heat stress during post-hatch period [5–10].

In an earlier study, Yahav et al. [11] reported that TM at 39.5°C for 3 hours (h)/day (d) from embryonic age (E) E11–E16 improves the chicks' thermotolerance acquisition. A similar effect was confirmed by [12–16] with TM at 39.5°C for 12 h/d from E7 to E16. Recent studies have also confirmed a long-lasting effect on thermotolerance acquisition in chicks at hatch or 1-day-old chicks, for instance, Piestun et al. [17] and David et al. [18] both with TM at 39.5°C for 12 h/d from E7 to E16, Al-Zghoul et al. [19] with TM at 38.5, 39, 39.5 and 40°C for 18 h/d from E12 to E18 and Al-Zghoul et al. [20] with TM at 39°C for 18 h/d from E10 to E18.

The effect of TM on hatchability in several studies has been reported to differ extensively, with hatchability after TM being higher [11, 21, 22], reduced [9, 12, 13, 23, 24], and not affected [25, 26]. Also, studies from different researchers have shown contradicting results on chick quality parameters, and hatch or chick weight after TM exposure was increased [22, 23, 27], decreased [28], and not affected [21, 25, 29].

Production of optimal quality chicks depends on controlling incubation conditions and understanding the insights into the complex interaction among them. Although various studies have reported the benefits of TM, results are still contradicting and depend on timing, duration, and level of TM. With this background, we systematically reviewed the recent literature regarding the effect of thermal manipulation on embryonic development, hatchability, and hatching quality of broiler chicks.

2. Search strategy and selection of publications

The search for potentially eligible publications was conducted using electronic databases Web of science, PubMed, and Scopus. The eligibility was based on the title, abstract, and keywords, and only included articles published in English language. Filters were applied in terms of publication date (2015-01-01 to 2021-09-30 in Web of Science, PubMed, and Scopus).

The developed strings used in the literature search from three databases included: (thermal OR temperature) AND (manipulation) AND (incubation OR embryogenesis OR embryo development) AND (broiler OR chickens OR poultry).

The identified studies from each database were exported to Microsoft Excel workbook to document bibliographic information (author names, title, and publication year). The same software was used to manage and exclude duplicate studies.

Thereafter, the reviewers examined the titles and abstracts, followed by examining the full version of the selected potential studies. At this point, each study was read to extract the aimed set of information. Therefore, the eligibility criteria for the final study selection included:

1. Animal materials are broiler chickens.

2. Study that entirely manipulated incubation temperatures (i.e., studies involving eggshell temperature manipulation or that combined incubation temperatures with other strategies were excluded).

3. Study reports TM age and a control group.

4. Study reports at least one of the following: hatchability, hatch rate, embryo mortality rate, body weight at hatch, body temperature at hatch, and chick quality.

The initial literature search identified a total of 229 articles (125 in Web of Science, 59 in PubMed, and 45 in Scopus database). After the elimination of 89 duplicate articles, 140 studies were available for analysis. Other 72 studies were excluded after evaluating the titles, thus a total of 68 articles were eligible for abstract screening. At this point, 30 studies were excluded because they did not meet the defined criteria, which resulted in 38 studies being available for full-text evaluation. From this, 7 studies were removed based on the previously defined criteria. Finally, 31 studies were selected and included in the definitive systematic review as shown in **Figure 1**.

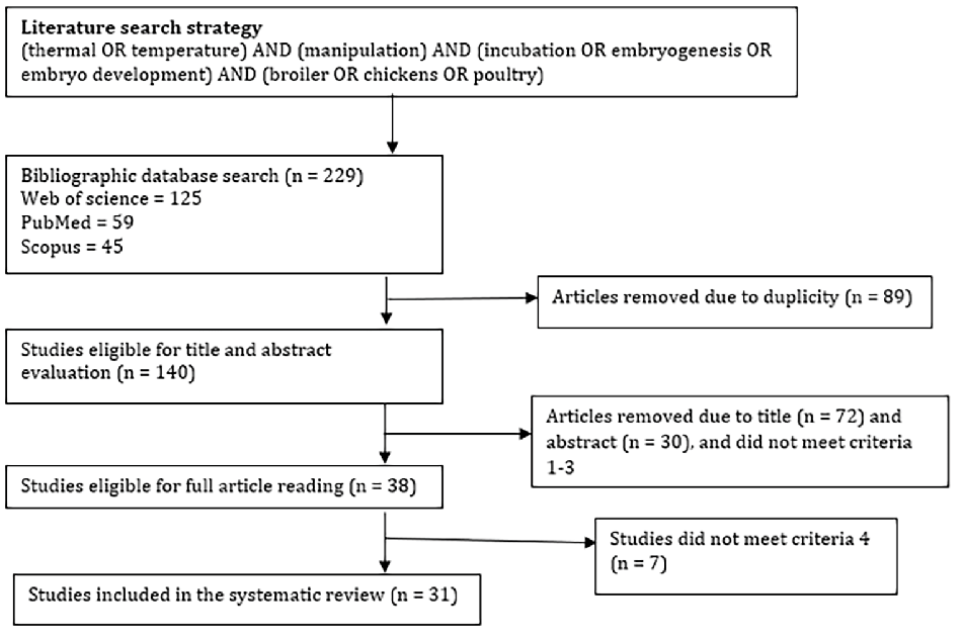

Figure 1.
A flowchart of the summarized study search procedure.

3. General characteristics of the studies included in the systematic review

Table 1 summarizes the information that was extracted from the final selected studies. The studies in the systematic review were published between 2015 and 2021, and 31 papers were finally included. Intermittent TM profile (3–18 h/d) was used in most studies (24 papers, 77%) compared with continuous (24 h/d) (6 papers, 19%),

Reference	Control group temperature profile	TM profile	TM age/ embryonic day (E)	Outcome
Piestun et al. [17]	37.8°C	Intermittent (12 hours (h)), continuous (24 h)/day (d) at 39.5 °C	E7–E16	There was no negative effect of intermittent TM on embryo development. Continuous TM negatively affected embryo weight, and significantly decreased the ratio of embryo weight to egg weight from E16 to E21.
Al-Zghoul et al. [19]	37.8°C	39°C for 9, 12, and 18 h/d	E12–E18	TM significantly reduced hatchability compared to control.
Al-Zghoul et al. [30]	37.8°C	39°C for 9, 12, or 18 h/d	E12–E18	1-d-old chick body temperature was higher in 9 h TM than the other TM and control groups.
Wilsterman et al. [26]	37.5°C	38.6°C	E0–E5, E5–E18	They found no differences in hatchability and hatch weight among the groups.
Rajkumar et al. [31]	37.5°C	39.5°C for 3 h/d	E16–E18	TM did not have a significant effect on hatchability and hatch weight.
Janisch et al. [32]	37.8°C	High (38.8°C) and low (36.8°C)	E7–E10, E10–E13	Low TM resulted in significant increase in hatch weight compared with high TM and the control group.
Krischek et al. [33]	37.8°C	High (38.8°C) and low (36.8°C)	E7 and E10, E10 and E13	Low TM significantly decreased embryo weights (body, liver, heart) compared to high TM and control, which were similar at some level.
Aminoroaya et al. [34]	37.6°C	39°C for 3 h/d	E12–E14, E15–E17	TM did not significantly affect hatch weight and there was no significant difference among the groups in hatchability.
Elmehdawi et al. [35]	37.4°C	38.4°C	E18–E20	TM did not negatively affect hatchability, chick weight, chick length, rectal temperature, and chick quality score compared to control.

Reference	Control group temperature profile	TM profile	TM age/ embryonic day (E)	Outcome
Almeida et al. [36]	37.5°C at 60% RH	Low (36°C), or high (39°C) at 60% RH	E13–E21	Differences between control and high TM were not significant for incubation period but it was longer in cold TM treatment. Differences between control and low TM treatment for hatchability were not significant but it increased in high TM group.
Al-Zghoul et al. [37]	37.8°C	39°C for 9, 12, and 18 h	E12–E18	TM slightly increased embryonic body weight in comparison to control on E18. No significant difference between TM groups was observed from E12 to E16.
Narinç et al. [38]	37.8°C at 55% RH	39.6 °C at 60% RH for 6 h/d	E0–E8, E10–E18	TM significantly lowered hatchability and chick quality but lowest in late TM treatments compared with control. No statistically significant difference was observed between TM and control groups for hatch weight.
Morita et al. [39]	37.5°C	Low 36°C and high 39°C	E13–E21	TM did not have a significant effect on hatch weight compared with control. Hatchability was increased in high TM but lower and similar in low TM and control treatments.
Al-Rukibat et al. [40]	37.8°C	38.5°C and 40°C	6 h at E16, 9 h at E17, and 12 h at E18	TM did not influence hatchability.
Zaboli et al. [41]	37.8°C	39.5°C for 12 h/d	E7–E16	TM reduced hatchability by 4%, reduced hatch weight, and delayed hatch time (6 h later) compared with control.
Al-Zghoul [42]	37.8°C	38.5, 39, 39.5, and 40°C for 18 h/d	E12–E18	1-d-old chick body temperature was higher with TM at 38.5°C than the other TM and control groups.
Vinoth et al. [43]	37.5°C	40.5°C for 3 h/d	E15–E17	TM had no significant effect on hatchability. 1-d-old chick weight did not differ between the groups.
Al-Zghoul et al. [44]	37.8°C	39.5°C for 18 h/d	E10–E18	There was no difference between the groups in hatchability, but hatch weight was significantly reduced in TM group compared with control.

Reference	Control group temperature profile	TM profile	TM age/ embryonic day (E)	Outcome
Al-Zghoul and El-Bahr [45]	37.8°C	38.5, 39, 39.5, and 40°C for 18 h/d at 56% RH	E12–E18	While TM at 38.5 and 39°C did not influence the hatchability, TM 39.5 and 40°C lowered hatchability compared with control group. Similar embryonic weights were found for all the groups.
Dalab and Ali [46]	37.8°C	39°C for 18 h/d	E7–E11, E11–E15, E15–E18, E7–E18	TM significantly influenced the hatchability. Early TM significantly improved hatchability versus control. Late and long TM adversely affected hatchability and chick quality compared with control.
David et al. [18]	37.8°C	39.5°C for 12 h/d	E7–E16	Significantly decreased hatch body temperature was found in TM chickens.
Saleh and Al-Zghoul [47]	37.8°C	39°C for 18 h/d	E10–E18	TM significantly reduced hatchability but did not significantly affect body weight of 1-d-old chicks compared to control.
Amjadian and Shahir [48]	37.8°C	39.5°C for 3 h/d	E11–E16	There was no significant effect of TM on hatchability and embryonic mortality and TM did not influence chick body weight and hatch body temperature.
Saleh et al. [49]	37.8°C	39°C for 18 h/d	E10–E18	No significant difference was identified in embryonic mortality, hatchability, and hatch weight between the control and TM treatment. TM led to significantly decreased hatch body temperature.
Tarkhan et al. [50]	37.8°C	39°C for 18 h/d	E10–E18	TM significantly reduced hatchability and body temperature in 1-d-old chicks compared with control but no significant increase in weight was found in 1-d-old chicks between the groups.
Nyuiadzi et al. [51]	37.6°C	37.6°C at 56%, and interruption of 15°C for 30 min at 81% RH.	E18–E19	No significant effect of TM on hatchability was observed. At hatching, body temperature was higher in TM than in control chicks, but hatch weight was not affected by TM.

Reference	Control group temperature profile	TM profile	TM age/ embryonic day (E)	Outcome
Basaki et al. [52]	37.5°C	Low (33°C) and high (41°C) for 3 h/d	E15–E17	Survival rate, embryos with deformities, hatchability, and hatch weight were not significantly different between groups.
Rocha et al. [2]	37.5°C	Low (36°C) and high (39°C) for 6 h/d	E0–E5	Hatch weight in low TM chicks was higher than control and high TM.
Khaleel et al. [53]	37.8°C	36 and 39°C for 18 h/d	E7–E16	TM did not significantly affect hatchability.
Brannan et al. [54]	37.5°C	39.5°C for 12 h/d	E7–E16	TM increased and decreased embryo mortality and hatchability, respectively but had no influence on hatch weight.
Yalcin et al. [55]	37.8°C	38.8°C for 6 h/d	E10–E14	TM did not affect relative embryo weight on E19 and hatchability but found strain differences in hatch weight.

Table 1.
Overview of thermal manipulation studies during incubation of broiler chicken eggs.

and a combination of both intermittent and continuous in only one study [17], in which no depressing effect of intermittent (12 h/d) TM between E7 and E16 on embryogenesis was reported, however, continuous (24 h/d) reduced embryo weight from E16 to E21.

Among the 24 intermittent TM studies, 20 articles reported the TM effect on hatchability, of which 65% found no significant effect, 30% being reduced, and a comparative study by Dalab and Ali [46] reported increased and decreased hatchability at different embryonic age. Meanwhile, embryo and/or hatch weight and chick quality were reported in 18 articles, of which 12 (67%) found no significant effect, three studies reported increased [2, 38, 50], and three reported reduced effect of TM on embryo and/or hatch weight and chick quality [42, 45, 46].

Embryo mortality was reported in four of 24 intermittent TM studies, of which three studies observed no significant difference in embryo mortality between intermittent TM and control groups [48, 49, 52]. However, Brannan et al. [54] identified increased embryo mortality in intermittent TM groups compared with control group.

Hatch or chick body temperature was reported in eight of 24 intermittent TM studies, which included no significant effect [48, 52], increased [30, 42, 51], and reduced [18, 49, 50].

From the six continuous TM studies, the application increased [39], and had no significant effect on hatchability [26, 35, 36] compared with control groups. Moreover, continuous TM increased [32], decreased [33] and had no significant effect on embryo, chick weight, or chick quality [26, 35, 39]. Furthermore, Elmehdawi et al. [35] reported no negative effect of continuous TM on hatch body temperature compared with control treatment.

In all the studies, the set standard incubation temperature was the control treatment, which was compared to TM treatments. 37.8°C was used as the standard incubation temperature in most studies (20 papers, 65%), followed by 37.5°C (8 papers,

26%). Only Aminoroaya et al. [34], Nyuiadzi et al. [51], and Elmehdawi et al. [35] used 37.6 and 37.4°C, respectively.

While most studies (23 papers, 74%) only used high-temperature TM (i.e., 1–3°C above the set standard incubation temperature), 7 papers were comparative studies of low and high-temperature TM, and only one study used low-temperature TM at 37.6°C but on 18 and 19 d of incubation, embryos were subjected to short cold exposure of 15°C for 30 minutes [51]. The above authors found no significant effect of TM from E18 to E19 on hatchability and hatch weight, but hatch body temperature was elevated compared with control group (37.6°C). In addition, studies involving low-temperature TM, the adjustments varied between 1 and 4.5°C below the set standard incubation temperature.

Three studies compared various high-temperature TM; both 38.5 and 40°C did not have any effect on hatchability [40]. Meanwhile, Al-Zghoul and El-Bahr [44] compared 38.5, 39, 39.5, and 40°C for 18 h/d from E12 to E18 and observed that 38.5 and 39°C did not impact hatchability however, 39.5 and 40°C reduced hatchability compared with control group (37.8°C). The latter TM setup was used by Al-Zghoul [42], who reported increased 1-d-old chick body temperature at 38.5°C compared with other TM and control treatments.

Three studies compared TM duration, which Al-Zghoul et al. [19] and Al-Zghoul et al. [37] identified depressed hatchability and increased embryo body weight on E18, respectively, at 39°C regardless of TM duration (9, 12, or 18 h/d) from E12 to E18. Using similar TM profile to Al-Zghoul et al. [19], Al-Zghoul et al. [37], and Al-Zghoul et al. [45] reported elevated body temperature in 1-d-old chicks at 9 h/d TM duration compared with other TM durations and control (37.8°C) treatment.

Among the seven comparative studies, low-temperature TM resulted in increased hatch weight [2, 32], reduced embryo weights [33], and both low and high-temperatures were not significantly different in hatch weight, embryo mortality, and deformed embryos [52]. Meanwhile, high-temperature TM resulted in higher hatchability [36, 39], and both low and high-temperature treatments did not impact and significantly differ in hatchability [52, 53]. Furthermore, continuous TM was used in most comparative studies [32, 33, 36, 39] compared with intermittent [2, 52, 53].

The main RH used in control and low-temperature TM studies was 56% and, 65% in high-temperature TM studies. In **Table 1**, we only reported RH of four studies (four papers, 14%) that used the different RH protocol from the above.

The embryonic age at the time of TM varied between E7 and E18 in most studies (24 papers, 77%), followed by E18–E20 and E0–E8 (three papers each) and one study by Morita et al. [39] reported the timing of TM from E13 to E21, which resulted in no significant influence on hatch weight but higher hatchability in high-temperature TM than low-temperature treatments.

Only seven studies compared embryo age at the timing of TM; hatchability and hatch weight were not affected by high-temperature TM at E0–E5 and E5–E18 [26], and E12–E14 and E15–E17 [34]. Also, Al-Rukibat et al. [40] showed no effect on hatchability after TM at 38.5 or 40°C for 6 h at E16, 9 h at E17 and 12 h at E18. However, TM at 39°C for 18 h/d at early embryonic age (E7–E11) significantly enhanced hatchability but in late (E11–E18) and long-term (E7–E18) negatively affected hatchability and chick quality compared with control (37.8°C) [46]. Janisch et al. [32] reported significant increase in hatch weight with low-temperature TM between (E7–E10) and (E10–E13) and Krischek et al. [33] found a significant decrease in embryo weights at low-temperature TM at E7–E10 and E10–E13 compared with high-temperature and control treatments (37.8°C). Narinç et al. [38] identified

significantly depressed hatchability and chick quality in late (E10–E18) TM compared with early (E0–E8) and control (37.8°C) treatments however, all treatments were not statistically different in hatch weight.

4. Discussion

This present review used a methodological approach to conduct a comprehensive literature search, which enabled a logical interpretation of the recent results obtained from broiler chicken incubation published studies. Thus, the effects of thermal manipulation on incubation performance, hatchability and hatching quality of broiler chicks could be examined.

4.1 Thermal manipulation and thermotolerance acquistion

The hatchery industry is expected to change dramatically with increasing demand for quality chicks and production efficacy. It is well established that incubation conditions significantly influence incubation and post-hatch performance besides, hatching quality in chickens [4]. During perinatal stage (critical period), incubation conditions may result in persistent variations in the epigenetic programming of different body systems and their roles in chickens [56]. One condition of interest is incubation temperature, which when manipulated by short or long-term may induce epigenetic adaptation thus enhancing development and maturation of particular body systems and their functions, which begins during the early periods of embryonic development [10].

TM is well known for inducing improved thermotolerance acquisition (thermoregulatory functions) in chickens, which is evidenced by reduced body temperature at hatch and during the first days post-hatch. Intermittent manipulation in incubation temperature between different embryonic ages resulted in thermoregulatory functions being boosted; 3 h of 39.5°C/d from E16 to E18 [21], 39.5°C for 12 h/d at E7–E16 [15, 16, 24], and 60 minutes exposure to 15°C at E18–E19 [57]. In our review, the similar effect was confirmed by [49, 50] at 38.5–39.5°C for 18 h/d from E10 to E18 and, David et al. [18] at 39.5°C for 18 h/d from E7 to E16. It's clear that thermotolerance acquisition in broiler chickens can be enhanced by application of TM between E7 to E18, a period that is termed the critical stage and, the ideal embryonic age for TM.

It is scientifically proven that successful TM should be between E7 and E18, a period which enables efficient alternation in threshold stimulus of the regulatory systems during the development and maturing of the thermoregulatory mechanism (hypothalamus-hypophysis-thyroid axis) and the stress control (hypothalamus-hypophysis-adrenal axis) [9, 11, 12, 58]. It is clearly reported that thermotolerance acquisition is improved via reduced plasma triiodothyronine (T_3) concentrations and basal metabolism, accompanied with lowered body temperature [15, 16, 24]. In addition, T_3 is the thyroid hormone of interest in the last week of incubation because it is vital for increasing extra energy requirements during hatching [14].

However, some recent studies have reported that TM increased [30, 51] and had no influence on hatch body temperature [48, 52]. These differences may be associated with the possible elevation or similarity in hormones that regulate metabolism (T_3) and growth (GH) leading to elevated or similar metabolic rate and heat production, accompanied with elevated and similar body temperature in thermal manipulated chickens and both thermal manipulated and control treatments, respectively [36, 39].

4.2 Thermal manipulation and embryo, hatch, or chick weight

Short-term (intermittent) alteration in incubation temperature during varying age of embryogenesis can boost muscle growth and development at hatch and in the first weeks post-hatch (early period (E0–E5) [22]; mid-term (E16–E18) [59]; long-term (E12–E18 and E10–E21) [60]). In the current review, the similar effect was identified with short and long-term TM (36.8°C for 24 h/d from E7 to E13 [32]; 39°C for 9–18 h/d from E10 to E18 [37, 50]; 36.°C for 6 h/d from E10 to E5 [2]), which was indicated by increased embryo, hatch and 1-day-old chick weight. In addition, it is evidenced that TM has significant effect on proliferation and differentiation of satellite cells, and thus growth and development of embryonic and chick muscles [61].

TM at 39.5°C for 12 or 24 h/d from E7 to E16 result in accelerated myoblast proliferation and cell differentiation, which is evidenced by increased myoblast number (25–48%) in the pectoral muscle and increased expression of myogenin in embryonic muscles, respectively [17]. Similarly, Al-Zghoul et al. [37] and Al-Zghoul and El-Bahr [44] found upregulation of MyoD, myogenin, insulin-like growth factor 1 (IGF-1), and growth hormone (GH) after TM at 38.5–39°C for 9–18 h from E12 to E18 in embryos and 1-d-old chicks. Furthermore, a linear increase in embryo breast muscle weight with embryonic age was observed but significantly elevated in the TM-treated embryos compared with controls during the second quarter of embryogenesis. The interpretation of the above findings explains the possible reasons for elevated embryo, hatch and 1-day-old chick weight after TM. However, the ability of myoblasts to proliferate declined in the embryos after TM compared with embryos incubated at 37.8°C in the last quarter of incubation [17]. Furthermore, Piestun et al. [59] reported increased muscle hypertrophy in thermal manipulated embryos at 39.5°C for 3 or 6 h/d from E16 to E18. This was evidenced by upregulation of myogenin, and IGF-1 mRNA expressions in TM embryos compared with control treatment.

Studies by Zaboli et al. [41], Al-Zghoul et al. [45], and Dalab and Ali [46] reported depressed embryo, hatch, or chick weight due to intermittent high-temperature TM, which partially agrees with Piestun et al. [14]. However, Piestun et al. [14] reported that only continuous (24 h) elevation in incubation temperature (39.5°C) from E7 to E16 negatively affected embryo growth and development and hatch weight. Although the above variation could have resulted from differences in factors such as breed or strain, flock age, incubation layout, and embryo age at the time of TM, the contradicting results due to TM length may suggest a strong gap for continuous studies on the length of TM.

In the current review, TM did not influence embryo, hatch, or chick weight in 67% of the intermittent TM studies that reported the above parameter. This result has been attributed to a possible similarity in plasma T3, T4, and GH leading to similar metabolic growth rate and heat production, which result in incubation duration and chick body weight being similar in both thermal manipulated and control treatments [36, 39].

Interestingly, Janisch et al. [32] and Rocha et al. [2] observed increased hatch weight at low-temperature TM compared with high-temperatures. This result may be associated with the variations based on factors such embryo age at TM, strain, and incubation temperature profile. However, it is well established that yolk weight is a critical factor that accounts for 20% of hatch weight [62]. At low incubation temperatures, nutrient metabolic rate, and the embryo's ability to draw liquids from the yolk sac are reduced, which result in increased yolk weights at hatch [63], and consequently, elevated hatch weight.

4.3 Thermal manipulation and hatchability

The effect of TM on hatchability in the present review is contradictive, with 65% of 20 studies that reported hatchability found no significant effect, 30% being reduced, and a comparative study by Dalab and Ali [46] reported increased and decreased hatchability with intermittent TM at different embryonic age. Also, earlier studies regarding TM and hatchability have shown contradicting results, for instance, Yahav et al. [11] and Piestun et al. [22] reported significantly increased hatchability with TM at 39.5°C for 3 h/d from E8 to E10 and 38.1°C for 24 h/d from E0 to E5, respectively. Yahav et al. [29] identified no effect on hatchability at 38.5°C for 3 h/d from E8 to E10. Piestun et al. [24] found decreased hatchability with TM at 39.5°C for 12 h/d from E7 to E16.

It is well documented that hatchability is depressed by overheating embryos however, length, strength, and embryo age at the time of high-temperature TM determine the effects of the application on hatchability [25]. Reduced hatchability has been associated with reduction in corticosterone concentrations at internal pipping after TM at 39°C for 2 h/d from E13 to E17 [64]. Continuous TM at 39.5°C from E7 to E16 depressed embryonic growth and development, which was accompanied by lower hatchability compared with intermittent and control treatments [14]. Low hatchability was associated with reduced development of pipping muscle (musculus complexus) on E18 and E19 day, which muscle is stated to have a significant role during hatching [14].

Meanwhile, embryo mortality rate and incubation duration or hatching time have been associated with hatchability. Brannan et al. [54], for instance, revealed increased embryonic mortalities (mid and late) after TM, which periods of development overlap with the plateau in eggshell temperature during TM at 39.5°C from E7 to E16, consequently, reduced hatchability. In addition, the above authors stated that fluctuating effect of TM on hatchability is associated with harmful levels of incubator temperature on embryo development besides, flock age, genotype, incubation design, etc. Almeida et al. [36] reported longer incubation period at low-temperature TM, which was followed by reduced hatchability compared with standard incubation and high TM.

Furthermore, reduced hatchability is linked to decreased chick quality, which is a well-known indicator for incubation challenges and investigated for assessment of incubation conditions [65]. While Elmehdawi et al. [35] identified no negative effect of high-temperature (38.4°C) TM from E18 to E20 on hatchability and chick quality, Dalab and Ali [46] observed lower hatchability and chick quality after TM at 39°C for 18/h from E15 E7 to E18. Similar to hatchability, the effect of exposure of embryos to low or high temperatures on chick quality is thought to depend on length and level of TM besides the stage of embryo development at the timing of TM [25].

Tzschentke [10] reported that slight increase in incubation temperature is expected to yield no depressing effects of TM in the last stages of embryogenesis, a period in which the development of mechanisms that regulates temperature in peripheral and central nervous systems, besides other body systems and their roles are completed. This could be the possible reason for no significant effect of TM on hatchability in most studies in the current review.

4.4 Thermal manipulation and eggshell temperature

Studies by Morita et al. [39] and Amjadian and Shahir [48] identified that exposure of embryos to high temperatures increased eggshell temperature in comparison to standard incubation temperature. The eggshell temperature reflects embryo body temperature [66]. The air temperature and heat transfer between the egg and the

incubator affect embryo body temperature, however, the correlation between heat production by the embryo and heat loss by the incubator determine the embryo temperature [67, 68]. It is established that the rate of chicken embryo heat production is proportional to increase in embryo development thus embryo body temperature reflects embryo development [69]. This could explain the longer incubation duration for low-temperature TM compared with control and high-treatments observed by Almeida et al. [36] and Morita et al. [39]. Earlier, Willemsen et al. [70] found significantly higher eggshell temperature (41.1°C) in high-temperature (40.6°C) compared with 35.5°C in low-temperature (34.6°C) thermal manipulated embryos, which was significantly reduced in comparison to 38.3°C of control temperature (37.6°C) from E17 to E18. Similarly, Piestun et al. [24] found that eggshell temperature was higher in thermal manipulated eggs at 39.5°C compared with standard incubation (37.8°C), accompanied by elevated hatching process of 6 h earlier. However, between E19 and E21, the eggshell temperature decreased although both the thermal treated and untreated eggs were placed in the same hatcher. Delay in hatching period has been linked to depressed metabolism in embryos after exposure to lower incubation temperatures than the standard [25].

In the current review, 65% of studies used 37.8°C as the standard incubation temperature, which also acted as the control treatment. During TM, any elevation in incubation temperature (above 37.8°C), RH is adjusted to 65% to eliminate excessive water loss from the eggs [14]. In addition, setting RH at 60% from E0 to E21 was thought to reduce the influence of RH on embryogenesis and embryonic mortality [36].

5. Conclusions

Thermal manipulation is an important approach that has been deeply studied due to its role in alleviating the effects of heat stress on broiler chickens. The success of this application depends on duration and strength of exposure in addition to embryo age at the timing of TM. The ideal embryonic stage for TM is between E7 and E18, in which thermoregulatory roles are enhanced. While intermittent TM has no adverse effects on embryonic development, hatchability, and hatching quality of broiler chicks, continuous TM depresses the above parameters. High-temperature (39–39.5°C) TM accelerates hatching time, shortens the incubation period, but has no significant effect on embryoinc development, hatchability and chick quality compared to low TM and incubation at standard temperature (37.8°C). Interestingly, in some studies, TM below 37.8°C was shown to increase chick weight at hatch compared with TM above 37.8°C. Furthermore, there is need for more comparative studies between low and high-temperature TM and the duration of TM because on addition to the available studies being insufficient, their results are also controversial. Additionally, a meta-analysis to provide an insight into contradicting results of TM application is thought of as a sound option. Also, there is need to continue studies on TM to identify the exact duration and intensity of TM and embryonic age to obtain higher hatchability and improved chick quality.

Conflict of interest

The author declares no conflict of interest.

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Chapter 8

Use of Additives and Evaluation of the Quality of Broiler Meat

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Abstract

In the poultry industry, the demand for safe and quality meat in the market has increased considerably. The type of feed used and the management of poultry have a significant impact on the safety and quality characteristics of poultry meat. The use of additives that increase productivity and improve meat quality has generated much research. Nanoparticles, prebiotics, and probiotics have been used as growth promoters to increase and improve growth rate, performance, immunity, resistance to pathogens, as well as to improve meat quality. The type and level of these additives incorporated in the diets influence the animal's development and meat quality parameters. The aim of the study was to report the results of scientific research on the use of food additives used in broiler nutrition and their effect on meat quality.

Keywords: nanoparticles, prebiotics, probiotics, broilers, quality meat

1. Introduction

Broiler production is based on increasing meat quality, improving the characteristics of the chicken meat. Characteristics such as appearance, texture, juiciness, watery, firmness, tenderness, odor, and flavor of the meat are important for the consumer's judgment before and after purchasing a meat product. However, quantifiable meat properties, such as water-holding capacity (WHC), shear force, drip loss, cooking loss, pH, and shelf life, are indispensable for the processing of meat products with added value. Many research was carried out as an alternative to improve the quantity, quality, and homogeneity of farm animals and their products. The use of additives can contribute to improving animal performance and meat quality parameters. One of these alternatives is the supplementation of nanoparticles, probiotics, and prebiotics in the diet of broiler chickens.

In this review, general aspects of the use of nanoparticles, probiotics, and prebiotics in poultry feed are reported.

2. Nanoparticles

Nanomaterials are being used in agriculture, feed, and food [1]. Some are stable at high temperature and pressure [2] and can be easily assimilated into the digestive system [3]. The action mode of the nanoparticles depends mainly on many factors,

such as particle size, sizes smaller than 300 nm spread in the blood, but particles smaller than 100 nm reach tissues [4]. Thus, there is better interaction with other biologically active substances due to a larger surface area *in vivo* [5]. Other factors, such as the solubility of particles and fillers, are important. Nanoparticles can be administered by ingestion or inhalation and exercise their actions in different ways [6]. According to Gao and Matsui [7], nanoparticles have unprecedented properties, such as large specific surface area, high surface activity, many taut centers, and high catalytic efficiency. There are indications that nanoparticles and minerals can increase absorption [8].

Silver nanoparticles exhibit a strong antimicrobial effect [9]. On the other hand, the use of nano-minerals, such as nano-selenium, nano-chromium, or nano-zinc, can improve the parameters of animal production, their healthiness, and the quality of the products obtained from them, research has shown better effects in relation to the inorganic salts of these elements and chelates used on a large scale in the animal feed industry [10]. Considering this, in the livestock industry, research was conducted to improve the composition and quality of meat. Thus, the enrichment of feed with nanoparticles and their effects on meat properties were evaluated.

2.1 Growth performance

Selenium (Se) influences the physiological function and growth performance of animals and humans [11, 12]. Thus, it is necessary for various enzymes that are active in all cells. Dietary supplementation with selenium can increase growth performance in broilers [13, 14]. Studies show that the use of nano-selenium in supplementation improved weight gain and feed-conversion rate [15, 16], higher percentages of breast and drumstick, and a lower percentage of abdominal fat [15]. Some studies indicated that supplementation of 0.30 mg/kg of selenium improves growth performance [17–20]. According to Zhou and Wang [19] supplementation of diets with 0.30 mg/kg of nano-selenium with organic sources of selenium was effective in increasing growth performance and feed-conversion rates of broilers [16, 19]. Other studies showed no effect of nano-selenium supplementation in the diet in relation to body weight gain [21] and growth performance [22].

Zinc (Zn) is essential with widely variable functions in many important enzymatic processes of glucose, protein, and lipid metabolism and production and secretion of hormones [23]. It is nutritionally essential for the development and maintenance of growth performance in broilers [24]. The permitted level of Zn for poultry diets, as recommended by the National Research Council [25], is 40 mg/kg. However, high zinc content in the diet can lead to excess zinc in the feces, which causes environmental pollution [26], affects the balance of other trace elements in the body, and can reduce the stability of vitamins and other nutrients [27]. The substitution of the inorganic source of ZnO by nano-ZnO or combined nano-ZnO and Zn promoted the growth of broilers, increased the absorption of Zn and antioxidant status without negative influence on the distribution of selected minerals in broiler tissues [28]. Other studies have shown that nano-Zn supplementation improved weight gain and feed efficiency [29, 30], decreased cholesterol levels [31], decreased abdominal lipids [30] as well as improved the meat quality of broilers [30]. A concentration of 2.5 ppm of nano-ZnO can improve the performance of broilers [32]. Concentrations of 20 and 60 mg/kg of nano-ZnO can promote body weight gain [33].

Silver (Ag) has been considered antibacterial made by humans and can be used as an additive instead of antibiotics due to its antibacterial properties and adaptability

to biological systems [34]. Nanosilver was destructive in the influence on pathogenic intestinal microorganisms and induced better nutrient absorption, improvement in feed intake, weight gain, and feed efficiency of broilers [35]. However, the study conducted by Ahmadi [36] showed that when Ag-NPs were introduced in diets there was no improvement compared to control treatment, performance, body weight, feed intake, feed-conversion rate, and feed efficiency of broilers during a 42-day experimental period. This effect may be a result of Ag-NPs could affect organisms in the intestine (intestinal microflora). Nanosilver is an effective elimination agent against a broad spectrum of Gram-negative and Gram-positive bacteria [37], including antibiotic-resistant strains [38, 39].

Manganese (Mn) have an important role in bone development, normal nutrient metabolism, and biochemical processes, such as pyruvate carboxylase, superoxide dismutase, and glycolyltransferase [40, 41]. Levels of 100–400 mg/kg supplemental manganese sulfate (MnSO4) decreased abdominal fat deposition [42, 43] and the level of malondialdehyde (MDA) in the broiler muscle, reducing lipoprotein lipase activity and increasing the activity of superoxide dismutase containing Mn (MnSOD) [42]. According to Brooks et al. [44] supplementation of Mn 20–500 mg/kg in diets for broilers did not affect BWG (weight gain) or FI (feed intake). Several other studies found no effects of dietary levels of Mn on growth performance [40, 42, 45–48].

Chromium (Cr) is important for physiological and nutritional activity [49]. It has potent hypocholesterolemic and antioxidant properties. It helps in the metabolism of fats, carbohydrates, and protein in animals; manifests itself in reducing the amount of glucose and cholesterol in the blood; helps in reducing fat deposits; and stimulates the formation of muscle tissue [50]. Kumari et al. [51] reported higher weight gain, the feed-conversion efficiency in the diet can produce lean meat with decreased muscle cholesterol and fat percentage for dietary supplementation with nano-Cr (400–1600 ppb). In the study conducted with chromium and nano-chromium supplementation and under thermal stress the results showed better performance, including weight gain and feed-conversion rate of broilers [52].

2.2 Meat quality parameter

The pH influences the quality of meat that reflects the change in acidity in the fermentation process of muscle tissue and speed of glycogen fermentation after slaughter, the stable pH value is conducive to normal maturation of muscles [53]. After slaughter, a rapid decrease in pH in the muscle results in the denaturation of the myofibrillar protein with the decrease in protein solubility, obtaining a poor WHC and greater drip loss [54], decreased juiciness, and intense muscle coloration [55].

The increase in the pH value observed after selenium supplementation indicates a delay in the metabolic conversion of glucose into lactic acid in the postmortem muscle [18]. The breast muscles of chickens that received nano-Se supplementation showed higher pH values after 45 min of 6.17 and after 24 h pH value 5.85 [18]. Similar results were reported for chicken breast meat with a Met-Se diet [56]. Mohammadi et al. [16] used dietary sources of Se and REO (rosemary essential oil) and did not observe effect at pH 4 h after slaughter.

Studies by Liu et al. [24] showed that Zn supplementation increases the pH value (5.88–6.06) after 24 h in the thigh muscle independent of the Zn source. A similar study was conducted by El-Hack et al. [30] who observed an increase in the pH value from 5.5 to 6.0 when supplemented with nano-ZnNPs. However, pH values of

6.15–6.25 were observed in chicken meat when supplemented to diet with nano-Zn (ZONPs - 10–50 ppm zinc oxide) [57]. Already lower pH values of 5.63–5.69 for chicken meat using nano-ZnO supplementation (2.5–40 ppm) were reported by Hussan et al. [32]. Supplemental Zn significantly increased pH values in broiler muscle [24, 57]. In agreement, Selim et al. [58] reported that chickens fed ZONPs reduced the pH of the breast muscle and thigh by 6.8%. ZONPs at 40 ppm reduced color and overall acceptability compared to control. According to Selim et al. [58], the use of ZONPs at 40 or 80 ppm did not affect the sensory evaluation of chicken meat, including texture, aroma, color, and general acceptability.

Shokri et al. [48] reported higher pH values for broilers fed diets supplemented with nano-Mn when compared with control, pH values 6.41–6.44 for breast and pH values of 6.50–6.83 for thigh after slaughter. After 4 h of slaughter, pH values of 6.15–6.24 for breast meat and thigh pH values of 5.99–6.20 were observed. Lu et al. [42] reported that the added Mn content had no effect on water-retention capacity and pH values in the thigh muscles and intramuscular fat in the chest and thigh muscles.

In a study conducted by Hashemi et al. [59], there were no differences in pH values after slaughter between control poultry and poultry fed nano-Ag, pH values from 5.38 to 5.78 for breast muscle were observed.

According to Sams and Mills [60], the normal pH values at the end of the post-mortem process are between 5.60–5.80 and 5.78–5.86, respectively. However, according to Soeparno [61], normal pH values would be in the range of 5.3–6.5. The high muscle pH makes the meat more susceptible to bacterial deterioration, while the low muscle pH increases the shelf life of chicken meat [62].

Some evidence indicates positive correlations between WHC and pH and a negative correlation between WHC and humidity [55]. In fact, Young et al. [63] explained that there is no good relative correlation between pH and water-retention capacity, and the lower overall final pH did not result in an overall decrease in water-retention capacity.

According to Huff-Lonergan and Lonergan [64], meat oxidation could decrease sensitivity to hydrolysis, weaken protein degradation, and reduce water reserves among myofibrils, which would increase meat juice loss by influencing softness and water-retention capacity.

Selenium is an essential trace element that positively regulates the antioxidant defense mechanism and is vital for the body's intra- and extracellular antioxidant systems [65]. Research shows improvement in antioxidant properties [18, 19, 65]. For levels from 0.15 to 0.3 ppm using different sources of if there was an improvement in oxidation levels [20].

MDA is one of the final products of the peroxidation of polyunsaturated fatty acids in cells and is a marker of oxidative stress [66]. Concentrations of 0.3–0.5 mg/kg of nano-Se in diet supplementation were effective to improve oxidation resistance by reporting lower MDA concentration in broiler samples fed diets supplemented with nano-Zn [18, 65]. The storage under cooling of the chest and thigh muscles is supplemented with Se observed a decrease in MDA concentration [16]. In addition, El-Deep et al. [17] reported a reduction in lipid peroxidation (MDA content) in broilers under high ambient temperature.

Changes in carcass characteristics may be due to increased tissue zinc residue, the effect of zinc on the antioxidant status and the oxidative enzyme, and especially the antioxidant function and water-holding capacity of muscle [24, 33, 58]. In the study by El-Hack et al. [30] the activity of liver enzymes and malondialdehyde (MDA)

decreased in the groups treated with nano-Zn (ZnNPs). Supplementation of broilers with 25 and 50 mg/kg of nano-Zn showed lower TBA values [67].

Hashemi et al. [59] observed an increase in MDA levels with an increase in nano-Ag supplementation levels. Protein oxidation can lead to the production of intermolecular bonds, including disulfide, dityrosine, and other intermolecular bridges to form the aggregation and polymerization of proteins [68].

Jankowski et al. [69] indicated that the antioxidant system worked properly when Mn was added in the form of nanoparticles, which can be attributed to the increase in the activity of Mn-SOD, GPx, and CAT. Lu et al. [70] reported that broilers fed diets supplemented with Mn presented low concentrations of MDA in the thigh muscle. Similar results were obtained when nano-Mn was added to the diets causing a reduction in the concentration of MDA in the thigh muscle in storage under refrigeration [48]. In contrast, Bozkurt et al. [71] reported that MDA concentrations increased in broilers fed diets supplemented with Mn. Already Bulbul et al. [72] reported that organic and inorganic sources of Zn and Mn decreased oxidative stress in laying hens.

According to Ognik et al. [73], a dose of 10 mg/kg in the form of NP-Mn_2O_3 induced large-scale lipid oxidation reactions. The reduction of Mn content, regardless of the form used, is disadvantageous, since it weakens the defense of the antioxidant system, which can induce oxidative processes in cells. In addition, the increase in dietary levels of Mn from 0 to 200 mg/kg in the diet regardless of the source showed lower values of TBARS [42, 43, 45, 70]. According to Kim et al. [43], high levels of Mn in the diet can be considered to improve carcass quality, preferably from the nano-Mn source because it presents higher bioavailability of Mn.

The oxidation of lipids in the breast muscle is a representative factor that reduces the quality of meat [74], and Mn is indicated as a necessary element for the production of SOD (superoxide dismutase) to increase antioxidant capacity and improve meat quality in chickens [41]. Oxidative changes in intramuscular lipids and products were determined based on TBARS [75, 76]. Thus, it has been demonstrated that the use of Mn in diets in organic (manganese methionine) or inorganic (MnO) forms increases MDA, glutathione peroxidase, and nitrogen oxide in chicken meat exposed to high-density stocking stress [71].

Studies report that Mn significantly reduced MDA levels in broilers [42, 70] and turkeys [77]. This may be due to the change in the activity of MnSOD (superoxide dismutase) in the mitochondria of muscle cells because MnSOD plays an important role in delayed lipid peroxidation of the cell membrane. However, there is an increase in the effect of MDA concentration with high Mn in the diet in the longissimus thoracis of pigs [78].

The activity of GSH-Px (glutathione peroxidase) affecting the oxidation state of myofibrillar protein could affect drip loss [79]. Studies report lower drip losses in breast meat of chickens who were fed nano-Se [19, 65] and organic Se [21, 80].

The pH drop retard leads to reduced protein denaturation, and consequently, to reduced drip and cooking loss [81], thus improving the water-retention capacity of meat. The use of nano-Se showed a decrease in drip loss [18].

Chicken meat with low pH has been associated with low WHC, which results in loss of cooking and drip loss. The lower pH decreases the ability of muscle proteins to bind to water, causing the shrinkage of myofibrils [82].

Some studies with nano-Se supplementation have observed an increase in meat water-retention capacity [83, 84]. In contrast, Mohammadi et al. [16] in the study reported that using dietary sources of Se and REO (rosemary essential oil) had no effect on water retention capacity (WHC) in the thigh and breast muscles in broiler.

Meat color and drip loss are important indices for assessing meat quality and are closely related to the oxidation state in muscles. The color of the meat is determined by the oxidation state of myoglobin [24]. The use of supplementation with if improves antioxidant capacity and thus could increase the content of myoglobin, thus improving the color of meat [83, 85]. In addition, when phospholipids in cell membranes are oxidized, changes in cellular permeability occur, leading to decreased water-retention capacity of the muscle.

Drip loss is commonly used as an indicator of the water-retention capacity (WHC) of meat. The lower drip loss reflected the higher content of water-soluble nutrients and the increase in meat juiciness [86]. Lower drip losses of the breast muscle were observed when supplemented with nano-Zn (ZONPs 25–50 mg/kg ZnO) [57]. On the other hand, Liu et al. [24] and Selim et al. [58] reported that additional nano ZONPs decreased drip loss in broilers. Similarly, Saenmahayak et al. [87] reported that drip loss increased significantly in the muscles of broilers fed zinc supplemented diets.

The decrease in drip loss in the breast muscle [78] can be attributed to a stable pH value [88].

Regarding water-retention capacity (WHC) no difference was observed in supplementation with nano-Zn [32, 87]. In contrast to our results, Yang et al. [89] recorded an increase in breast muscle WHC with the addition of inorganic zinc in broilers.

The physical and chemical properties of proteins, including solubility, hydrophobicity, WHC, and even nutritional value can be modified by protein oxidation [90]. In postmortem muscle, protein oxidation has been gradually recognized as an important factor for meat quality. During postmortem storage, the muscle has a decreased ability to maintain its antioxidant defense system, and this can cause an increased accumulation of reactive oxygen and nitrogen species [91]. Improved antioxidant status can promote the maintenance of cell membrane integrity [65], which can be explained by the results of water-retention capacity. According to Hashemi et al. [59], there was no significant difference for breast in WHC value, while thigh supplementation with nano-Ag resulted in higher levels of WHC, which may be due to the low level of protein oxidation.

The quantifiable properties of meat are indispensable for processors involved in the manufacture of meat products, such as water-retention capacity (WHC), shear force, drip loss, cooking loss, pH, shelf life, protein solubility, and fat-binding capacity [92].

Muscle pH had a significant positive correlation with water-retention capacity (WHC), and WHC had a significant correlation with an a* value [55].

In addition, thighs with nano ZnNPs lower loss by cooking [67].

The color of meat is an indicator of quality, which represents its freshness for consumers [93]. Some studies did not report differences in the color of the breast muscles for supplementation with nano-Se compared to the control [18, 56, 83]. Meanwhile, Boiago et al. [94] observed a decrease in L* values of breast muscles for broilers fed diets supplemented with Met-Se Se, which may be related to a reduction in moisture on the meat surface because of increased water-retention capacity [95].

El-Hack et al. [30] reported lower L* values for breast meat from chickens treated with dietary supplements of nano-Zn ZnNPs and did not observe differences for a* values. For thigh meat, the different treatment groups with nano ZnNPs did not affect the values of L* and a* [67]. However, for the value of b* there was an increase for the thigh muscle [24, 67] and the breast muscle [24, 30, 89].

According to Hashemi et al. [59] in the treatments of zeolite, nano-silver (50 and 75 ppm) there was no significant difference for the color parameters parameter L*, a*, and b* in the breast muscle.

Lipid oxidation can promote the accumulation of metmyoglobin, such as brown pigments, in meat [94, 96]. The increase in the yellowing of meat may be due to the increase in the formation of oxymyoglobin [97]. In addition, lipid oxidation is associated with the destruction of meat pigments, such as carotenoids [98]. Some researchers have also demonstrated that there is a significant negative correlation between the color values of clarity of breast meat and the pH of breast meat [62]. Color is the most important characteristic for the appearance of meat [99], which is influenced by sex, genotype, and breed; moreover, it relates to the pH value [100].

Softness, described as shear force, is an important indicator of consumer acceptability and is determined by the structural properties of various proteins and fats in muscle [101]. Nano-Se supplementation showed a decrease in the value of the shear force of the breast muscles and lower cooking loss [18].

Baowei et al. [85] reported that SS supplementation in the 0.3 g/kg diet reduced the hardness of the goose's breast muscles. Results indicate that the supplementation of broiler feed with organic Se or nano-Se leads to improvement of meat quality, in relation to the addition of inorganic Se [102].

Many studies have shown that IMP (inosine 5′-monophosphate) contributes to the sensory perception of meat [101]. A higher IMP content was observed in chickens supplemented with nano-Se may be associated with a better quality of Guangxi Yellow chicken meat [19].

Zn supplementation increased the content of intramuscular breast muscle fat in broilers independent of the source of Zn [24]. Hodgson et al. [103] observed that higher levels of intramuscular fat caused a significant decrease in shear force. Liu et al. [24] showed that Zn supplementation decreased the shear force of the thigh muscle and breast muscles, regardless of the source of Zn.

Texture parameters such as succulence, softness, and flavor obtained lower values when using a diet with nano ZnNPs [30].

Regarding the texture profile of broiler breast meat for hardness, cohesiveness, gumminess, and chewiness were influenced by the treatment with nano Ag (NZ75 higher values), while adhesiveness and springiness were not influenced [59]. For the thigh muscle texture profile of broilers, hardness, adhesiveness, and cohesiveness were not influenced, however there was a difference in springiness for supplementation with nano Ag [59]. Results may be related to water-retention capacity (WHC), a quality parameter related to the meat softness process, which is an important parameter in the sensory evaluation of meat [104].

Yang et al. [77] reported the use of Mn in the duck diet, they observed a significant increase in intramuscular fat and decreased shear strength, showing similar results in studies conducted by Yang et al. [89] in broilers.

Meat softness is a factor used to evaluate the acceptability of the consumer of cooked meat [99] and is generally associated with the content of MIF and muscle fiber structure [105]. Shear force is a reliable indicator that inversely represents the softness of the meat.

3. Probiotic, prebiotic, and simbiotic

Probiotics are considered live microbial supplements that beneficially influence the host by improving intestinal microbial balance [106], stimulating metabolism, reducing the risk of infection by opportunistic pathogens [107], tend to improve levels of body antioxidants, which can improve the health of broilers [108]. The

study has shown that dietary probiotic supplementation increases growth rate, feed efficiency, and immunity in chickens [109], improve chicken meat quality, such as WHC, tenderness, and oxidative stability [110], increase weight gain and feed-conversion ratio, improve antioxidant capacity in organs and muscle tissue in heat-stressed chickens [111]. Probiotics used in animal nutrition include groups of bacteria, yeasts, and fungi, such as *Lactobacillus acidophilus, L. lactis, L. plantarum, L. bulgaricus, Lactobacillus casei, L. helveticus, Lactobacillus salivarius, Bifido bacterium* spp., *Saccharomyces cerevisiae*, and *S. boulardii* [112–114].

The prebiotic is a nondigestible feed ingredient that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host [115]. The prebiotics are used as substrates for survival and multiplication of probiotics in a lower gut region that act as symbiotics [116]. Some prebiotics are composed of diverse sugar units. Therefore, each prebiotic may influence the animals differently [117]. Prebiotics such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and mannan oligosaccharides (MOS) are considered preventive agents, as they can select a gastrointestinal microbiota that not only benefits the host but can serve as a barrier to the colonization of pathogens [118]. Besides, feed additives, such as probiotics, prebiotics, and symbiotics have been proposed as a nutritional strategy to improve the resilience of animals against heat stress [119].

3.1 Growth performance

Probiotics have a beneficial effect on the host animal, improving its intestinal microbial balance [120]. This creates a healthy intestinal environment with increasing counts of healthy bacteria and suppresses intestinal pathogens, thereby improving digestion and nutrient utilization [121]. According to Al-Shawi et al. [122], the animal not only requires an optimal amount of food but also must improve the digestibility of the food to maximize growth. Some studies have reported lower feed intake in broilers fed with probiotics [123–126]. Thus, increased nutrient absorption in broilers produces lower feed intake to maintain their nutrition needs [127]. Amerah et al. [123] reported that the inclusion of *B. subtilis* in the feed improved the feed-conversion ratio by reducing feed intake.

The inclusion of *Bacillus* species as a dietary supplement increased performance [126, 128] and reduced mortality [126, 129]. A similar result has been reported when using *S. cerevisiae* as a supplement [124]. Probiotics can improve the performance of chickens by improving the immune response [130]. However, Amerah et al. [123] found no beneficial effect on body weight gain (BWG) in broilers with the inclusion of *B. subtilis* in the diet. Other studies have reported no effect or minimal effect of probiotics on the growth performance of broilers [131, 132].

Bai et al. [133] evaluated the feeding of broilers with *B. subtilis* in the diet and reported higher average daily gain (ADG) and lower feed-conversion ratio (FCR). On the other hand, broilers with the inclusion of *S. cerevisiae* in the diet improved the weight gain and the feed-conversion ratio (FCR) [125, 134–136]. Studies have shown that feeding with a dose >1.0% of *S. cerevisiae* diets produces higher body weight, low feed-conversion ratio compared to chickens fed a low dose of yeast [125, 137]. In contrast, in other studies, body weight gain and feed-conversion ratio (FCR) were not influenced by supplementation with yeast in the basal diet [138–140].

According to Patel et al. [141], the effectiveness of probiotics is influenced by the selection of the most efficient strains, manipulation of genes, combination of several

strains and the combination of probiotics, and synergistically by the action of the components. However, the use of multiple strains may improve the effectiveness of probiotics as they beneficially affect the host by enhancing growth-promoting bacteria with competitive antagonism against pathogenic bacteria in the gastrointestinal tract [142].

In the broilers fed with multi-strain probiotics, such as *L. acidophilus*, *B. subtilis*, *S. cerevisiae*, *Enterococcus faecium* [113], *A. oryzae* [124], *L. casei*, *Bifidobacterium thermophilum*, *Enterococous faecium* [121], and *Clostridium butyricum* [114], body weight gain, better feed-conversion rate (FCR) [113, 121, 124], and a lower percentage of abdominal fat was observed [124]. In quail, the highest final body weight (BW) values were recorded in groups T5 (probiotic bacteria—*B. toyonensis*) and T10 (probiotic bacteria—*B. toyonensis* and *Bifidobacterium bifidum*). Thus, in the termination period, the use of a higher level of probiotics (T5) reported an increase in body weight gain (WG) [143].

Therefore, the effectiveness of the application of probiotics varies depending on several factors, such as probiotic strains, dosage of administration, method of administration, diet composition, age and breed of birds, and management conditions [114, 131, 132, 136, 144].

The positive prebiotic effect on growth performance can be due to the ability of prebiotics to enhance lactobacilli and bifidobacteria populations, and these beneficial bacteria compete with harmful bacteria for colonization [145].

Prebiotic diet reported higher carcass weight, carcass yield, and breast muscle weight [146, 147], and an increase in body weight gain [148].

The study observed a significant effect of diet on feed conversion, and control birds showed poor feed conversion. The rearing system also affected weight gain and feed intake, so confined birds had better weight gain and feed intake [149]. Birds fed diets supplemented with probiotics and prebiotics showed greater body weight and weight gain, whereas feed intake was greater in control birds. Similar studies, diets with prebiotic treatment and probiotic alone, reported better responses for body weight gain and FCR compared to the use of symbiotic treatment [150]. Several other studies also showed that the addition of probiotics or prebiotics alone or in combinations as synbiotics in feeds had no effect on the feed intake of broiler chickens [151]. On the other hand, dietary synbiotic supplementation can increase the breast muscle weight of broilers in comparison with those fed the basal diet [152].

3.2 Meat quality

The physicochemical properties of meat are important and can determine its storage or further processing. They are interconnected and influence the sensory quality of meat. Thus, the use of probiotics can influence these parameters [153]. Meat quality is also a very important parameter for the effect of dietary treatment in broiler studies. The supplementation of probiotics in basal diets had beneficial effects on quality broiler meat [128, 133].

The decreased pH relates to the generation of lactic acid through the anaerobic pathway, and probably promotes the denaturation of myofibrillar proteins, and reduces the ability of these proteins to maintain water [154]. Wang et al. [155] observed the decline in pH, however, pH24h (6.01) was increased by *S. cerevisiae* supplementation in relation with the control (5.80). Similar results have reported the inclusion in the diet of *S. cerevisiae* [156] and mixture *Pediococcus acidilactici* and *S. cerevisiae* [157]. Other studies showed that yeast products or culture improved the

meat quality of broilers and pigs by decreasing the yellowness and stabilizing the pH of meat [102, 158, 159]. However, the study of fresh quail meat observed an increased pH value with the probiotic treatment in the diet of groups T4 (*B. toyonensis*-BT) for T6 (*B. bifidum*; 6.71–6.83) and decreased for T2 (lowest level of BT, pH 6.02) in comparison with the control (6.31) and the remaining groups (6.33–6.43) [143].

On the other hand, Cramer et al. [160] observed that *B. subtilis* supplementation and heat stress did not influence the extent of pH decline up to 4 h postmortem, so the pH of breast muscles from control or probiotic slightly decreased. Yet, the probiotic mixture (*E. faecium*, *P. acidilactici*, *Bifidobacterium animalis*, and *Lactobacillus reuteri*) feeding levels and cyclic heat exposure for 6 h postmortem did not influence the extent of pH decline up to, so the pH of breast muscles from control or probiotic slightly decreased [161]. Similarly, the study reported that heat stress does not affect the initial temperature decline (from 15 min to 24 h postmortem) of breast muscles at commercial slaughter conditions [162]. Cramer et al. [160] observed a significant interaction on ultimate pH at 24 h postmortem, increased ultimate pH values 5.90 and 5.95 by thermoneutral and heat stress, respectively, of broiler breast (*B. subtilis* supplementation). Similar results were observed by Aksit et al. [163], Zhang et al. [144], and Kim et al. [161]. Heat stress can lower the initial and final pH of chicken breast muscles, heat stress normally increased lactate accumulation and the rate of postmortem glycolysis due to the high activity of glycolytic enzymes, such as pyruvate kinase and lactic dehydrogenase [144, 164]. According to Cramer et al. [160], probiotic supplementation could alleviate the pH decline of breast muscles from heat-stressed broiler by likely affecting the rate of postmortem glycolysis metabolism. Some studies reported that microbial probiotic supplementation could increase the ultimate pH of broiler breast muscle [156, 165, 166]. Already Aksu et al. [156] observed an increase in pH values 6.24–6.31 of chicken breast muscle when used 0.2% *S. cerevisiae* in the diet. In another study, Zheng et al. [166] included *E. faecium* supplement in feed chickens and found that breast muscle had a higher ultimate pH value (6.11) than that from control (5.77). Hence, the high ultimate pH of breast muscle might be related to the downregulating effect of probiotic supplementation on glycolytic enzymes that could alleviate an increase in glycolytic metabolism induced by high ambient temperature [161, 166]. Nonetheless, some studies have reported no effect on pH values on broiler beast meat when using *B. subtilis* (6.67–7.03) [133] and *S. cerevisiae* (5.6–6.16) [167].

In cooking loss in broiler no difference was reported, regardless of probiotic feeding levels [165, 168, 169]. Also, the drip, cooking, and thaw losses on breast muscle were not observed by Benamirouche et al. [157]. However, some studies observed that heat stress increases drip and cooking loss [79, 144] of the breast meat [167]. In contrast, lower cooking loss was observed in *S. cerevisiae* supplement diet [155], and lower drip and cooking losses of broiler breast muscles were observed when using the probiotic in broiler [133, 160]. An increase in drip loss in broiler muscle under heat stress has been attributed to protein denaturation or loss of protein functions due to a rapid decline in pH when carcass temperature is high [144, 164]. Broilers fed probiotic supplement diet showed higher breast meat WHC than broilers fed without probiotic [165, 167, 170]. A similar result was observed in quail meat [143]. In meat quality, water-holding capacity, including drip loss and cooking loss, are crucial because some nutrients could easily lose during exudation by water loss [171]. Zhang et al. [144] suggest that the decreased final pH could be associated with the poor quality characteristic of breast muscle from heat-stressed broilers, especially on color, WHC, and tenderness.

Meat tenderness can be estimated by measuring the shear force; lower shear force indicates tenderer meat and was one of the crucial sensory qualities that influenced the consumer [172]. The shear force in breast and drumstick meats decreased with *S. cerevisiae* treatments [144] and *B. coagulans* in breast meat [128]. Suggests that the dietary supplementation of *S. cerevisiae* could improve meat tenderness of broilers [128, 135, 165, 173, 174]. However, in other studies, no beneficial effects were observed [144, 175]. Heat stress and feeding broilers with probiotics no influence the shear force of the broiler breast muscles [160]. Greater tenacity was reported by Zhang et al. [144] in treatment with heat stress, while Lu et al. [176] found heat stress had no effect on the shear force of chicken breast meat. In addition, these findings indicate that probiotic mixture supplementation observed no influence on WHC and shear force of breast muscle from chickens exposed to cyclic heat [161]. The pH and water-holding capacity of the meat are important quality attributes; high pH broiler breast meat has higher water-retention capacity than lower pH meat, resulting in increased tenderness [177].

No interactions between probiotic feeding levels and display storage time on CIE L^* (lightness), CIE a^* (redness) breast muscles from chickens exposed to cyclic heat challenge were found, except for CIE b^* (yellowness) [160, 161]. The quail meat color a^*, b^*, and L^* values were decreased by probiotics treatment with all levels studied as compared to the control group [143]. Haščík et al. [178] observed an increase in a^* and b^* values of the thigh and an increase in the L^* values of breast and thigh cuts in birds fed probiotics alone or in combination with pollen. In contrast, Haščík et al. [179] reported that a^* values in breast muscle were increased, whereas the values of L^* and b^* for broiler breast meat were not altered because of *Lactobacillus fermentum* supplementation. The L^* values change has been previously correlated with low ultimate pH and poor WHC [55].

The MDA concentration shows the intensity of the lipid peroxidation rate in the body and indirectly shows the degree of damage by tissue peroxidation [155]. Some studies have reported an antioxidant effect of probiotic feeding on lipid oxidation, suggesting that the improved meat quality might be closely connected with its enhanced antioxidant capacity by yeast supplementation in broilers [110, 133, 135, 155, 156, 160, 169, 170, 180]. Thus, it can improve the quality parameters of broiler meat under heat stress. However, in a study by Kim et al. [161] no effects on lipid oxidation stability were observed.

Sensory evaluation test results for lightly cooked breast meat, there was an improvement in the odor of chicken meat fed with *S. cerevisiae* supplement. Other sensory attributes show no influence between treatments [167]. According to the previous studies, there was widespread agreement about sensory quality and intramuscular lipid content [181]. Apart from that, the result of Nakano [182] suggested that the fat in the meat was converted into the favorable fat in the presence of probiotics for preferable sensory qualities. In addition, supplementing probiotics in basal diets had beneficial effects on the meat quality of broilers [128, 133].

Broiler chickens subjected to heat stress can induce a lower final pH with variation in meat color, water-holding capacity (WHC), and meat tenderness [81, 144, 163], resulting in lower acceptability of the meat by the consumer. In this sense, poultry farming strives to mitigate the negative effects of heat stress on poultry production, to reduce economic losses. The main concerns about the use of these bioactives are their efficient administration under fully controlled conditions.

Heat stress not only impairs muscle growth and structure [160, 183], but also influences meat quality, decreasing the pH value, water-retention capacity and redness, and increasing skeletal muscle lightness in chickens [5, 184–186].

Some studies suggest that the glucose level in skeletal muscles may be influenced by heat stress, causing an accumulation of lactic acid in muscle tissue [144], and a rapid decline in pH with low pH values final [5, 144, 160]. Tavaniello et al. [147] and Maiorano et al. [187] no reported influenced pH 24 (5.76 and 5.87) fully fit within the pH range accepted for commercial poultry meat [178, 179]. Already lower values of pH 24 h postmortem were observed under heat stress conditions with and without the use of symbiotic compared to the control.

The redness a∗ value was reduced in breast meat from prebiotics treatment compared to control [147]. Yet, L∗ and b∗ values were similar between the experimental groups. Dietary symbiotic addition reduced L* value. However, it did not affect the b* value [152]. The a* values were lower for the thermoneutral symbiotic, but higher a* values were reported for heat stress and symbiotic. Other studies reported that heat stress can increase L* and reduce a* and b* of breast meat [119]. This could be due to the denaturation of sarcoplasmic proteins, which results in the scattering of light [144].

Less drip and cooking losses were observed, and there was no effect on breast muscle shear force in broilers fed symbiotic compared to those fed a basal diet [152]. However, sob heat stress increased drip loss and cooking loss and decreased shear force in broilers when compared with those under thermoneutral temperature [152]. Similar results were found in broiler exposed to heat stress, which observed decrease in the WHC [119].

Broilers exposed to heat stress reported higher MDA concentration but lower activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the breast muscle. Compared with broilers fed the basal diet, symbiotic supplementation decreased MDA content and increased GSH-Px activity of breast muscle in broilers [152].

4. Conclusions

Meat quality is influenced by several factors, such as food. The use of additives to improve meat characteristics was evaluated. It was shown that the type of additive, the quantity, and the method of application are important parameters for obtaining chicken meat with desirable characteristics for the consumer and for the industry in obtaining meat-derived products.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

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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The poultry industry has been continuously evolving in multiple research and management areas, including hatchery practices, disease control, genetic selection, intestinal physiology, and nutrition, among others. For instance, it has been observed that amino acid requirements tend to change based on the different environmental and immunological challenges that an animal is undergoing. In addition, a continuous improvement in feed efficiency and growth performance has also affected meat quality parameters. Additionally, research on the impact of hatchery settings and environmental conditions on embryonic development and performance parameters is crucial for an economically feasible production, considering the importance of animal welfare as well. This book provides a compilation of recent scientific information on different pivotal areas of the broiler industry.

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