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Research article

Microbiology, induction, and management practices to mitigate lameness caused by bacterial chondronecrosis with osteomyelitis in broiler chickens

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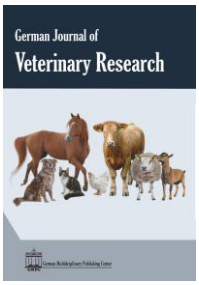

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Abstract

Bacterial chondronecrosis with osteomyelitis (BCO) lameness is the most prevalent skeletal disorder in poultry, implicated by the inherent risk of the high growth rate of modern broilers. The non-synchronization of massive body mass accretion vs maturation of the structural bone induces vulnerability to bone microfracture and immunosuppression, leading to bacterial necrosis in the skeletal and thoracic vertebral bones, leg paralysis, and eventual death. BCO lameness also imposes animal welfare issues and confers food safety risks. A study in 20 broiler farms in Australia recorded 28% BCO lesions of the necropsied birds, while a study in commercial flocks in Europe reported 19% moderate-to-severe lameness symptoms. The high incidence of lameness in commercial broiler farms reduces production value and leads to a huge economic loss in the poultry industry. Managing BCO lameness in broiler poultry is more challenging than that of other species because the peak of lameness incidence emerges at the marketing age of broilers. Moreover, curative treatment of the affected broilers is arduous due to their larger populations but shorter production cycles. Thus, condemnation of clinically lame birds has been a common practice in broiler farms to prevent the transmission of highly contagious BCO throughout the broiler populations. This practice decreases the production value and increases the economic forfeits. Therefore, presenting intervention measures for managing and controlling BCO in broiler poultry is necessary. Knowledge of etiological agents, pathogenesis, detection, and experimental lameness models to evaluate the efficacy of the intervention measures is highly important in presenting approaches to mitigate BCO lameness. Thus, this review focuses on microbiology studies of BCO, models of creating experimental lameness, and intervention measures mitigating BCO lameness in broiler chickens.

Keywords: Chondronecrosis, Osteomyelitis, Lameness, Broilers, Mitigation

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Introduction

The supply and demand for chicken meat have significantly increased worldwide. Global chicken meat production in 1961 was 7.56 million tons, and it is estimated to increase to 139.19 million tons in 2025 (Uzundumlu and Dilli, 2022). The poultry industry has successfully presented fast-growing broilers to meet the high demand for chicken meats through genetic selection of the traits of interest, such as growth rate, feed conversion rate, and breast quality. The growth rates of modern broiler chickens significantly differed from the chicken meat types in the 1970s. Modern broiler chickens can grow by approximately 300%, from 25g per day to 100g per day (Knowles et al., 2008; Zuidhof et al., 2014). The efficiency of the broiler's growth to gain high muscle mass has detrimentally impacted the locomotion system and led to animal well-being concerns (Knowles et al., 2008). The structural bone hardly sustains excessive body weight accretion, leading to several bone pathologies, such as deformity, weakening, infections, contusion, and osteoporosis (Rath et al., 1999). The rapid growth of modern broilers imposes an inherent risk of bacterial chondronecrosis with osteomyelitis

(BCO) lameness, the most predominant musculoskeletal disorder in broilers and mostly in a severe state (McNamee and Smyth, 2000). BCO lameness is characterized by poorly mineralized columns of cartilage in the proximal growth plates of the skeletal bones, causing osteochondrotic clefts, micro-fractures, and eventual chondronecrosis (McNamee et al., 1998; Riddell et al., 1983; Wideman, 2016; Wideman and Prisby, 2013). Insufficient blood supply to the growth plate or vascular congestion implicates the progression of severe chondronecrosis. Subsequent pathogen infiltration in the wound sites exacerbates the development of severe chondronecrosis (McNamee et al., 1998; Prisby et al., 2014; Wideman and Prisby, 2013; Ytrehus et al., 2007).

BCO lameness is the most common locomotion disorder in the US, Australia, Canada, and Europe. The prevalence of lameness in commercial broiler flocks in Bulgaria is up to 15%, causing up to 10% mortality (Dinev, 2009). A study estimated that 27% of broilers in the UK suffered skeletal problems in the pre-slaughter period, with 3.3% of them causing complete paralysis (Knowles et al., 2008). The latest data presented that 19% of birds in European commercial broilers demonstrated moderate-to-severe lameness signs (Granquist et al., 2019). In Australia, a study recorded 28% BCO lesions of the observed birds from 20 commercial broiler farms (Wijesurendra et al., 2017). Typically, BCO incidences in broilers are noticeable at approximately 4 to 5 weeks of age; when the growth plate experiences accelerated development at the same time, broiler density increases in the barn (McNamee and Smyth, 2000). BCO develops quickly in 2 to 5 days and can lead to death within 24 – 48 hours after reaching a severe paralysis state (Wideman, 2016). Condemnation of clinically lame birds at the marketing age has become a common practice in broiler farms to prevent BCO outbreaks (McNamee et al., 1998; McNamee and Smyth, 2000). A daily record in Western Canada presented the incidence of birds culled due to lameness ranging from 0.46 to 4.08% (Riddell and Springer, 1985). Reckoning the annual chicken meat production in the US to approximately 20 million metric tons, with a regular 4% loss. It is estimated that the annual production forfeit for the US poultry industry due to skeletal disorders is approximately 0.8 million metric tons (Chan et al., 2022). Overall, the disturbance of the bone and musculoskeletal systems in the rapidly growing broilers affects the performance, well-being, morbidity, and mortality of the birds (Choppa and Kim, 2023).

Thus, developing diagnostic and intervention strategies mitigating lameness disease in broiler poultry is urgently needed. Current diagnostic tools assessing the incidence of lameness in poultry are still limited. While the utilization of Infrared thermography (IRT) has been introduced by Weimer et al. (2019), this approach is beyond the scope of this review since most current lameness studies still emphasize necropsy and microbiological investigation. Findings acquired by such examinations are paramount to developing mitigation measures by employing experimental models to measure the efficacy of the treatments. Some studies have reviewed the etiology, pathology, clinical presentations, and intervention measures to reduce BCO lameness in broilers. Nevertheless, no literature provides the integral mapping of diagnostic tools determining the BCO cases and its causative agents and experimental models assessing the effectiveness of the developed intervention measures to reduce BCO. The current review focuses on microbiology studies, models of inducing experimental lameness, and several intervention strategies to prevent and mitigate leg disorders in the broiler poultry industry.

Microbiology studies of BCO

The genus *Staphylococcus* is frequently associated with bone infections, leading to numerous bone disorders, particularly BCO lameness (Szafraniec et al., 2020). The first BCO incidence discovered in Australia in 1972 was initially implicated by *S. aureus* infection (Nairn and Watson, 1972). Later, BCO progression involves multiple bacterial attacks in the cartilage of the long bones, although *Staphylococcus* spp. is the most predominant species isolated from BCO lesions (Alharbi et al., 2024a; Asnayanti et al. 2024a; Asnayanti et al., 2024c; Jiang et al., 2015; Kense and Landman, 2011; McNamee and Smyth, 2000; Wideman and Prisby 2012). In this regard, microbiology studies identifying bacterial species associated with BCO and their pathogenic implication are critical for developing intervention measures combatting BCO based on the targeted organism. Here, we present the approaches employed for bacterial identification of the lame

birds and major bacteria recovered from pathogenic BCO lesions.

Initially, pathogen identification in infectious diseases relies on culture-dependent methods. Culture-based identification employs bacterial propagation in a certain culture media coupled with phenotypic characterization, such as morphology and staining, through microscopy and biochemical testing (Jiang et al., 2015; Petrosino et al., 2009). In the studies of bacterial BCO in chickens, isolates were derived from intestinal tissues, liver, blood, BCO lesions in the joints or thoracic bones, and feces (Al-Rubaye et al., 2015; Ekesi et al., 2021; Jiang et al., 2015). Overall, culture-based identification enables intensive characterization of the targeted bacteria, including their metabolism, mode of action, and infectious dose (Jiang et al., 2015; Petrosino et al., 2009). However, non-culturable bacteria, which are fastidious and slow-growing but play a significant role in disease pathogenesis, may be missed using this method.

Later, pathogen identification, including those associated with BCO, adopts genotype identification methods using nucleic acid sequencing. 16S rRNA gene is the most predominant biomarker for bacterial identification because it is essential for ribosomal activity across bacterial species. The 16S rRNA gene contains variable regions interspersed between conserved sequences that are unique to each bacterial species, facilitating differentiation between closely related organisms. This gene also has a relatively slow evolution rate which is suitable for phylogenetic classification. Thus, 16S rRNA gene sequencing can present complete information on bacterial communities, including the diversity, composition, and structure of the community (Janda and Abbott, 2007; Patel, 2001; Tringe and Hugenholtz, 2008). Pathogen identification based on the 16S rRNA gene applies to both culture-dependent and culture-independent methods.

Next, non-culture-based identification, such as molecular profiling of the 16S rRNA gene sequences, provides a powerful technique for comprehensively analyzing the structure and diversity of microbial communities (Jiang et al., 2015; Singh et al., 2012). The16S rRNA gene sequencing can identify bacteria directly from clinical samples without the need for culturing. Non-culture-based identification has been enhanced with the development of next-generation sequencing technologies such as sequencing by oligonucleotide ligation and detection (SOLiD), 454 pyrosequencing, and Illumina sequencing. The next generation sequencing is a powerful tool for studying the composition of complicated microbial communities of a vast collection of non-culturable samples from diverse environments (Hong et al., 2010; Jiang et al., 2015; Qin et al., 2010; Vahjen et al., 2010). The 454 FLX and Illumina are the most predominant platforms employed for microbial community studies. Illumina sequencing has 50–150 bp reads and generates up to 1.5 billion reads per run, whereas the 454 FLX technology has 250–400 bp reads and produces ~1 million reads per instrument run. The longer reads of 454 FLX technology enable high-resolution phylogenetic analysis for determining operational taxonomic units (OTUs) and phylogenetic distances between OTUs (Droege and Hill, 2008; Kennedy et al., 2014; Luo et al., 2012; Poza et al., 2012; Werner et al., 2012). Nevertheless, the shorter Illumina reads can be made up by the paired-end (PE) methods in which each entity is sequenced from both the 5' and 3' ends (Luo et al., 2012; Werner et al., 2012). The regions of the 16s rRNA gene, such as regions V3 and V6 with less than 200 bp reads, can be masked by the overlapping PE reads (Bartram et al., 2011; Gloor et al., 2010; Jiang et al., 2015).

A culture-based identification method using the 16S rRNA gene sequencing mapped the taxonomy of bacteria present in the blood, liver, and femur of healthy broilers. The results demonstrated that *Enterobacteriaceae*, *Enterococcaceae*, and *Staphylococcaceae* were prevalent in blood and liver isolates, while femoral head isolates overrepresented *Escherichia/Shigella* and *Enterococcus* spp. (Adriaensen et al. 2024). Meanwhile, studies on lame birds using the culture-dependent method identified the presence of staphylococci, e.g. *Staphylococcus xylosus* and *S. simulans*, *Escherichia coli*, *Mycobacterium avium*, *Salmonella* spp., and *Enterococcus* from lame bones (Al-Rubaye et al., 2015; Dinev, 2009; Ekesi et al., 2021; House et al., 2024; Kense and Landman, 2011; Shwani et al., 2020; Stalker et al., 2010). The initial molecular identification of the bacteria associated with BCO using the 16S ribosomal DNA sequencing was conducted on the samples isolated from blood, proximal femoral

heads, and proximal tibial heads of experimental lame birds. The finding of this study revealed that *Staphylococcus* spp., in particular *Staphylococcus* agnetis, represents the main species identified from BCO lame samples (Al-Rubaye et al., 2015). The latest studies confirmed this finding that more than 50% of the bacteria associated with BCO identified based on the 16S rRNA gene biomarker belong to the genus of *Staphylococcus* (Alharbi et al., 2024a; Asnayanti et al., 2024a; Asnayanti et al., 2024c). *S. agnetis*, *S. cohnii*, *S. saprophyticus*, *S. hyicus*, *S. simulans*, *S. gallinarum*, and *S. lentus* are several coagulase-positive staphylococci that are abundant in our research farm (Al-Rubaye et al., 2015; Ekesi et al. 2021; Jiang et al., 2015; Mandal et al., 2016; Shwani et al., 2020), which is in agreement with a study from skeletal lesions identifying *S. agnetis*, *S. cohnii*, *S. epidermidis*, *S. hyicus*, and *S. simulans* (Szafraniec et al., 2020). In addition, a study identified the bacteria attributable to BCO by the 16S rRNA gene sequencing of lame birds at three commercial broiler production houses in Arkansas presented *E. coli*, *Staphylococcus* spp., and *Enterococcus* spp., as the most abundant bacteria isolated from blood and BCO lesions of lame birds. The bacterial isolates were geographically dependent and varied among the production sites (Ekesi et al., 2021).

Furthermore, using the non-culture-based approach, molecular profiling of the hyper-variable region V6 of the 16S rRNA gene from the femoral and tibial lesions using Illumina sequencing showed that *Staphylococcus* spp. was overrepresented in the BCO lame birds. At the same time, the bacterial communities present in all samples comprised 90.9% *Proteobacteria*, 6.1% *Firmicutes*, and 2.6% *Actinobacteria* (Jiang et al., 2015). Next investigation of blood microbiota associated with BCO lameness was performed by deep sequencing of 16S RNA genes. The study presented that the chicken blood microbiota comprised 60.58% *Proteobacteria*, 13.99% *Bactroidetes*, 11.45% *Firmicutes*, 10.21% *Actinobacteria* and 1.96% *Cyanobacteria*. This study concluded that the blood microbiomes of healthy birds were significantly different from those of lame birds. *Staphylococcus*, *Granulicatella*, and *Microbacterium* were substantially discovered in lame birds (Mandal et al., 2016). Overall, both culture- and non-culture-dependent studies present the map of the complex microbiota of healthy and lame chickens, facilitating the studies of bacterial role in BCO pathogenesis.

The coagulase-positive staphylococci, particularly *S. aureus*, has long been attributed to human osteomyelitis disease. Most of the strains of *S. aureus* isolated from humans and animals have membrane proteins such as collagen-binding protein, protein A, fibrinogen- and fibronectin-binding proteins, and bone sialoprotein (Josse et al., 2015; Kavanagh et al., 2018; Olson and Horswill, 2013; Urish and Cassat, 2020). Capsules and membrane proteins of S. aureus are suggested to play important roles in their virulence and adherence to the chicken's cartilage (Sompolinsky et al., 1985). Protein A prevents the accumulation of neutrophils besieging the bacteria and obstructs the complements opsonization through the Fc portion of immunoglobulin (Colburn et al., 1980). Versatile *Staphylococcus* can exhibit some virulent factors to attack or escape host defense. In general, three main categories of virulence proteins produced by *Staphylococcus* are pore-forming toxins (PFTs), exfoliative toxins (ETs), and superantigens (SAgs) (Fraser and Proft, 2008; Oliveira et al., 2018).

The next bacteria frequently associated with locomotor disorders and septicemia in chickens is *Enterococcus cecorum* (Huang et al., 2023). Generally, *E. cecorum* is a normal flora in the intestine of mammals and birds (Devriese et al. 1991). Since 2002, *E. cecorum* has been reported as a causative agent of arthritis and osteomyelitis lameness outbreaks in broiler flocks in the U.S. (Dunnam et al., 2023) and Canada (Stalker et al., 2010). Recent data from France presented that Enterococcus represented 12.9% of the poultry pathogens in 2020 (Souillard et al., 2022). The strains of *E. cecorum* recovered from the gut of lame birds are clustered away from those of healthy birds (Huang et al., 2023).

BCO lameness typically develops in the proximal end of the femur and tibia of the long bone and the fourth thoracic (T4) vertebra (McNamee et al., 1998; Nairn and Watson, 1972; Wideman et al., 2013). 80% of BCO lameness incidence occurs in the proximal femur and tibia. In our studies, *Staphylococcus* was the most isolated bacteria in the proximal femur and tibia of lame birds (Alharbi et al., 2024a; Asnayanti et al., 2024a; Asnayanti et al., 2024c). In commercial flocks, one out of nine lame birds demonstrated microscopic lesions and necrotic

degradation of the thoracic vertebrae, leading to complete paralysis (McNamee et al., 1998; Wideman, 2016). BCO develops in the thoracic vertebrae is called spondylitis or vertebral BCO, representing approximately 20% of BCO lameness cases. *Enterococcus* has been the most frequently found on birds exhibiting spondylitis symptoms. Typically, clinical signs of broilers with spondylitis demonstrated swollen hocks leading to a kinky back or sitting on the hocks with the leg extended forward (Wideman, 2016). Figure 1 presents some clinical symptoms of BCO lameness affecting rapid-growth broilers. Generally, broilers developing BCO lameness show clinical signs such as apathy to external stimuli, limping gait, pale eye sheet, pale crown, ruffled feather, kinky back, and paresis (Mutalib et al., 1983; Nairn and Watson, 1972; Wideman, 2016). If BCO lesions occur in the head of the long bones, the birds dip down one wing or both wings to bolster their locomotion (Thorp et al., 1993). In summary, microbiology studies of bacterial BCO enable further understanding of the disease symptoms and pathogenicity which are important for the development of detection and intervention measures combatting BCO lameness in broilers.

Figure 1: Diagnosis of BCO lame birds showing general symptoms of sick birds including head down (1), apathy towards external stimuli (2), pale eye sheet (3), ruffled feathers (4), pale crown (5), as well as specific lame signs including kinky back with posterior extended to the front (6), and wing deep down to support the locomotion (7).

Intervention strategies mitigating BCO lameness

Controlling musculoskeletal disease in broilers is a great challenge in poultry farming because the peak lameness rate emerges at the marketing age, and curative treatment of the affected broilers is arduous due to their larger populations but shorter production cycles. Nevertheless, opportunistic bacteria embroiled in BCO pathogenesis exhibit the risk of highly contagious BCO. Currently, culling clinically

lame birds is a common practice in broiler farms to prevent widespread BCO throughout the farms. This practice results in decreased production value and increased economic losses. Therefore, presenting intervention measures for managing and controlling BCO in broiler poultry is necessary to reduce the incidence of BCO and halt disease transmission. Considering the complexity of the etiology and pathology of BCO lameness, different measures concerning breeding, nutrition, and housing management are critical to mitigate this locomotor problem for sustainable chicken meat production (Waldenstedt, 2006). Foremost, the genetic selection of broiler breeders is the first measure to promote musculoskeletal health, which can be attained through balanced breeding for skeletal health and production traits (Dunnington et al. 1987; Havenstein et al. 2003). The next critical measure is the management of the production house, particularly environmental control for lighting, temperature, and humidity (Tahamtani et al., 2020). Extreme environmental states such as high temperature, high humidity, poor ventilation during the summer, and freezing or wet bedding during winter intensify the prevalence of locomotion problems in broiler poultry (Buijs et al., 2012). Thus, strict control for temperature, humidity, and ventilation setup is indispensable to stave off the emergence of leg abnormalities in broiler farms (Tahamtani et al., 2020). Here, we discuss the importance of the lighting regime and barn designs to promote the leg health and well-being of broilers. We also emphasize the importance of nutrition intervention to prevent skeletal disorders in broilers.

Lighting regimes

Lighting of production houses has been intensively studied to assess its impacts on skeletal problems, feed intake, and growth rate (Charles et al., 1992; Kristensen et al., 2006). The intensity and duration of the light or the ratio of the photoperiod and scotoperiod are several variables commonly manipulated to optimize the lighting regimes. Several studies have been carried out to modify the most popular 23 h light: 1 h dark lighting regime. Altering the photoperiod: scotoperiod of 23L:1D to more natural light: dark regime reduced leg abnormalities of broilers (Sorensen et al., 1999). A study comparing the 23L:1D control and elevated lighting program presented that the elevated lighting decreased the incidence of leg disorders and sudden death syndrome compared to the 23L control. Practically, the elevated lighting program represented the gradual increase of the photoperiod from 6 to 23 hours from 4 to 35 days of age. This study also reported that males were more affected by the lighting treatment than females (Classen et al., 1991). An investigation of continuous and intermittent lighting regimes found that intermittent lighting conferred higher final body mass than continuous lighting schedules (Buyse et al., 1996). Another study investigated the effects of three lighting schedules: 24L, 2L:1D, and 2L:6D for the first 4 days of newborn broiler chicks on leg bone growth. The 24L regime resulted in a larger femur and tibia diameter but relative asymmetry of tibia and femur lengths. This finding indicated that a longer duration of light has a positive impact on leg bone growth in the first 4 days.

Nevertheless, the asymmetrical shape of the tibia and femur represents developmental instability (van der Pol et al., 2015). Thus, the duration of light and the transition state from the photoperiod to the scotoperiod significantly influence skeletal bone health. The next pivotal aspect of lighting is light intensity. An investigation aimed to compare the broiler response to high-intensity light (150 lx) versus that of low-intensity light (50 l×) for 8 weeks. The birds grown with low-intensity light had higher body weight than those of high-intensity light (Charles et al., 1992). Overall, the duration and intensity of the light regime are critical to prevent locomotion problems. The lighting regime highly influences the physical activities of fast-growing broilers, which enhance bone density and decrease the deformities of the skeletal bones.

Barn designs

Barn dimensions and designs promoting flexible physical movement and maintaining proper bird density are key factors in ameliorating bone health and animal well-being. Broilers older than 3 to 4 weeks of age are suggested to grow at a maximum of 12 birds/m^2 barn. A study investigated the well-being and health parameters of broilers reared at a density of 10 birds/m^2 but at different floor spaces: 100 birds/10 m^2, 300 birds/30 m^2, 1000/100 m^2, and 5000 birds/500 m^2. The results presented that the incidence of tibial dyschondroplasia was

more severe at the large group size (5,000 birds/500 m^2 pen) than at the 100 m^2 floor. This finding inferred the deleterious effects of large group sizes (Kiani and von Borstel, 2019). Pen enrichments using barrier perches and angular ramps were also developed to encourage the physical training of broilers. A study reported that pen enrichments improved the biophysical characteristics of the tibia and promoted the movement and active behaviors of the broilers.

Nevertheless, this treatment compromises body weight gain because of higher activity and lower feed intake (Güz et al., 2021). Managing the position of the feeders and drinkers is also essential to minimize the risks of skeletal disorders. Lengthening the distance between feeders and drinkers effectively encourages the birds to walk to access water and feed. Moreover, it is important to regulate the height of the water-feeding lines according to their age to promote locomotor activity (Güz et al., 2021). Furthermore, restraining body mass accretion positively affects body conformation, bone-building processes, and locomotor activity because of weight load reduction (Đukić-Stojčić and Bessei, 2011). Therefore, balancing the rate of body weight gain and systematic physical movement or exercise represents a pivotal measure to maintaining skeletal health.

Feed supplementations

Feed treatments, particularly supplement inclusion in the broiler diet, is a strategic approach to mitigating musculoskeletal issues in broilers because it can be applied on a large scale without extra animal handling that potentially induces stressful conditions for the birds. Such treatments can be carried out by managing and manipulating the quantity and quality of feed ingredients that are critical for skeletal bone health, presenting alternative nutrient sources, and managing optimum feeding duration. Protein and amino acids, calcium (Ca), phosphorus (P), trace minerals, vitamins, and probiotics are some essential nutrients and diet supplementations potentially utilized in feed treatments to alleviate BCO in poults (Liu et al., 2023; Mateos et. al., 2002; Nääs et al.f, 2012; Wideman 2016).

Probiotics

Adding probiotics into a broiler's diet has become an emerging practice in broiler poultry and provides great potential to alleviate skeletal disorders in broilers (Neveling and Dicks, 2021). Several studies have demonstrated the advantageous impacts of probiotics on intestinal integrity, immune defense, leg health, and growth performance of broilers (Fazelnia et al., 2021). Four lameness trials assessing the effect of the Biomin probiotic PoultryStar containing 4 bacterial strains: *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, and *Lactobacillus reuteri* on the lameness occurrence in broilers presented lameness reduction by approximately 44%-71% (Wideman et al., 2012). Adding the same probiotic product plus prebiotic-fructooligosaccharides in the broiler's feed also evoked positive impacts on the bone integrity of heat-stressed broilers, thereby mitigating leg problems (Yan et al., 2019). Additionally, commercial probiotics containing *Bacillus licheniformis* and *Bacillus subtilis* reduced lameness incidences by 37.86% and 34.20%, respectively (Alrubaye et al., 2020b). A study on a commercial probiotic containing *E. faecium* showed no significant difference in response to the two dosage treatments. Low dosage (4.0×10^8 Colony Forming Unit (CFU)/chick) and high dosage (2.0×10^9 CFU/chick) demonstrated lameness reduction by 37.93% and 50.57%, respectively ($p>0.05$) (Do et al., 2024). Broilers fed with Bacillus subtilis diet for 56 days also recorded substantially lower BCO rates, less severe lesions, and lower FCR but higher body mass gain compared to the control treatment (Owen, 2017). The probiotic treatments are suggested to maintain the homeostasis of the intestinal microbiota, enhance improving intestinal function, and modulate the absorption of nutrients, calcium, phosphorus, and hormones, which are critical to bone health (Liu et al., 2023; Xu et al., 2022).

Vitamin D_3

Vitamins are natural organic compounds of foods that are critical for animals' development, growth, and reproduction. Although vitamins are required in small amounts, some vitamins are inadequately synthesized in the body, rendering the necessity of vitamin supplementations (Ward, 2023). In poultry, supplementation of vitamin D_3 has become a general practice because of intensive poultry farming indoors without enough sunlight exposure, minimizing the self-production of natural vitamin D. Inadequate levels of vitamin D in broiler chickens can impair bone health causing leg disorders (Broz and Ward

2007). Broilers are commonly supplemented with vitamin D3 in the metabolic forms of 25-hydroxyvitamin D_3 ($25(OH)D_3$) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) because vitamin D receptors have a higher affinity toward metabolite forms vitamin D_3 than vitamin D_3 (Yousefzadeh et al., 2014). A study on the effect of a commercial HyD product on the incidence of lameness concluded that adding 25-hydroxy vitamin D_3 to the drinking water presented a 34.58% lameness reduction compared to the negative control (Wideman Jr et al., 2015b). We also assessed the impact of a commercial Panbonis feed derived from *Solanum glaucophyllum* on BCO lameness. Panbonis contains approximately 15% of a glycoside analog of $1,25(OH)_2D_3$ (Bachmann et al., 2013; Gili et al., 2016), with a longer retention time in the intestines (Bachmann et al., 2013; Mathis et al., 2016; Zimmerman et al., 2015). This study concluded that 1 µg/kg of Panbonis is the optimum concentration for $1,25(OH)_2D_3$-glycosides supplementations. The feeding strategy for $1,25(OH)_2D_3$-glycosides was also investigated in this study. The finding demonstrated that early feeding of 1 µg/kg of Panbonis for the first 28 days conferred equal protection against BCO lameness to the feeding of 1 µg/kg of Panbonis for the entire trial (56 days) (Asnayanti et al. 2024a). Bioactive $1,25(OH)_2D_3$ have been proven to play pivotal roles in bone calcification, cell growth and differentiation, endocrine regulation, and immune responses (Cantorna 2010; Girgis et al. 2013; Girgis et al. 2014; Hewison 2012; Kongsbak et al. 2013; Kreutz et al. 1993; Kutuzova and DeLuca 2007; Nagpal et al. 2005; Norman 2008; White 2010). Overall, the optimum concentration and feeding strategy are critical for vitamin D_3 supplementation because excessive vitamin D_3 addition in the feed can be contra-productive to bone health.

Trace minerals

The next nutrient commonly supplemented to the broiler's diet is trace minerals (TM), particularly manganese (Mn), zinc (Zn), and copper (Cu). These TM play pivotal roles in the immune system, synthesis of connective tissue, and bone development (Junior et al., 2019; Kidd, 2004; McKnight et al., 2020). Mn enhances breast muscle, bone development, and growth performance (Li et al., 2024). Mn also modulates the immune system through the activation of heterophils, macrophages, and natural killer cells (Son et al., 2007). The increasing incidence of tibial dyschondroplasia in chickens resulted from decreasing Mn levels in the body (Liu et al., 2023; Wang et al., 2021). Equally, Zn is also required for bone and collagen synthesis, body mass accretion, and immune defense (Caterson et al., 2000; Krane and Inada, 2008; Pimentel et al., 1991). Low Zn level significantly affects nutrient absorption in the gut. Next, Cu has been proven to enhance bone metabolism (Muszyński, 2018). Cu is also pivotal to the synthesis of collagen fibers, and inadequate Cu intake disrupts bone mineralization in chickens (Brink et al., 1992). A study of the effects of a commercial product Availa-ZMC containing organic trace minerals – Zn, Mn, Cu – on the incidence of BCO demonstrated different results based on the challenge model used. In the trial using the wire flooring model, 0.1% of Availa-ZMC supplementation reduced lameness by 28.79%, whereas 0.15% of Availa-ZMC decreased lameness by 13.64%. In the study conducted by *S. agnetis* challenge in litter floor, 0.1% of Availa-ZMC supplementation resulted in a 40.96% lameness reduction, while 0.15% of Availa-ZMC decreased lameness by 37.35% (Alrubaye et al. 2020a). The latest study assessing the strategy of TM feeding time found no significant difference in lameness reduction resulting from the supplementation of 0.15% of Availa ZMC for 56 days from the same treatment for the first 28 days (Alharbi et al., 2024a). Overall, these studies present evidence that the concentration of TM supplementations and the timing of feeding impact its potency in protecting broilers from BCO lameness.

Protein and amino acid contents

Fulfilling protein and amino acid requirements for a broiler's diet is necessary for the increased protein turnover and health of rapid-growth broilers (Heijmans et al., 2022). Adequate essential amino acid and protein intake are required for energy synthesis, supporting the metabolic system, and nurturing temperature homeostasis. To gain optimal growth, modern broilers need 3.17-3.48g digestible lysine (Dlys)/Mcal of dietary amino acid intake. Surplus protein intake results in the deposit of uric acids and urates in the skeletal joints and internal organs, causing arthritis, leg deformation, and eventual lameness (Maharjan et al., 2020). Currently, the sources of proteins for boiler feed

are competing with protein sources of food for human consumption, particularly corn and soybean crops. Several attempts aimed to present alternative protein sources meeting the demand for protein as well as diverting the dependency on corn and soybean (Liu et al., 2023). Microalgae has been a promising nutrient source for poultry feed due to its high protein content, a wide range of bioactive compounds, and a huge potential for massive production (Janssen et al., 2022; Neumann et al., 2018; Yusuf et al., 2016). A study assessing the impact of 5% *Spirulina platensis* supplementation in a reduced concentration of crude protein demonstrated a slightly increased cumulative lameness by 6.3% compared to the negative control (Asnayanti et al., 2024c). This finding is contradictory to the results of the inclusion of 10% *Spirulina platensis* in the broiler diet. Supplementation of 10% *Spirulina microalgae* in the reduced crude protein of broiler feed minimized systemic inflammation and bacterial leakage from the liver but enhanced footpad quality and the pigmentation of skin, breast, and thigh in broiler chickens (Mullenix et al., 2022; Mullenix et al., 2021). These findings indicated the dose-dependent activity of microalgae-derived proteins.

In addition to the feed additive assessments, the effect of the treatment of enrofloxacin containing fluoroquinolone antimicrobial on the incidence of BCO lameness in broilers was also examined. Adding 10 mg enrofloxacin/kg body weight (BW)/day to drinking water reduced lameness by 35,9% (Wideman Jr et al. 2015a). To conclude, the success of the supplementation of broiler feeds against BCO lameness in broilers depends on the nature of the bioactive effects on bone health, gut health, and the immune system. Optimum concentration, optimum timing, and administration approaches of the supplements are critical to the determination of the treatment's efficacy.

Overall, the treatment of vitamin D3 generated from *Solanum glaucophyllum* seems a promising strategy to control BCO lameness in broiler poultry because of its robust data on the optimum concentration and supplementation timing. Nevertheless, all intervention measures presented above provide their respective benefits in terms of efficacy, costs, and practicability. They do not exclude one another, yet they might interact synergistically to reduce lameness in broilers (Alharbi et al., 2024a; Alrubaye et al., 2020a; Asnayanti et al., 2024c; Do et al., 2024; Mullenix et al., 2021; Mullenix et al., 2022; Wideman Jr et al., 2015b). Combining the intervention treatments is anticipated to present collaborative effects on mitigating BCO lameness. Furthermore, determining the most practical and cost-effective treatment may not necessarily be approachable because, first, their assessments and available data were not completely comparable. Second, the quantitative value of the lameness reduction was achieved from the interaction between the environment and the treatments. Thus, the choice for the treatment of BCO depends on the preference of broiler producers.

Induction of experimental BCO lameness

A reliable and reproducible model to create experimental lameness in broiler chickens emulating lameness outbreaks in commercial farms is paramount to investigate the etiology, pathogenesis, and intervention strategies controlling BCO in broiler poultry. Nevertheless, the incidence of BCO lameness in research populations is unpredictable and sporadic, driving the necessity to present strategies for inducing experimental BCO lameness in broiler flocks. Creating experimental BCO can be conducted on a small scale or a large scale of broiler flocks, depending on the purpose of the study. While the approach of inducing lameness can be achieved by direct pathogen exposure and mechanical induction (Wideman 2016). This review presents strategies for inducing different scalability of experimental BCO lameness.

Small-scale lameness induction

Generally, studies on BCO lameness with experiments requiring less than approximately 250 birds may need a small-scale lameness induction. This approach was highly used in the early phase of studies of BCO lameness when the investigation mainly addressed the etiology, pathogenicity, and clinical manifestations of the disease. A direct pathogen inoculation is suitable for creating a limited experimental BCO in broilers. Direct bacterial administration into experimental chicken hosts can be conducted by oral gavage, intraperitoneal, intravenous, and subcutaneous injections, and through respiratory routes (Alderson and Nade, 1987; Alrubaye et al., 2020a; Alrubaye et al., 2020b; Daum et al., 1990; Emslie et al., 1983; Martin et

al., 2011; McNamee et al., 1999; Wideman and Pevzner, 2012; Xu et al., 2018).

Intravenous administration of *S. aureus* into 29 days of age, birds successfully developed lameness symptoms after two days of injection (Emslie et al., 1983). In several studies, intravenous injection of *Staphylococcus* spp. in broilers and turkeys successfully created experimental BCO lameness (Daum et al., 1990; Emslie et al., 1983). Septic arthritis and localized osteomyelitis were generated by subcutaneous inoculation of S. aureus recovered from clinical lame birds into the hock joints of 4-week-old broilers (Alderson and Nade, 1987). Aerosol infection through the respiratory route of birds in the isolator also successfully created experimental BCO (McNamee et al., 1999). Martin et al. (2011) reproduced experimental Entrococcal spondylitis (ES) in male broilers by comparing three challenge routes: oral gavage (108), intravenous (IV; 103), and air sac (AS; 103). The observation demonstrated 30.3% gross lesions in the orally challenged group and 2.9% spinal lesions in the intravenously challenged birds. This finding indicates bacterial escape from the gut, translocating to the bloodstream, and circulating to reach the vertebral growth plate (Martin et al. 2011).

In addition to the direct bacterial inoculation, injection of immunosuppressive drugs such as dexamethasone, glucocorticoid, and corticosteroid can also trigger femoral head necrosis in chickens (Xu et al., 2018), osteomyelitis lesion in turkey (Huff et al., 1998) and epiphyseolysis in broilers (Durairaj et al., 2012). Wideman and Pevzner (2012) performed three independent experiments comparing intramuscular injections of saline and 0.9 to 1.5 mg of dexamethasone (DEX)/kg of BW in the broilers. The results presented 0 to 8% and 24 to 68% of tibial head necrosis in saline- and DEX-injected birds, respectively. Nevertheless, the DEX-injected birds also demonstrated growth inhibition and some lesions that are non-pathognomonic for BCO, such as fatty necrosis of the tibiae and avascular femoral head necrosis. Overall, these studies were capable of inducing clinical lame symptoms in limited numbers of birds (Durairaj et al., 2012; Huff et al., 1998; Wideman and Pevzner, 2012; Xu et al., 2018).

In summary, the methods used for direct challenge administration or direct pathogen exposures allow us to precisely measure the concentration of the challenge agents, both bacterial and immunosuppressor, that are applied to the experimental chicken hosts. However, this approach is hardly applied to large numbers of birds because it is much more laborious, time-consuming, and requires skilled manpower to handle the birds one by one. Overall, this approach is fit for more closely controlled studies of host-pathogen interaction, etiological agents, or pathology of infectious diseases, particularly BCO lameness.

Large-scale lameness induction

Studies evaluating the efficacy of intervention treatments mitigating BCO lameness in broilers with experiments requiring more than approximately 250 birds will need a large-scale lameness induction. In this regard, the larger the number of animals involved, the more reliable the obtained results become. Here, we present three strategies for triggering a large-scale experimental lameness in experimental broiler flocks by using pathogen exposure and mechanical stress models.

Wire flooring model

The development of experimental BCO lameness in broilers using a wire floor model was initiated by Wideman et al. (2012). The wire floor model employs elevated wire pens with feeders on one end and water pipelines on the other end, as illustrated in Figure 2A. The wire-flooring chamber is a 3 m × 1.5 m rectangular pen with a net size of 5 cm^2. The flat wire panels are elevated on 30-cm brick, allowing the feces to come through and deposit underneath the panel (Alrubaye et al., 2020a; Asnayanti et al., 2024b; Wideman et al., 2012; Wideman, 2016). The wire floors induce mechanical stress on susceptible joints instigated by unstable footing while traversing back and forth from feeders and waterers, thereby promoting the formation of osteochondrotic clefts and micro-fractures in the proximal epiphyseal-physeal growth plates of the joints. The mechanical power of the wire floor imposes double pressures on the high-body-mass broilers, leading to immunosuppression and leaky gut, thereby allowing pathogen translocation from the intestinal lumen into the bloodstream and circulating to the bones. Concomitant suppression of the wire forces and the vulnerable skeletal bones of rapid-growth broilers promotes substantial pathogen infection,

which is quiescent within the bird or in the environment. Eventually, this circumstance causes lameness outbreak in the birds reared on the wire floor pens without direct pathogen administration (Gilley et al., 2014; Jiang et al., 2015; Wideman Jr et al., 2014; Wideman Jr et al., 2015b; Wideman et al., 2012; Wideman, 2016; Wideman and Prisby, 2013). The susceptibility of broilers toward this model is also influenced by the genetic characteristics of the birds (Wideman, 2016). The peak of the BCO outbreak created by the wire floor model emerged after 5 weeks of age and powerfully triggered BCO lameness rates of up to 85% (Asnayanti et al., 2024b). This model has been successfully employed to assess several feed additives, probiotics, and antibiotics mitigating BCO lameness in broilers by using the BCO infection group in the wire floor as a positive control group (Alrubaye et al., 2020a; Wideman Jr et al., 2015a; Wideman Jr et al., 2015b; Wideman et al., 2012). Overall, this model has been a golden method for creating BCO lameness in research flocks. Unfortunately, it is unrepresentative of naturally occurring BCO in commercial flocks, as commercial broiler farms do not utilize wire floors, thus decreasing research translatability. Furthermore, the use of a large quantity of wire-flooring chambers in a huge-scale trial necessitates laborious work to assemble, disassemble, and clean out the system in between trials.

Bacterial challenge in litter floor model

The second model of inducing a substantial scale of experimental lameness is bacterial challenge administered in the drinking water of the broilers grown on the litter floor chambers. The litter floors are made of a 3 m × 1.5 m square pen with wood-shaving litter bedding (Alrubaye et al., 2020a; Alrubaye et al., 2020b). Similar to the wire floor, this model also installs feeders and water pipelines in the opposite direction, encouraging the bird to walk back and forth to access the water and feed, as illustrated in Figure 2B. The experimental birds are challenged with bacteria attributable to BCO on days 21-22 of age. Bacterial solutions are diluted in 20 L of clean water per carboy to a final concentration of 105 CFU/mL (Alrubaye et al., 2020a; Alrubaye et al., 2020b). The carboys are connected to the waterer on days 21-22 of age and intermittently shaken throughout each day to prevent bacterial aggregate in the carboy (Do et al., 2024). *S. agnetis* and *S. aureus* were employed as bacterial challenge agents in this model to evaluate the effect of feed additives, probiotics, and vaccines on the incidence of lameness, and they successfully generated cumulative lameness up to 65% (Al-Rubaye et al., 2017; Alharbi et al., 2024b; Alrubaye et al. 2020a; Do et al., 2024). A study revealed that there was no significant cumulative lameness resulting from the administration of approximately 10^5 CFU/mL of *S. agnetis* at 10, 20, or 30 days of age for 24 hours (Alrubaye et al. 2020b). Although this model has successfully reproduced BCO lameness in a large-scale lameness trial, direct bacterial exposure to the environment remains a great concern because it has the potential to cross-contaminate other chicken flocks.

Aerosol transmission model

The aerosol transmission model is the latest approach to induce a large-scale BCO lameness in broilers introduced in this review (Asnayanti et al., 2024b). This system combines the strengths of wire floor pens to trigger naturally occurring BCO lameness and airborne transmission of the disease to the experimental birds reared on the litter floor pens. The wire floors are used as incubator chambers for BCO development by persistently imposing torque and shear stress on susceptible leg joints of broilers possessing inherent risks of over-body weight broilers. The effectiveness of the aerosol transmission model is determined by the array of the system presented in Figure 2C. One wire-flooring pen is set up upstream of each line of chambers. If the study runs two lines of treatment pens, thus, two wire floor pens are required as the BCO source group. A line of litter floor pens is assembled downstream of the wire floor pen for birds assigned to randomized treatment groups. Empty space between the wire floors and the litter floors is allotted for a buffer zone to prevent direct contact between the BCO source birds in the wire pen and the experimental birds in the litter pens to ensure equal treatment to all groups. Exhaust fans are installed at the end of the barn to control the air circulation assisted by automatic inlet ventilation on the sides of the house. The air circulation aerosolizes the bacterial agents of BCO and transmits them throughout the house. Cool pads on the side of the wire floor, maintaining the temperature, occasionally impose additional pressure on the birds on the wire floors, exacerbating the progression of the

BCO source group. The overall design allows the aerosol transmission of pathogens from the BCO source group in the upstream pens downwind to the litter pens. The reproducibility of this model was proven through four independent experiments, resulting in a 54-84% cumulative lameness in the BCO source group on the wire floors and a 60-70% cumulative lameness in the litter groups with no statistical difference (Alharbi et al., 2024b; Asnayanti et al., 2024a; Asnayanti et al., 2024b; Asnayanti et al., 2024c; Perera et al., 2024). The inclusion of only one or two wire floors in this model reduces laborious work yet is capable of inducing a large-scale lameness mimicking BCO outbreaks in commercial broiler farms where the BCO lameness starts from a few birds and rapidly spreads throughout the house. Therefore, this model is an ideal approach to evaluating the efficacy of intervention measures mitigating BCO in broilers, particularly the potency of the prophylactic drugs, feed supplements, and vaccines in reducing BCO lameness in broilers.

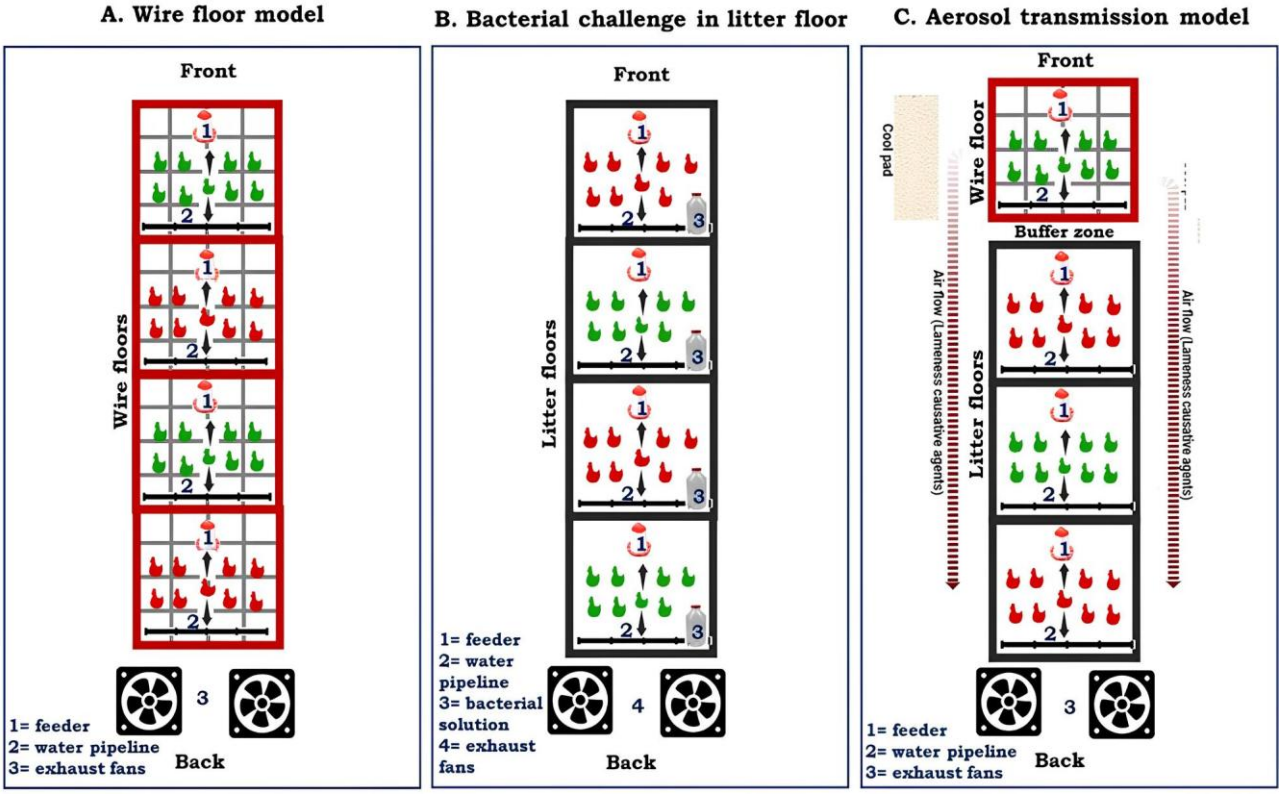

Figure 2: Three models of creating a large-scale experimental BCO lameness in broilers. A. Broilers reared in wire floor pens, B. Direct bacterial challenge of broilers reared in the litter floor pens, and C. Aerosol transmission model combining wire and litter floors. Part C of Figure 2 was adopted from Asnayanti et al. (2024).

Conclusions

This review summarizes the microbiology approaches that are predominantly used to diagnose BCO lameness incidence and to determine bacterial causative agents of BCO lameness in broilers. *Staphylococcus spp.* and *Enterococcus spp.* are the most prevalent bacteria isolated from lame birds, making them pivotal targets for developing intervention strategies against BCO lameness. Intervention measures combatting lameness in broilers can be evaluated through observation or experimental models. In this review, the induction of the experimental lameness models is classified based on the scalability of lameness created. A small-scale lameness induction can be conducted through bacterial and immunosuppressor challenges in chicken hosts. A large-scale lameness induction can be obtained using three models: bacterial challenge in the litter floor model, wire floor model, and aerosol transmission model. Feed additive treatments discussed in this review were evaluated using large-scale lameness induction models, ensuring the feasibility of these treatments. In contrast, the effects of lighting regimes and barn designs on lameness were assessed through observatory methods. Intervention measures summarized in this review do not compete with each other, but they can collaboratively boost BCO lameness control in broiler populations. Efficacy, practicability, and cost-effectivity are essential consideration factors in choosing measures controlling BCO lameness. Overall, this review

presents insight into the predominant diagnostic tools, experimental BCO models, and intervention measures to address locomotion disorders in broiler chickens.

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