Title:

Enterobacteriaceae and *Enterococcaceae* are the dominant bacterial families translocating to femur heads in broiler chicks

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"This is an Accepted Manuscript of an article published by Taylor & Francis in Avian Pathology on 14 december 2023, available at: https://doi.org/10.1080/03079457.2023.2288872."

ABSTRACT

As a result of rapid growth of broilers, bacterial chondronecrosis and osteomyelitis has emerged in the last decade, with bacterial translocation from the gut to internal organs, including the femur head. In this study we isolated translocated bacteria in femur heads, blood and liver, during ageing of broilers, and identified the bacteria using 16 rDNA sequencing. We also provided histopathological descriptions of femur head lesions. Bacteria were isolated from blood, liver, and femoral head samples. In the femoral heads an age-related presence of bacteria was observed, with high prevalence at 2-days post hatch and no bacteria isolated from femoral heads of broilers older than 14 days. Bacterial identification using 16 rRNA gene sequencing demonstrated that bacteria present in blood, liver, and femur belonged to different taxonomic groups. Bacteria isolated from blood and liver samples belonged predominantly to the *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae*, while *Escherichia*/*Shigella* and *Enterococcus spp*. were the most prevalent taxa in femoral head samples. No bacteria were isolated from the femoral head of any of the birds with histopathological lesions. All 19-day and 22-day old birds in this study showed cartilage retention in both legs, and had signs of separation between the articular cartilage and the growth cartilage in at least one of both legs. This study shows that young clinically healthy broilers have a higher prevalence of bacteria in the femoral head compared to older broilers and that presence of bacteria in blood and liver was common during ageing of broilers.

Key words: broiler, bacterial chondronecrosis and osteomyelitis, *Enterococcaceae*, *Enterobacteriaceae,* bacterial translocation, gut health.

RESEARCH HIGHLIGHTS

- Large number of bacteria isolated from femoral heads of clinically healthy broilers.
- The prevailing taxa in femoral heads were *Escherichia/Shigella* and *Enterococcus spp*.
- Continuous presence of bacteria in blood and liver of clinically healthy broilers.
- *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae* prevail in blood and liver.

INTRODUCTION

Modern broilers are the result of years of genetic selection directed at economic traits, including rapid weight gain, yield of breast meat and feed use efficiency (Knowles et al., 2008; Tallentire et al., 2016). As a result of intensive genetic selection for these traits, broiler chicken growth has increased over 400% between 1950 and 2005 (Zuidhof et al., 2014). The majority of broiler chickens are reared in intensive production systems (Knowles et al., 2008), where they grow from approximately 40g at hatch to over 3.5 kg by 5 weeks of age. As a consequence of optimizing traits for production, chickens may suffer reduced welfare, with poor walking ability being a major concern (Alrubaye et al., 2020; Ferver et al., 2021; Knowles et al., 2008).

Bacterial chondronecrosis with osteomyelitis (BCO) is an important cause of severe lameness in fast growing broilers (Wideman, 2016). Over the last two decades the incidence of lameness caused by BCO has increased noticeably, with currently over 1% of all broilers grown to heavy weights being affected after 5 weeks of age (Wideman, 2016). The size and structural integrity of the broilers' skeletons are unable to keep up with the rapid increases in body weight currently expected in the broiler industry. Unsurprisingly, this has a detrimental effect on the developing cartilage, which results in cartilage microfractures in the growth plates of the proximal femur and tibia (Wideman, 2016; Wideman et al., 2015). These microfractures can be colonized by hematogenous opportunistic bacteria that can enter the circulation via translocation through the integument, respiratory system, or gastro-intestinal tract. The colonization of bacteria can cause obstruction of the local blood flow, trigger osteomyelitis, and finally result in necrosis of the cartilage (Wideman, 2016; Wideman et al., 2015,

2012). Bacterial translocation through the epithelial lining of the gastro-intestinal tract, and bacteremia, are of significant importance in the pathogenesis of BCO. Therefore, reducing the amount of opportunistic pathogenic bacteria that can pass the intestinal barrier, improving the integrity of the intestinal barrier, and supporting the host's immune system in order to efficiently eliminate translocated bacteria from the systemic circulation is of importance in the prevention of this disease.

To the best of our knowledge, there are no studies simultaneously investigating translocation of bacteria to the blood and liver, and invading the bone marrow of the femoral heads at different time points in early life of broilers as baseline data for further studies on the pathogenesis and control of BCO. We describe here the isolation of translocated culturable aerobic bacteria from femur heads, blood and liver, during the rearing of broilers, and identification of the bacteria using 16 rDNA sequencing. In addition, histopathological descriptions of femur head lesions are provided.

MATERIAL AND METHODS

Ethics statement. The study was performed in accordance with the EU Directive 2010/63/EU and followed the guidelines of the ethics committee of the Faculty of Veterinary Medicine, Ghent University.

Birds and housing. One hundred and twelve one-day-old unvaccinated male Ross 308 broilers were obtained from a local hatchery (Vervaeke-Belavi) and housed at the Faculty of Veterinary Medicine, Ghent University. They were housed in floor pens on wood shavings, with water and feed (composition: see table 1) provided *ad libitum*. The chicks were randomly divided over 8 pens with 14 birds per pen. Temperature was adjusted with age, with target temperatures set at 32°C for day 2, gradually lowering to 23°C by day 22. The photoperiod was adjusted from 24h light until day 3, to 23h light : 1h dark from day 3 until day 6, after which it was set to 18h light: 6h dark until the end of the trial.

90 **Table 1. Diet composition of the feed used throughout the study**

Base diet	**Percentage (%)**
Wheat	58.72
Soybean meal	26.4
Rapeseed meal	6
Sunflower oil	4.9
Monocalcium phosphate	1.65
Limestone	1.08
NaCl	0.38
Mineral premix	0.2
Vitamin premix	0.2
DL-Methionine	0.22
L-Lysine HCl	0.19
Threonine	0.06
Vitamin premix	**Concentration (g/kg)**
Calcium	317
All-rac-α-tocopheryl acetate	40
Niacin	30
Panthotenic acid	8.5
Riboflavin	4.3
Pyridoxine	2.7
Retinol	1.95
Menadione	1.6
Thiamine	1.6
Folic acid	1.1
Biotin	0.15
Cholecalciferol	0.0625
Cyanocobalamin	0.0085
Mineral premix	**Concentration (g/kg)**
Calcium	248
Zinc	55
Manganese	60
Iron	10
Copper	8
Iodine	0.625
Selenium	0.15
Magnesium	5
Sodium	1

Sample collection. During the trial birds were euthanized for sampling at 13 timepoints (day 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, and 22). On the first sampling day 2 birds from each pen (16 birds) were euthanized. Subsequently, one bird per pen (8 birds) was euthanized at each timepoint.

On day 2 to 4 the chicks were euthanized by CO_2 gassing, while from day 5 on, euthanasia was by intravenous injection of an overdose of pentobarbital. After euthanasia, the birds were weighted, and samples were collected.

On days 2, 3, 4, 6, 7, 10, 14, and 19, both hip joints were exposed and disarticulated, after which the femoral heads were cut in half with a sterile knife and the cut surface was swabbed (Biolab Inc, Alpharetta, GA, USA) for microbiological culture. Thereafter the femoral heads were fixed in phosphate buffered formalin.

At five different timepoints (day 5, day 8, day 12, day 16 and day 22) the following samples were taken: first, 1mL of blood was drawn from the jugular vein and added to 2µL heparin (5000 U.I/mL stock) in a 2mL eppendorf tube, then mixed by inversion. Subsequently, the liver was carefully dissected from the digestive system and collected in its entirety. Finally, femoral head samples were collected as described above.

Samples were stored at 4°C after collection. Sample processing was performed the same day.

Sample processing

Characterisation of bacterial translocation. Blood, liver and femoral head samples were plated for bacterial counts and bacterial identification.

Blood samples were cultivated on Colombia blood agar (Oxoid, Hampshire, UK) by spreading out 100µL blood-on-heparin using a sterile L-shaped spreader (VWR, Radnor, PA, USA) and incubation was done overnight at 37°C with 5% CO_2. After incubation the colony forming units (CFU) on each plate were counted to determine the CFU/100µL.

The livers were diluted with Hank's balanced salt solution (HBSS) (1g liver/ 2mL HBSS) and were homogenized. Subsequently a 1/10 dilution in HBSS was made. Six 20µL drops (120 µL) of both the 10^0 and 10^{-1} dilutions were plated out on TSA agar (Sigma-Aldrich) and incubated overnight at 37°C with 5% CO_2. After the incubation the total colony forming units (CFU) in all six 20µL-drops were counted and added together. To determine CFU/g liver, the following calculations were made:

$$CFU/gram_{liver} = \left(\frac{total\ CFU_{counted}}{6} * 50 * dilution\ factor\right) * 2$$

The swabs (Biolab Inc., Middlebury, CT, USA) collected from the inside of the femoral head were immediately inoculated onto Colombia blood agar (Oxoid) and incubated overnight at 37°C with 5% CO_2. After incubation the colony forming units (CFU) of each side were counted and added together for each animal.

Identification of the bacterial isolates using Sanger Sequencing of the 16S rRNA gene. To identify the bacteria in the blood, liver and femoral head samples, bacterial DNA was extracted and the 16S rRNA gene was sequenced. Bacterial colonies were harvested from their respective culture plates using a sterile 1µL inoculation loop (Biosigma S.p.A., VE, Italy) and DNA extraction was performed using 20µL of lysis buffer (0.25% SDS and 1M NaOH diluted in distilled water), after which the mixtures were heated at 95°C for 5 minutes, and centrifuged for a short spin. Subsequently 180µL deionized water (LiChrosolv®, Sigma-Aldrich) was added and the samples were centrifuged at 13 000 rpm for 5 minutes. The supernatant (DNA sample) was transferred and used in the following polymerase chain reaction (PCR).

A PCR-reaction was performed to amplify the 16s rRNA gene aiming to characterize the taxonomic identity of the translocated bacteria. The PCR-reaction mix contained 650 µL Polymerase Taq platinum (Biomix®), 17.5µL of both the forward (fD1; 5'-AGAGTTTGATCCTGGCTCAG-3) and the reverse primer (rD1; 5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1991), and 488µL 180µL water for chromatography (LiChrosolv®, Sigma-Aldrich). Each PCR reaction contained 2µL of the PCR-reaction mix and 18µL DNA sample. A negative control was included. The PCR amplification consisted of an

initial denaturation at 95°C for 5 minutes, followed by three cycles of 45 seconds at 95°C, 2 minutes at 55°C and 1 minute at 72°C and thirty cycles of 20 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C. The final extension was done at 72°C for 7 minutes.

DNA-samples were diluted in aqua HPLC (1/2 dilution) with an end volume of 25µL for Sanger sequencing. The 16S gene database of the Basic Local Alignment Search Tool (BLAST®, National Center for Biotechnology Information, Bethesda MD, USA) was used to determine the taxonomic group for each bacterial isolate.

Femoral head histology. To evaluate the morphology of the femoral heads, formalin fixed femoral head samples from both the left and the right leg, derived from 19-day old and 22-day old chickens, were decalcified with a decalcification solution based on Ethylenediaminetetraacetic Acid (EDTA) (pH 8) for 2 weeks, after which they were placed on 70% ethanol solution. Subsequently, the femurs were embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin for microscopic evaluation. The evaluation of femoral morphology was based on a scoring system quantifying separation of the articular cartilage from the growth cartilage (0 – no cleft; 1 – minimal cleft formation; 2 – moderate cleft formation; 3 – partial separation; 4 – complete separation), degeneration of the articular cartilage and the growth cartilage, scored separately (0 – no sign of degeneration; 1 – signs of degeneration/ necrotic lesions), cartilage retention (0 – no cartilage retention; 1 – cartilage retention), cellular depletion within the bone marrow (0 – no areas of cellular depletion; 1 – area(s) of cellular depletion), lymphoid hyperplasia within the bone marrow (0 – no lymphocyte follicles, 1 – focal presence of lymphocyte follicles, 2 – multifocal presence of lymphocyte follicles), and vascular regression within the growth plate (0 – no sign of regression; 1 – fibrin in vascular lumen). To obtain one value per chicken, the average score from the left and the right femoral leg sample was used. The sum of the scores for degeneration of articular cartilage and degeneration of growth cartilage was used as a measure of cartilage degeneration. The scoring system was based on lesions described by Wijesurendra *et al.,* 2017.

RESULTS

Bacterial translocation. At all 5 sampling points, bacteria could be detected in the blood and in the liver of some, but not all chickens. No age-related effects were observed in bacterial counts derived from blood and liver samples (figure 1a and 1b). The chicken's age affected the CFUs obtained from femoral head samples. On day 2, 87.5 % of the chicks had one or more CFU in the femoral samples, with a subset of animals having high counts (figure 1c). After day 12, not more than one CFU per bird was counted. On days 16, 19, and 22 all samples were free of bacteria.

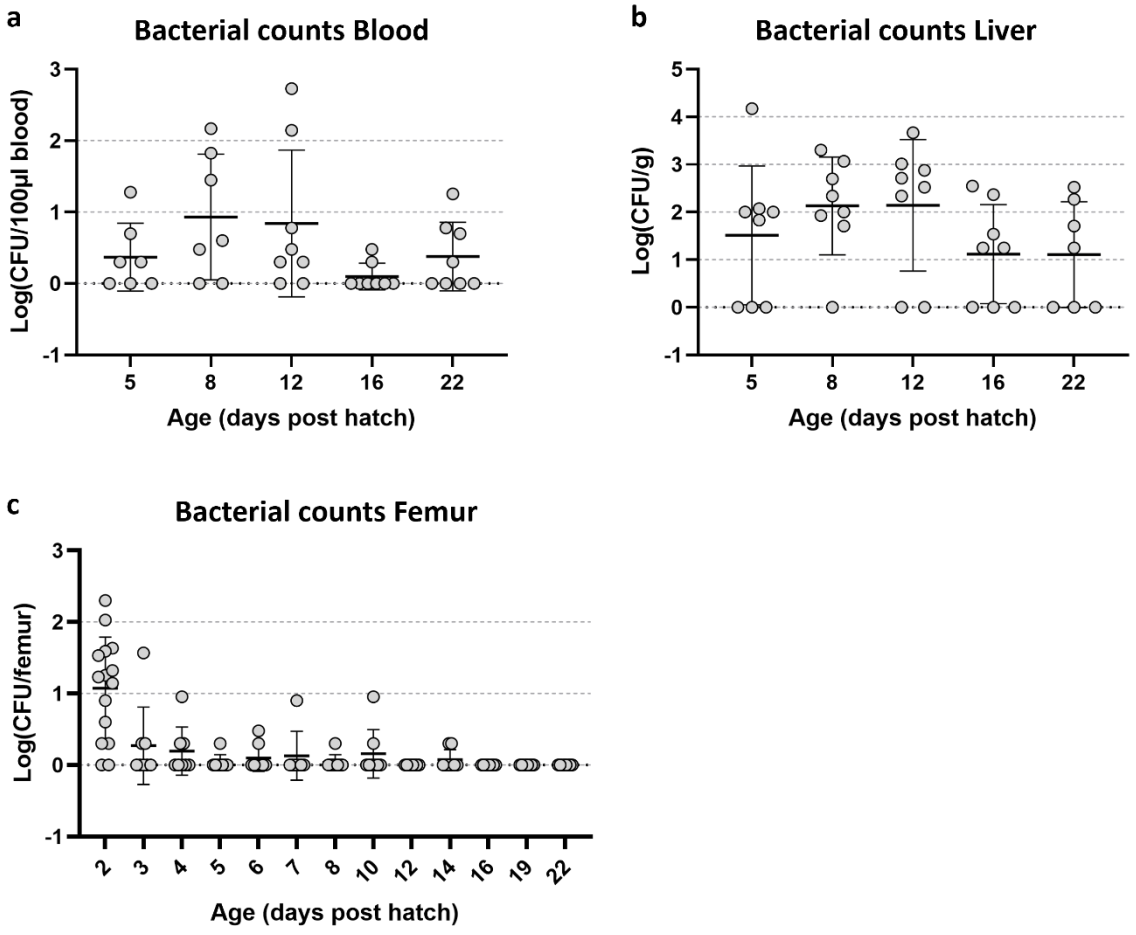


Figure 1. Colony forming units (CFUs) counted in samples from blood, liver and femur at different timepoints. Samples were collected from blood (a), liver (b), and femur (c) at different timepoints, and were cultivated overnight. Bacterial colony forming units (CFUs) were counted for each animal.

Taxonomic description of translocated bacteria. Nucleotide BLAST analyses revealed 22 bacterial taxa in the blood, liver, and femur. For some isolates it was not possible to differentiate between the species based on 16rDNA sequencing, which is why they are grouped together (separated by a division sign or backslash) in the following results.

Table displays the identified bacterial species and the tissue where they were isolated from.

183

184 **Table 2. Twenty-two different bacterial taxa were isolated from blood, femur, and liver. In the first 3 columns the taxonomy**

185 **is shown, and in column 4, 5, and 6 an 'x' marks whether that species was isolated from blood, femur, or liver, respectively.**

Bacterial taxa	Phylum	Family	Blood	Femur	Liver
Acinetobacter Iwoffii	Proteobacteria	Moraxellaceae			x
Acinetobacter Johnsonii/ A. haemolyticus	Proteobacteria	Moraxellaceae	x		
Acinetobacter radioresistens	Proteobacteria	Moraxellaceae	x		
Bacillus spp	Firmicutes	Bacillaceae	x	x	
Corynebacterium glyciniphilum	Actinobacteria	Corynebacteriaceae		x	
Enterococcus faecium/E. hirae/E. durans/E. pseudoavium/E. lactis	Firmicutes	Enterococcaceae	x	x	x
Enterococcus gallinarum/ E. casseliflavus	Firmicutes	Enterococcaceae	x	x	x
Enterobacter spp./ Citrobacter spp.	Proteobacteria	Enterobacteriaceae	x		
Enterobacter spp.	Proteobacteria	Enterobacteriaceae	x		
Enterococcus avium	Firmicutes	Enterococcaceae			x
Enterococcus faecalis	Firmicutes	Enterococcaceae	x	x	x
Enterococcus pallens	Firmicutes	Enterococcaceae	x		x
Escherichia/Shigella	Proteobacteria	Enterobacteriaceae	x	x	x
Klebsiella pneumoniae	Proteobacteria	Enterobacteriaceae			x
Lactobacillus spp.	Firmicutes	Lactobacillaceae	x		
Macrococcus caseolyticus	Firmicutes	Staphylococcaceae	x		x
Microbacterium spp.	Actinobacteria	Microbacteriaceae	x		

Neobacillus spp.	Bacillota	Bacillaceae	x		
Pedobacter spp.	Bacteroidota	Sphingobacteriaceae	x		
Peribacillus spp.	Bacillota	Bacillaceae	x	x	
Proteus mirabilis	Proteobacteria	Enterobacteriaceae			x
Staphylococcus warneri/S. pasteuri	Firmicutes	Staphylococcaceae	x		x

Figure 2 shows the fraction of the animals from which the bacteria were isolated at the different timepoints, but not the quantity in which the bacteria were present. Bacterial taxa that were isolated from only a single animal were not plotted on the graph.

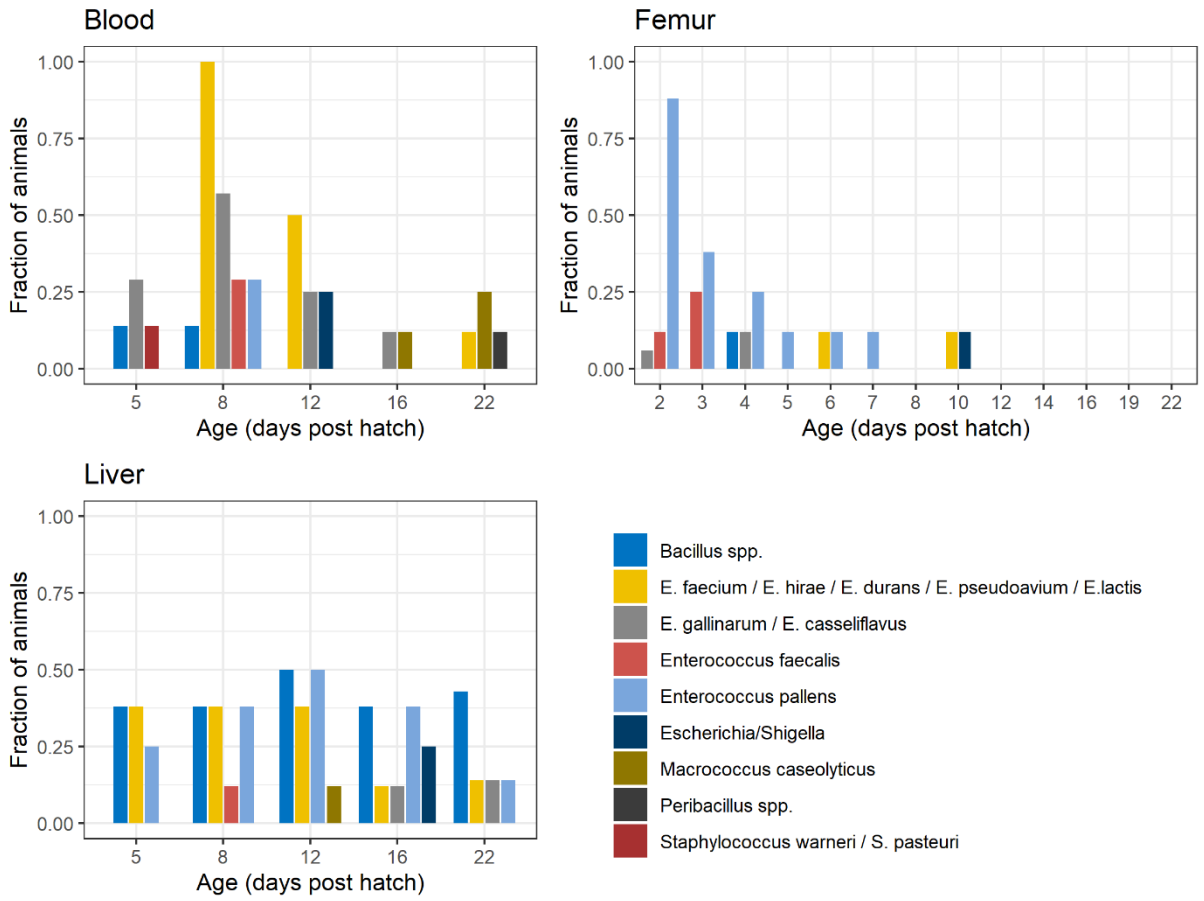


Figure 2. Fraction of animals showing bacterial growth in either the blood, liver or femur. All sampling days are plotted on the x-axis. If no data is plotted for a certain age, this indicates no growth was observed on that sampling day.

Femoral head morphology. In two birds femoral head separation (FHS) was detected macroscopically, one 12 days old (Figure 3) and one 22 days old bird.

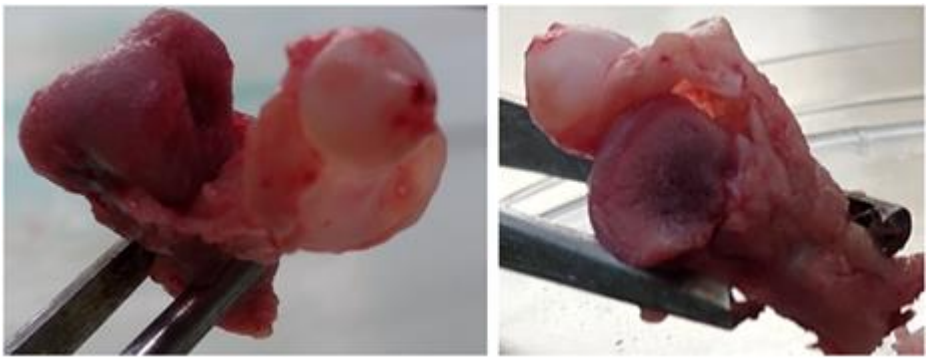
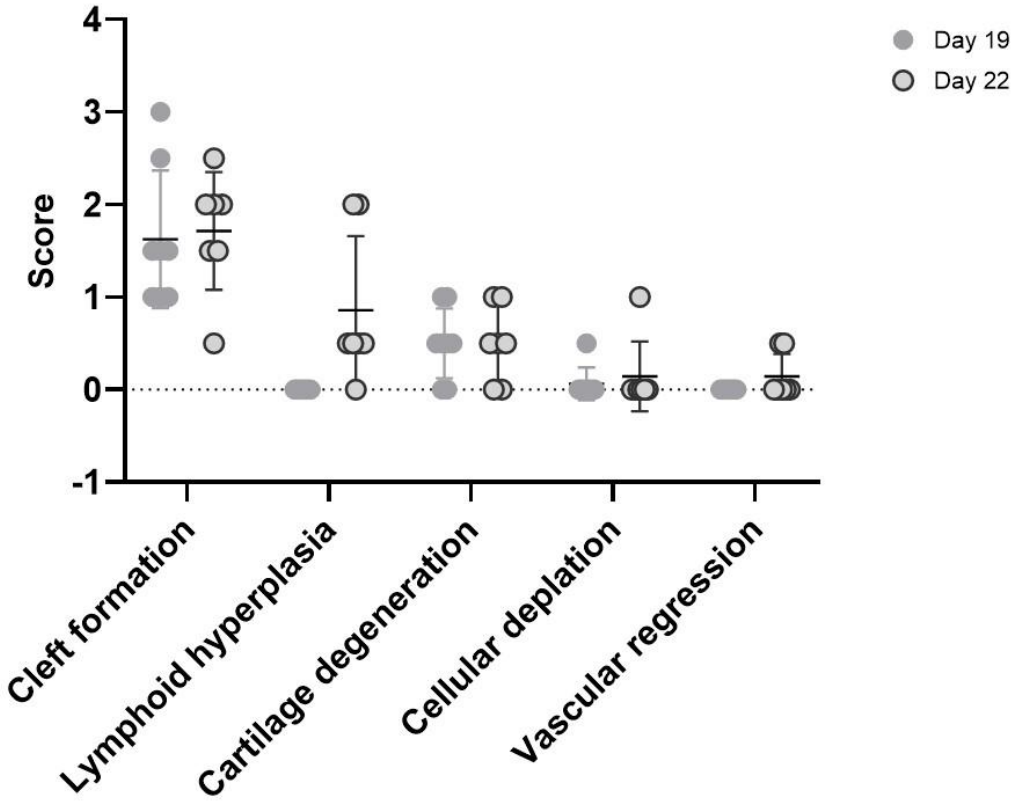


Figure 3. Femoral Head Separation in a 12 day old bird.

Microscopically all birds showed cartilage retention in both legs at both timepoints (figure 5). Separation of the articular cartilage from the growth cartilage, degeneration of the articular cartilage and the growth cartilage, cartilage retention, cellular depletion within the bone, lymphoid hyperplasia within the bone marrow, and vascular regression within the growth plate are reported in figure 4.



204

205 **Figure 4. Morphology of the femur in 19-day old and 22-day old broilers. Data are presented as the mean ± standard**
206 **deviation. Femur tissue was evaluated based on a scoring system quantifying cleft formation between the articular**
207 **cartilage and the growth cartilage (0 – no cleft; 1 – minimal cleft formation; 2 – moderate cleft formation; 3 – partial**
208 **separation; 4 – complete separation), lymphoid hyperplasia within the bone marrow (0 – no lymphocyte follicles, 1 – focal**
209 **presence of lymphocyte follicles, 2 – multifocal presence of lymphocyte follicles), articular and growth cartilage**
210 **degeneration (0 – no sign of degeneration; 1 – signs of degeneration/ necrotic lesions), cellular depletion within the bone**
211 **marrow (0 – no areas of cellular depletion; 1 – area(s) of cellular depletion), and vascular regression within the growth**
212 **plate (0 – no sign of regression; 1 – fibrin in vascular lumen).**

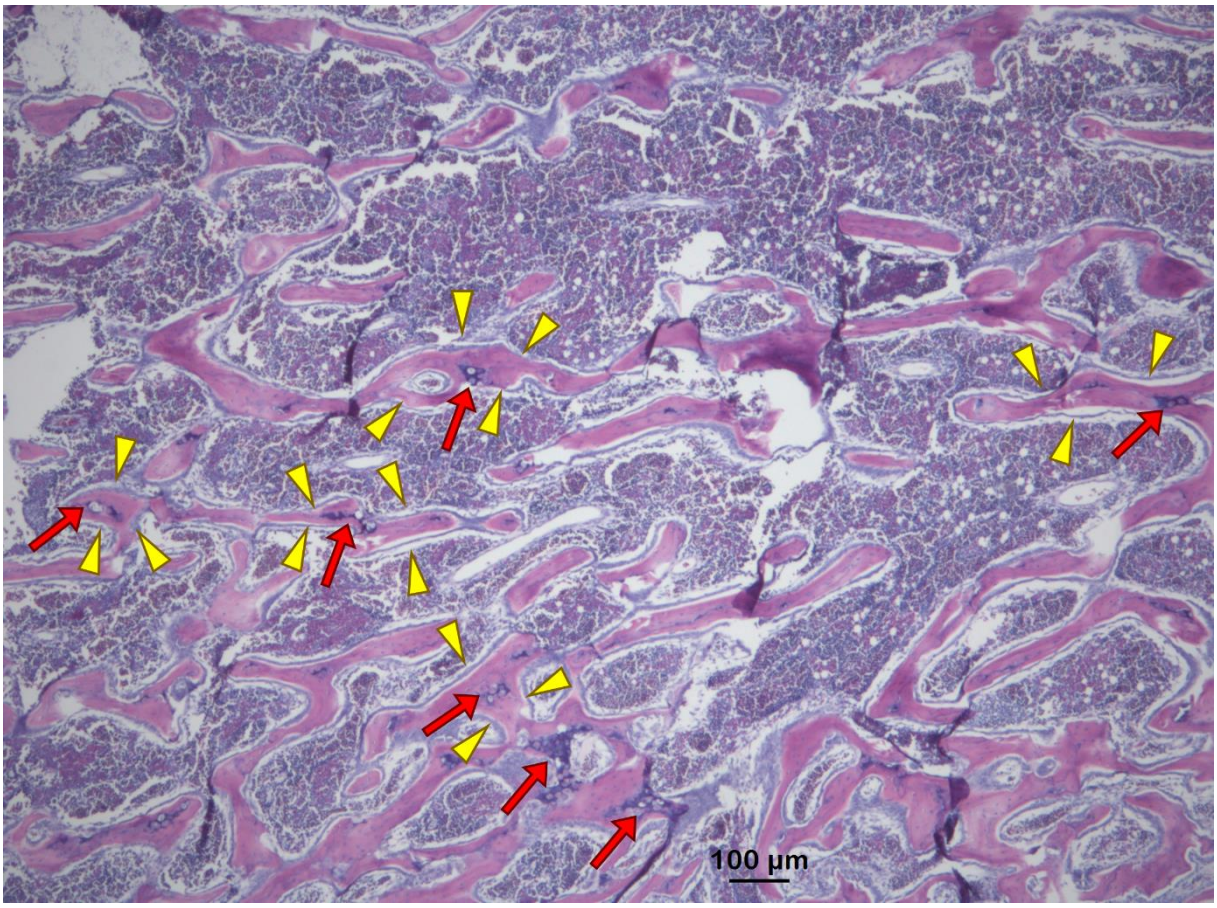


Figure 5. Retention of cartilage in osteoid. HE stained sample of a 22 day old bird. Several aeras of retained cartilage (red arrows) can be observed in within the osteoid (yellow arrowheads)

On both day 19 and day 22, 100% of the birds showed signs of separation between the articular cartilage and the growth cartilage in at least one of both femoral heads (**Error! Reference source not found.** 6). The presence of necrotic lesions (figure 7) or a more severe form of separation between the articular and the growth cartilage in a sample was not associated with the presence of lymphocyte follicles (figure 8a).

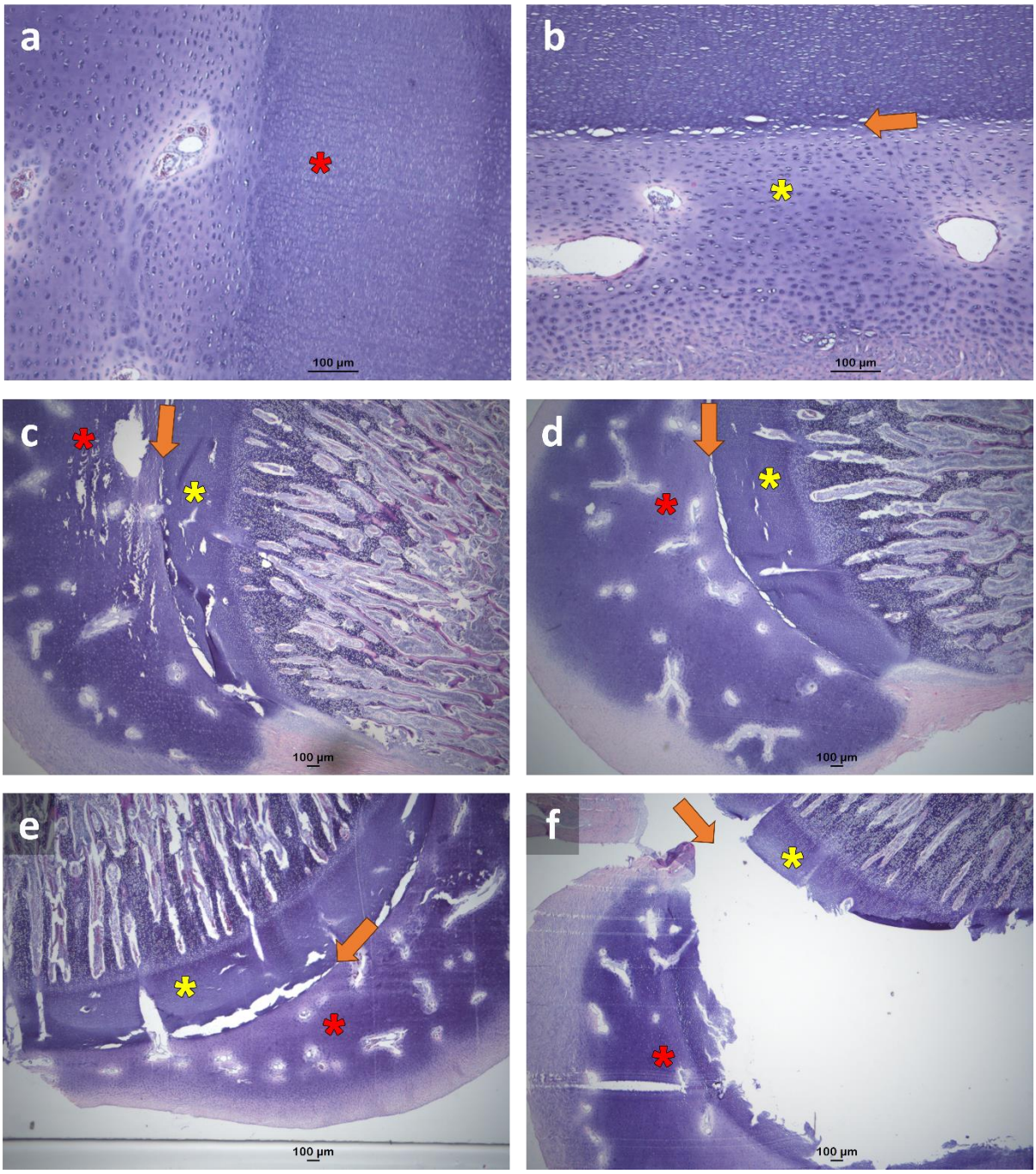


Figure 6. Degrees of separation between the articular cartilage and the growth cartilage in 22 day old broilers. a) No signs of separation (orange arrow) between the articular cartilage (red *) and the growth cartilage (yellow *). b) Formation of small clefts between the articular and the growth cartilage. c) Larger clefts between the articular and the growth cartilage. d) Larger clefts between the articular and the growth cartilage over an extended area. e) Partial separation between the articular and the growth cartilage. f) Complete separation of the articular cartilage.

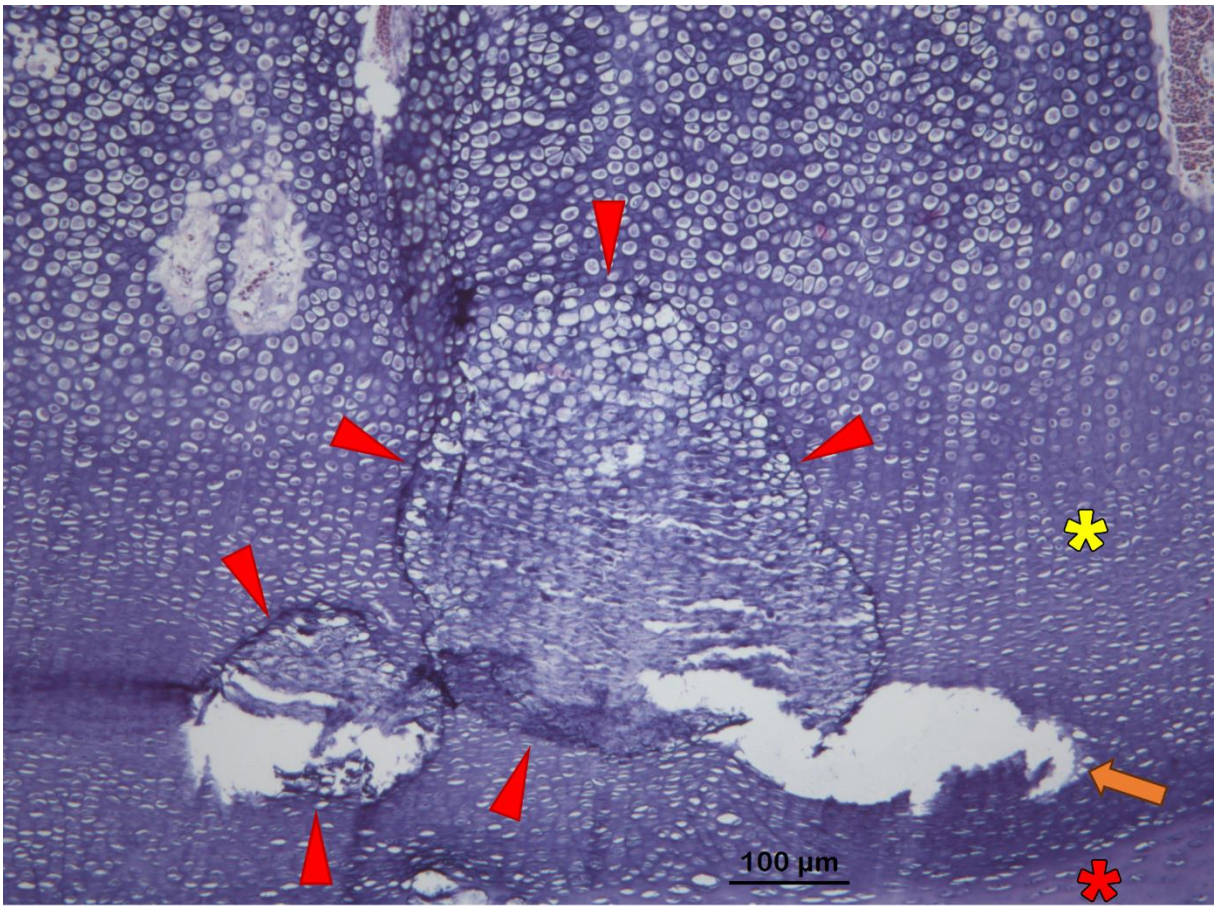


Figure 7. Necrotic areas in the growth cartilage of a 22-day old bird. Red arrow heads indicate two areas of necrotic cells. The orange arrow shows a cleft between the articular cartilage (red *) and the growth cartilage (yellow *).

Figure 8 shows the formation of follicles of lymphocytes (8a), areas of cellular depletion in the bone marrow (8b), and fibrin in the blood vessels of the growth plate (8c).

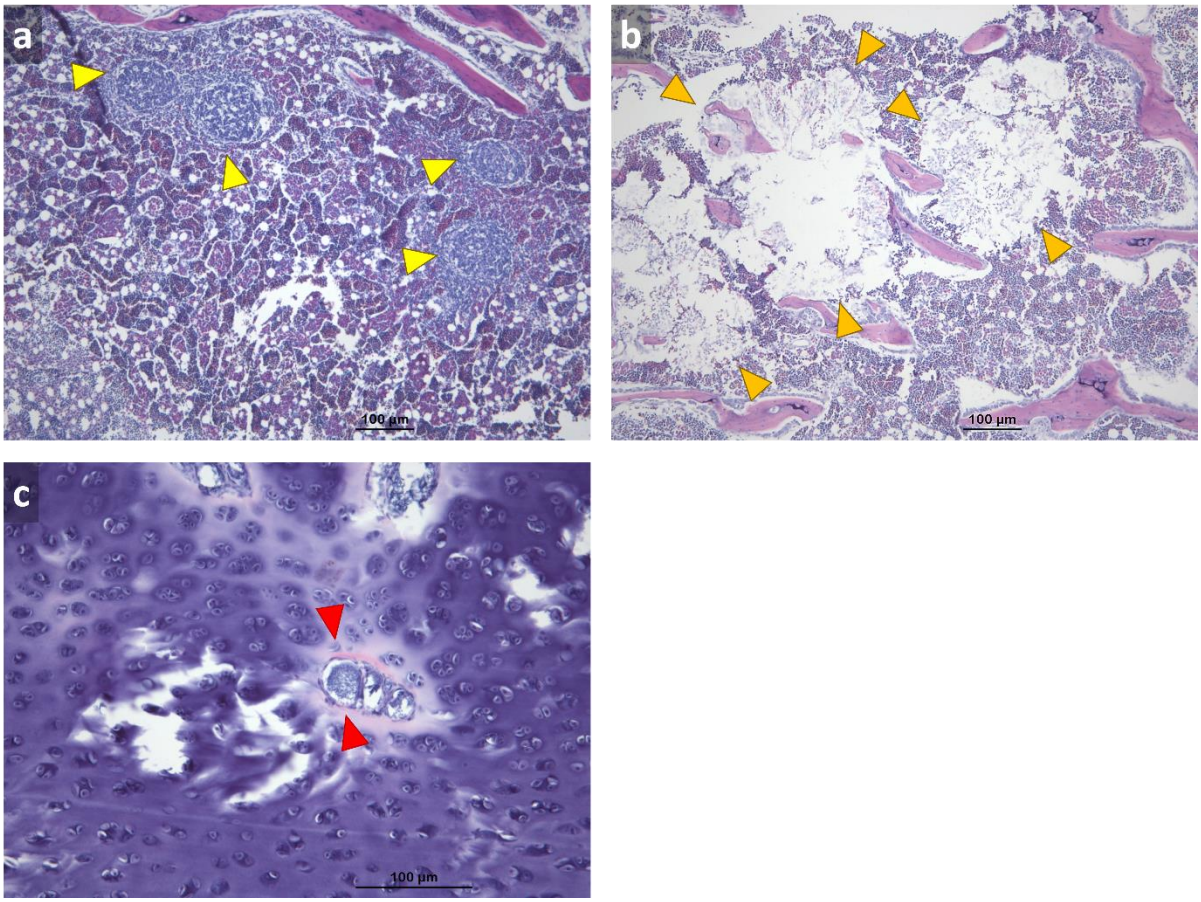


Figure 83. HE stained femur samples of 22-day old broilers. a) Lymphoid follicles in bone marrow (yellow arrow heads). b) Cellular depletion in the bone marrow (orange arrow heads). c) Fibrin in the blood vessels (red arrow heads).

DISCUSSION

Lameness in broilers is one of the main concerns in the poultry industry, resulting in reduced welfare and major economic losses (Hul et al., 2021). Bacterial chondronecrosis with osteomyelitis (BCO) is recognized as an important cause of lameness that may affect over 1% of broilers grown to heavy processing weights after 5 weeks of age (Wideman, 2016). In our study we examined early life bacterial translocation and femoral head morphology to better understand the underlying causes of bacterial chondronecrosis with osteomyelitis (BCO). The trial was performed with male broilers, as they tend to be more prone to developing osteomyelitis and chondronecrosis because of rapid growth.

Relatively large numbers of bacteria were present in the femoral heads of birds at two days post hatch compared to older birds. Bacterial chondronecrosis with osteomyelitis is the result of bacteria that enter the circulation by translocation through the respiratory and/or the intestinal mucosal barrier. Subsequently, these bacteria spread hematogenously and colonize the small blood vessels in the cartilage suffering from microtrauma in the long bones, most commonly the femur and the tibia (Wideman, 2016). In the present study, several chickens of all ages had bacteria in the blood and/or in the liver; however, this was not the case for the femoral samples. An age-related effect on the presence of bacteria in the femoral heads was observed**.** Almost all 2-day old chicks had bacteria present in the femoral heads. While some only had a few colonies, others had over 100 CFU's in the femoral samples. This number decreased drastically as the broilers got older and after the age of 2 weeks, no colonies were found in the femoral samples. These results indicate that bacteria may be present in the circulation, without noticeable colonization of the femoral head at later ages.

Bacteria present in blood, liver, and femur belonged to different taxonomic groups. Mandal et al. (2016) investigated the blood microbiota and argued that, despite the current belief that blood is sterile, there might be dormant and not-immediately-culturable forms of microbes present in the blood. In their study they found the chicken's blood microbiota dominated by Proteobacteria*,* followed by Bacteroidota, Firmicutes, Actinobacteria, and Cyanobacteria. The discrepancy in the abundance of these phyla in the blood and gut led to the belief that the blood microbiota may not be the result of bacteria passively present in the blood after translocation from the gut. Similar to Mandal et al. (2016), the bacteria isolated from blood samples in the present study belonged predominantly to the phyla Proteobacteria, Firmicutes, and Actinobacteria. Mandal et al.'s (2016) study was based on deep sequencing of 16S RNA genes on blood samples using a relatively high number of PCR amplification cycles. PCR is unable to differentiate live from dead bacteria. Therefore, the results may be an overestimation of the amount of bacteria present in the samples. The presence of bacteria in the blood without any obvious clinical manifestation was also observed in the present study. The origin of the bacteria present in the blood is unclear. It should be considered that there is a possibility that the

growth of microorganisms may have been inhibited by the use of heparin as anticoagulant (Rosett and Hodges, 1980). The liver serves as a filter that clears bacteria that have successfully penetrated the intestinal barrier (Balmer et al., 2014). Therefore, it could be expected that the taxa found in the blood, could also be found in the liver samples. However, not all bacteria found in the blood samples were found in liver samples, indicating that bacteria translocating through the intestinal barrier either did not pass through the liver, were not removed by the liver, or had a different origin.

Bacterial species most commonly associated with BCO lesions are *Enterococcus cecorum*, *Staphylococcus aureus* and *Escherichia coli*, often in combination with other micro-organisms (Ferver et al., 2021; McNamee and Smyth, 2000; Wideman et al., 2012). Souillard *et al.'*s retrospective study (2022) indicated that 77.9% of the locomotor diseases in France associated with *Enterococcus spp.* involved *E.* cecorum, many of which were associated with other bacteria such as *E. coli* and *Staphylococcus spp.*. Jiang et al., (2015) analyzed the structure and diversity of microbial communities in the proximal femora and tibia from both clinically healthy broilers and from lame broilers with BCO lesions, through molecular profiling of 16S ribosomal RNA. They detected complex microbial communities in all samples, even in the ones that appeared to be macroscopically normal. Interestingly, they were able to demonstrate major differences in the microbial communities between microscopically normal bones and bones with BCO lesions. In the bones with BCO lesions, the genera *Staphylococcus, Enterobacter, and Serratia* were overrepresented. In the current study, *Escherichia/Shigella* and *Enterococcus spp*. were the most prevalent taxa in femoral samples. No *Enterobacter spp.* were isolated from femoral samples; however, *Enterobacter spp.* were found in some blood samples. It is important to note that the absence of culturable bacteria in the present study compared to the findings of Jiang et al. (2015) could be associated with the bacterial culture methodology, while the identification methodology in Jiang et al. (2015) bypassed bacterial culture. Interestingly, in none of the birds where femoral head separation was observed macroscopically, any bacteria were isolated from the femoral samples. These findings are in accordance with the results of Wilson et al.(2020), whose study was also based on bacterial culture methodology. They found that in

most femoral head alterations (femoral head separation, femoral head transitional changes, and femoral head necrosis) no bacteriological evidence of BCO was present. Even in the most severe lesion category (severe femoral head necrosis), only 33% of samples contained culturable bacteria.

Causes of bacterial translocation of the bacterial agents are not fully understood, but early post-hatch intestinal permeability or loss of intestinal epithelial integrity because of intestinal pathogens at later age could be causative. Also factors that affect intestinal integrity, such as heat stress, can increase *E. cecorum* translocation in an infection model (Schreier *et al.,* 2022). While early-post hatch permeability is a normal physiological process to enable yolk antibodies to reach the bloodstream, intestinal integrity losses at later ages can be prevented in various ways, including dietary additives that support the intestinal microbiota or mucosal integrity (Alrubaye *et al*., 2020).

In conclusion, this study shows that young broilers have a higher prevalence of bacteria in the femoral head, mainly belonging to *Enterococcaceae* and *Enterobacteriaceae*. The results of bacterial isolates from blood and liver samples indicate presence of bacteria in the blood and liver without an obvious clinical manifestation. Histopathological lesions indicating some level of femoral head separation were seen in all 19-day and 22-day old birds in this study, not associated with bacterial colonization.

ACKNOWLEDGEMENT

The authors want to acknowledge the support of lab technicians of the Laboratory of Veterinary Pathology for making and staining tissue sections.

DISCLOSURE STATEMENT

The authors report there are no competing interests to declare.

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