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Examining Pathogenesis and Preventatives in Spontaneous and Staphylococcus-Induced Bacterial Chondronecrosis with Osteomyelitis in Broilers

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Examining Pathogenesis and Preventatives in Spontaneous and *Staphylococcus*-Induced
Bacterial Chondronecrosis with Osteomyelitis in Broilers

A dissertation submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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ABSTRACT

Bacterial chondronecrosis with osteomyelitis is the most important cause of lameness in broilers. This is important to poultry production, as it poses animal welfare issues, and causes a significant loss in revenue. The remediation of this disease requires the study of its etiology with fitting models and evaluating preventatives. The research reported herein covers genomic virulence analysis of BCO isolates, mainly *Staphylococcus aureus*, and *Escherichia coli* retrieved from lame birds. We found that *S. aureus* isolates were closest to chicken strains in Europe but may have been in the Arkansas area for a decade. Phylogenomics suggest our *S. aureus* is restricted to poultry, while the *E. coli* spans various hosts. This dissertation includes the analyses of mainly BCO isolates to determine virulence using the embryo lethality assay. Human *S. aureus* was the most lethal to layer and broiler embryos. *Staphylococcus agnetis* 908 that may induce lameness to >50% did not show virulence. This inconsistency among others compelled us to offer that embryo lethality assay may not be an effective tool for estimating the pathogenicity of BCO isolates. Furthermore, research reported herein covers the investigation of the feed additive *Availa-ZMC* for lameness reduction potential in broilers using litter and wire-flooring models for inducing lameness. *Availa-ZMC* (a mixture of organic trace minerals) resulted in a reduction of lameness by 20% in the wire-flooring model, and 25% in the challenge on litter flooring model. Finally, this dissertation reports on a Typhoid-Mary experiment in broilers raised on the wire flooring to determine whether broilers challenged with *S. agnetis* can transmit the bacterium to birds in the same pen thereby spreading BCO and lameness. The Typhoid Mary experiment shows that young broilers exposed to *S. agnetis* at an early age harbor the bacterium and if mixed with unexposed birds 10 days later can transmit the bacterium to their pen mates.

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Thank you, mom, for teaching me the value of education, and hard work. Thank you to my brothers, sisters, and friends. Your constant support got me this far.

DEDICATION

I dedicate this dissertation to the loving memories of my brother, Roy Emenike "Chicago" Ekesi.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLISHED ARTICLES

Chapter 4: Alrubaye A.A.K., Ekesi N.S., Hasan A., Elkins E., Ojha S., Zaki S., Dridi S., Wideman R.F., Rebollo M.A., Rhoads D.D. (2020). Chondronecrosis with Osteomyelitis in Broilers: Further Defining Lameness-Inducing Models with Wire or Litter Flooring, to Evaluate Protection with Organic Trace Minerals. Poultry Science….………………………………………………………………………In Press

Introduction

Literature Review

Introduction/ Literature Review

Chicken Domestication

Domestic Chicken—*Gallus gallus domesticus,* is the most widely distributed poultry species

(Miao., *et al*, 2013; Zhang., *et al*, 2017). Chickens have been important to human societies for

thousands of years. They are food; the meat and eggs serve as a reliable source of protein.

Chicken also served anthropomorphic purposes in entertainment (cockfights), religious practices,

and ornamentation. Domestic chickens make good biological and medical models. From an

archaeological perspective, domestic chickens are closely associated with humans; they have

been dispersed primarily by human activity. This makes chickens an important biological marker

of agricultural, cultural contacts, and trade between societies and civilizations (Mwacharo.,

2013A & B; Peters., 2016). There is evidence that multiple domestications of Red Jungle Fowl,

the primary parents of the most recent domestic chickens, began between the ending of the

Pleistocene and the beginning of the Holocene era in southern China, South Asia, and Southeast

Asia (Tixier-Boichard., *et al*, 2011; Miao., *et al*, 2013; Peters., *et al*, 2016; and Bosse, 2019).

Journals and archaeological evidence suggest that chickens got to Europe via southern (through

Greece and Persia) and northern (through china and Russia) trading routes (Crawford., 1990; and

Tixier-Boichard., 2011). Archaeozoological evidence suggests that domestic chickens were

raised in Africa, particularly ancient Egypt around 1307–1196 BC (Houlihan., 1986; Mwacharo.,

2013). Chickens showed up in Sudan 1650 BC, and Kenya around 800AD (Houlihan., 1986;

Marshall., 2000; Mwacharo., 2013). The sequence of events concerning the spread of chickens

across the rest of Africa is not fully understood (Blenck., 2000; Tixier-Boichard., 2011; and

Mwacharo., 2013). Mitochondrial DNA from 3000-year old chickens at the Teouma site

(Vanuatu) reveals that chicken spread from southeast Asia to Oceania between 1400–900 BC

(Storey., 2010; Miao., 2013). In the Americas, lineage and propagation of chickens are debatable (Maio., 2013). Some studies reported that DNA and carbon dating evidence suggest that Polynesian chickens were introduced in the Americas (Chile) in the pre-Columbian *AD 1304– 1424* (Storey., 2007; Maio., 2013). Storey, *et al*. (2007) suggested, based on dating and DNA evidence, that chickens were introduced to the Americas before the arrival of the Spanish or Portuguese, but they were of Polynesian origin. Gongora *et al*. (2008) also countered the Polynesian-Chilean American contact view citing that pre-Columbian chickens sequences lie among European/Indian subcontinental/Chinese haplotype rather than Polynesia.

Broiler Production in the USA

Growth of the broiler Industry

Broilers and layers are the two parts of commercial chicken production. Broilers yield meat and layers produce eggs. My dissertation focuses on broiler chickens. Our team worked to better understand the mechanisms of pathogenesis behind the incidence of lameness in the production of rapidly growing birds. We induced the disease using models. We tested formulations postulated to reduce bacterial chondronecrosis with osteomyelitis (BCO) leading to lameness in commercial broiler production farms. We also surveyed multiple commercial farms and sampled lame broilers. Our goal was to improve models for studying BCO, enhance animal health and welfare, improve meat quality, incorporate sustainable practices, and improve productivity.

The poultry industry in the United States of America is one of the most successful sectors in US agricultural production. Around the early 1900s, poultry production in the USA, like in most parts of the world, was mainly practiced in small non-specialized units using diverse breeds of chickens that already existed on the continent (Sainsbury, 2000; Muir & Aggrey, 2003). By

3

the late 1930s to mid-1940s, there was an explosion in poultry production in the US and across Europe (Sansbury, 2000). Various genetic improvement programs were introduced. Poultry breeders utilized line- and cross-breeding techniques adapted from plant breeders (Sansbury., 2000). After successful crossbreeds were introduced, the numbers of poultry breeding programs dwindled. The poultry breeders became streamlined and specialized to service large-scale poultry production (Sainsbury, 2000).

Over 50 years after poultry intensification, poultry production in the US and around the world shifted from a small scale non-specialized side activity to a global-scale specialized and integrated industry. This level of global integration drives international trade by ensuring standardization of poultry practices and products, particularly through shared policies and transfer of technologies (Sainsbury, 2000; Bessei, 2018). Integrated production generally involves contracting production to local farmers. In 2000, 57 million tons of chicken meat was produced around the world (Executive guide to world poultry trends, 2000). In 2019, world poultry meat production output was 128 million tons (Food and Agricultural Organization; Executive guide to world poultry trends., 2020). The US poultry industry produced about 9 billion pounds (4.5 million tons) of broiler meat in 1968, and about 56 billion pounds (28 million tons) in 2018—*a 600% growth in productivity* (Figure 1: USDA., 2019). In terms of monetary value, US poultry production was priced at 32 billion dollars and it is still growing (Figure 2).
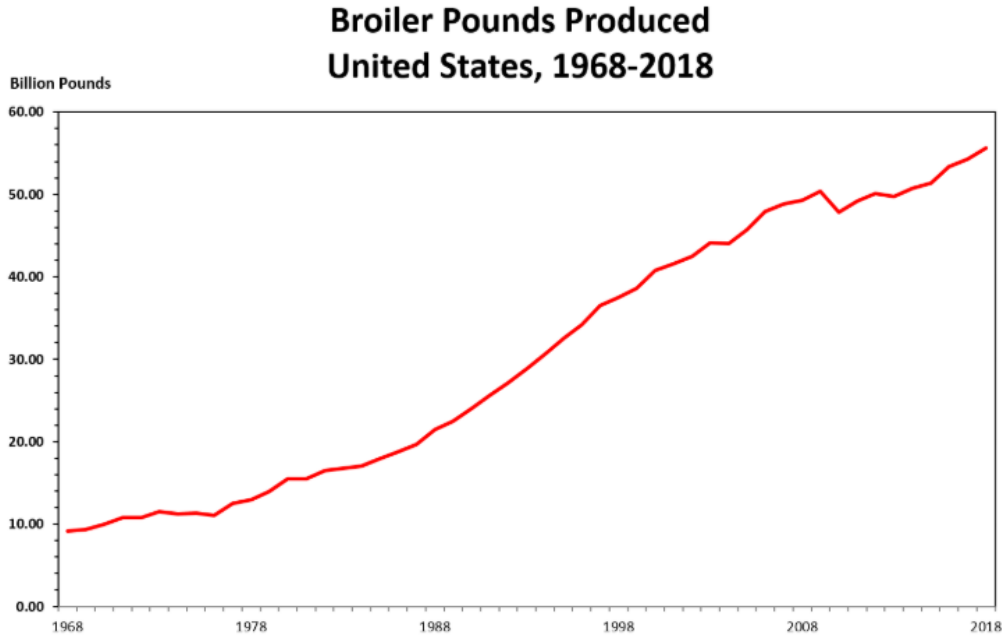
Figure 1. USDA Report: Broilers produced by the pound in the USA since 1968 (Image reproduced from USDA., 2019).
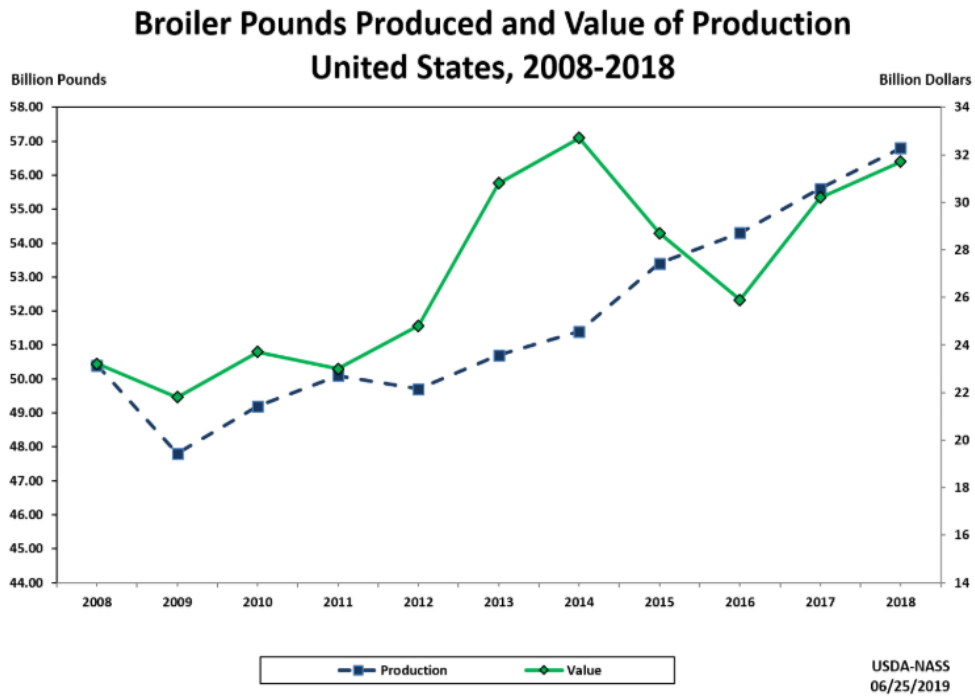
Figure 2. US broiler production in pounds with the estimated monetary value from 2008 to 2018 (Image reproduced from USDA., 2019).

Modern Broilers Selection and Breeding Techniques

Until the early 1900s, the only way to select and breed chickens was to identify the best breeder for the phenotype of interest and mate them for the next generation (Muir & Aggrey, 2003). However, technologies applied in poultry breeding have since advanced. The technologies were geared for (i) management of poultry reproduction, (ii) tracking of pedigrees, (iii) mating, and (iv) accurate utilization of true breeding values of potential candidates (Muir & Aggrey, 2003). Before the 1940s, breeding technologies were strictly aimed at producing purebred stock from pure-breeding lines. But shortly after breeding programmes were implemented, broiler producers began merging specialized lines and crossing them to make commercial production animals with distinct breeding goals (Sainsbury, 2000; Muir & Aggrey, 2003). Today, broiler products are usually three-way or four-way crosses between pure breeding lines over four generations (Muir & Aggrey, 2003; Paxton, 2010 Pollock, 1999; Sainsbury, 2000). A common generation and multiplication from pure breeding line to broiler products are described below:

(i)Pure-breeding line: Chickens are owned by primary breeder companies and kept on biosecure farms for selection programmes. Breeding companies maintain up to ten pure-breeding lines for their numerous broiler lines (Figure 3; Muir & Aggrey, 2003; Pollock., 1999).

(ii) Great-grandparent stock: These come from pure-breeding lines; they are used primarily to multiply the line and produce tens of thousands which are needed for generating the grandparent lines. They are subject to mass selection for selected traits. They are heavily controlled by the primary breeding companies. In figure 3, they have designated flocks A males and females, B males and females, C males and females, and D males and females (Muir & Aggrey, 2003; Pollock., 1999).

6

(iii) Grandparent stock: are the first-generation in the four-way ABCD cross. They are A males x B females and C males x D females from Great-grandparent stocks that are used to produce hybrid AB or CD hybrids parents. Hundreds of thousands of Grandparent stocks are distributed to the local distributor of parent stocks or integrated production companies (Muir & Aggrey, 2003; Pollock., 1999).

(iv) Parent Stock: are AB-hybrid males and CD-hybrid females. They are mainly owned and maintained by broiler production companies (Muir & Aggrey, 2003; Pollock., 1999).

(v) Broilers: are the commercial products of crossing parent stocks. They are meat-type chickens that are raised, slaughtered, processed for large scale meat consumption (Muir & Aggrey, 2003; Pollock., 1999).

Expectations for future meat demands often drive poultry breeding goals. Hence the intensification of artificial selection fuelling modern broiler production (Muir & Aggrey, 2003, Paxton, 2010; Pollock.,1999). In the 20th century, Poultry breeders kept the pace of production by adapting numerous vital selections and breeding technological innovations (Table 1, Muir & Aggrey, 2003).
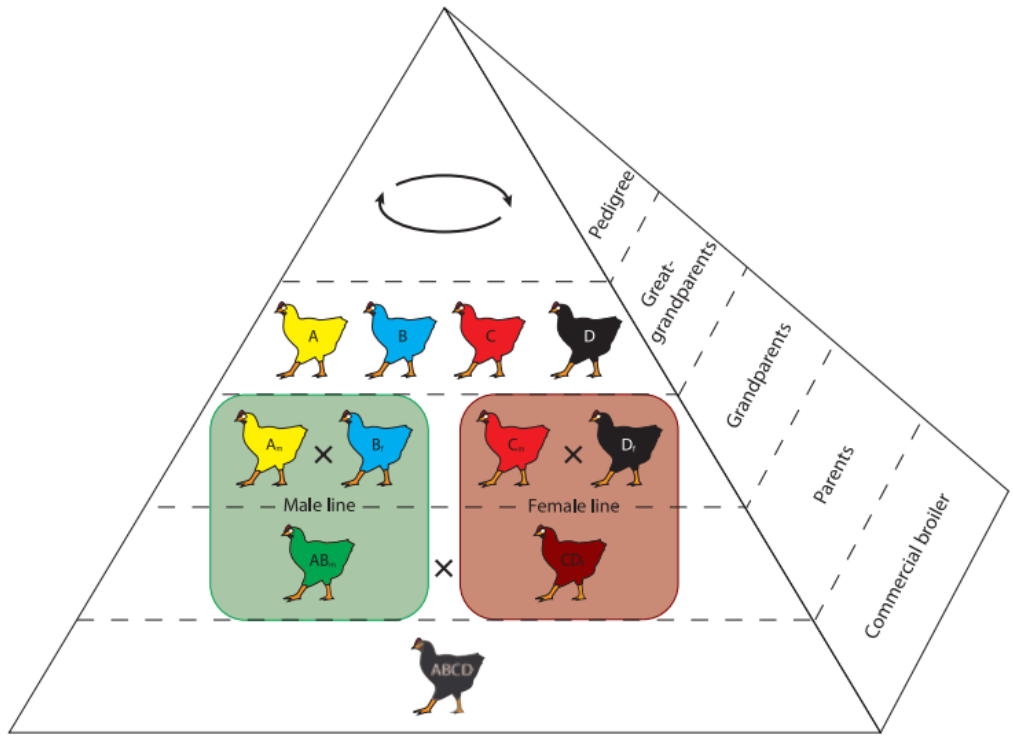
Figure 3: Example of generation and multiplication scheme in modern broiler production from elite/ pedigree to commercial broilers products. (Reproduced from Paxton., *et al*, 2010).

Table 1. Timeline for critical technologies employed for poultry breeding in the 20th century (Reproduced from Muir & Aggrey, 2003).

Technique	Decade of introduction (approximate)
Mass selection	1900
Trapnesting	1930
Hybridization	1940
Pedigreeing	1940
Artificial insemination	1960
Osborne index	1960
Family feed conversion testing	1970
Selection index	1980
Individual feed conversion testing	1980
BLUP breeding value estimation	1990
DNA markers	2000

Impact of Genetic Selection in Broiler Production: Pros and Cons

The poultry industry has seen great successes largely due to its ability to economically produce acceptable products (Anthony, 1998; Muir & Aggrey, 2003; Paxton., 2010; Tallentire, 2018). This success is the cumulative effect of selection intensities, shorter generation times, and lessened environmental impacts, among many other benefits (Anthony, 1998; Muir & Aggrey, 2003; Tallentire., 2018). It is important to explore the advantages and disadvantages that have surfaced from intensive selective broiler breeding.

Compared to the early 20th century, the modern market-weight broiler's time of production has substantially dropped. In the 1920s, it took an average of 112 days to raise 2.5 lb live-weight birds. In 2019, 6 lb live-weight birds can be raised in 47 days (Muir, 2013; NCC, 2020). The "Feed to Meat Gain" or the pound-amount of feed used to produce a one-pound live-weight broiler in 2019 was about 4.7 lb feed per 1 lb broiler (NCC, 2020). In 2019, the feed to meat gain was 1.80 lb per 1 lb of broiler produce with acceptable meat yield (Anthony, 1998; Muir, 2013; NCC, 2020). Modern broilers have been heavily selected for growth rate since the 1950s. By 2015, the growth rate has increased by 400% (Figure 4; Muir & Aggrey, 2003; Renema, 2007; NCC, 2020). In addition to meat quality and quantity, the monetary value and exports of broilers have been considerable (Anthony, 1998; Muir & Aggrey, 2003; USDA, 2019). The mortality rate of chickens has dropped from 18% to 5% since 1925 (Muir & Aggrey, 2003; NCC, 2020). Interestingly, broiler meat production has had a relatively lower impact on the environment compared to beef and pork production (Anthony, 1998; Tallentire., 2018). This is associated with a drop in the fossil fuel emission of greenhouse gases during feed production, combined with the reduced nutrient loss from poultry manure. These benefits increase even more in feed-efficient birds (Muir & Aggrey, 2003; Tallentire, 2016; 2018).

Although the poultry industry has recorded immense feats particularly as it concerns the artificial selection of desirable broiler traits. There are growing concerns over the negative effects of genetic selection in broiler production (Anthony, 1998; Hock., 2014; Muir & Aggrey, 2003). Hock (2014) counted 23 classes of organ system metabolic disorders important to broiler chickens and turkeys (Table 2; Hock, 2014). Some reports suggest that in rapidly growing birds, one of the main issues observed is increased carcass fat deposition (Anthony, 1998; Tumová., 2010). Then there are issues of broiler liveability, immune function, and reproductive complications at the breeder level (Anthony, 1998; Hock, 2014).
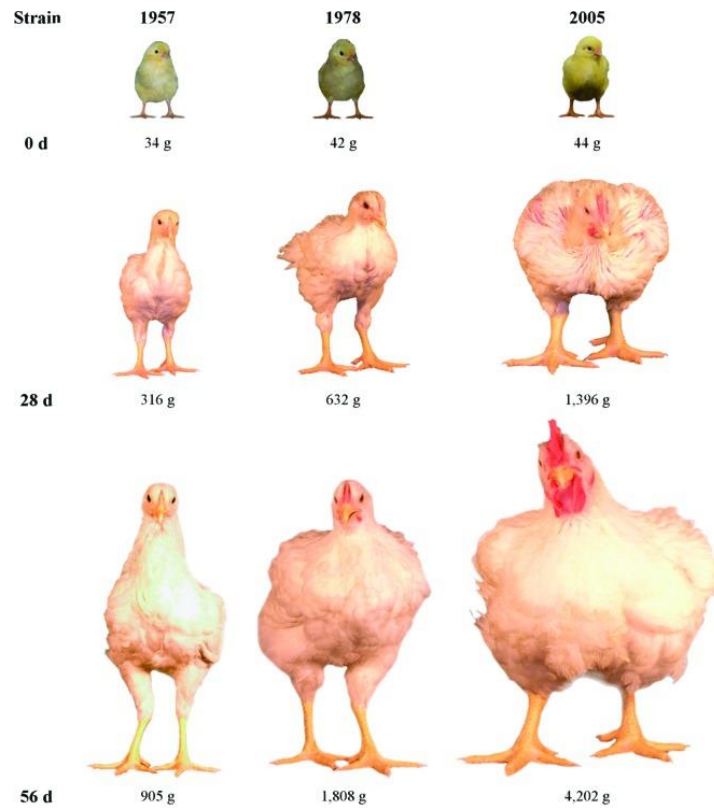
Figure 4: Age-related changes in the size of the University of Alberta Meat Control strains unselected since 1957 and 1978, compared to the Ross 308 broilers (2005) for Day 0, 28, and 56. (Image reproduced from Zuidhof., 2014).

Table 2. The number of idiopathic disorders of broiler chickens and turkeys reported in the literature (Reproduced from Hock, 2014)

Organ system	Number
Skeletal disorders	11
Muscle disorders	4
Integument	3
Cardiovascular disease	2
Reproduction	3

Around the globe, some broilers develop ascites, a condition caused by increased pulmonary pressure and resultant hypoxia that culminates in the accumulation of fluids in the peritoneal cavity resulting in abdominal swelling (Al-Zahrani, *et al*., 2019; Anthony, 1998; Parveen, *et al*., 2019; Wideman, 2000). More issues emanating and associated with the rapid growth rate of broilers include muscle abnormalities resulting from the production of high-yield birds heavy with meat that surpasses several metabolic and/or anatomical limits (Anthony, 1998). Concerning animal behavior, Bokkers *et al*. (2003) write that no difference was found in resting demeanor between fast- and slow-growing broilers raised to 13 weeks, as the birds seemed motivated to perform all kinds of behavior in a feasible environment. They noted, however, that for fast- and slow-growing broilers the ability to carry out certain behaviors became tasked with age, probably due to their weight (Bokkers., 2003). In the past 30 years, there has been an increased incidence of breast meat abnormalities like wooden-breast (WB), white-striping, and spaghetti-meat (SM) (SM) in broilers (Abash *et al*., 2016; Petracci *et al*., 2015; 2019; Sihvo *et al*., 2014; 2017). White stripes are recognizable by the accumulation of lipids and proliferation of connective tissue line up in the same direction as the striations of the muscles (Figure 5B-D; Petracci, *et al*., 2015; 2019). WB was first described in 2014 by Silvo, *et al* (Sihvo, *et al*., 2014).

WB mainly affects the pectoralis major and sometimes pectoralis minor. It presents a confined lesion at 2 weeks of age that develops as a fibrotic injury with a hardened and pale appearance in the pectoral muscles (Figure 5E-H; Abasht, *et al*., 2016; Petracci, *et al*., 2015; 2019; Sihvo, *et al*., 2014; 2017). SM affects broiler chicken pectoralis major muscles impairing its integrity. It is characterized by a soft consistency in the ventro-cranial segment due to poor adhesion of Musculo-fibers (Figure 5I; Petracci *et al*., 2015; 2019; Tasoniero, *et al*., 2020).

Selection for rapid growth is also associated with numerous skeletal defects that clinically are important to various degrees of locomotion. The group of locomotion difficulties resulting from skeletal diseases is called *Lameness*. Skeletal defects include tibial dyschondroplasia, epiphyseal ischaemic necrosis, epiphyseal separation, skeletal fracture, valgus-varsus deformity, angular bone deformity, twisted leg, spondylolisthesis (kinky-back), gastrocnemius tendon rupture, among others (Havenstein *et al*., 1994; 2003; Julian, 1998; Muir & Aggrey, 2003).
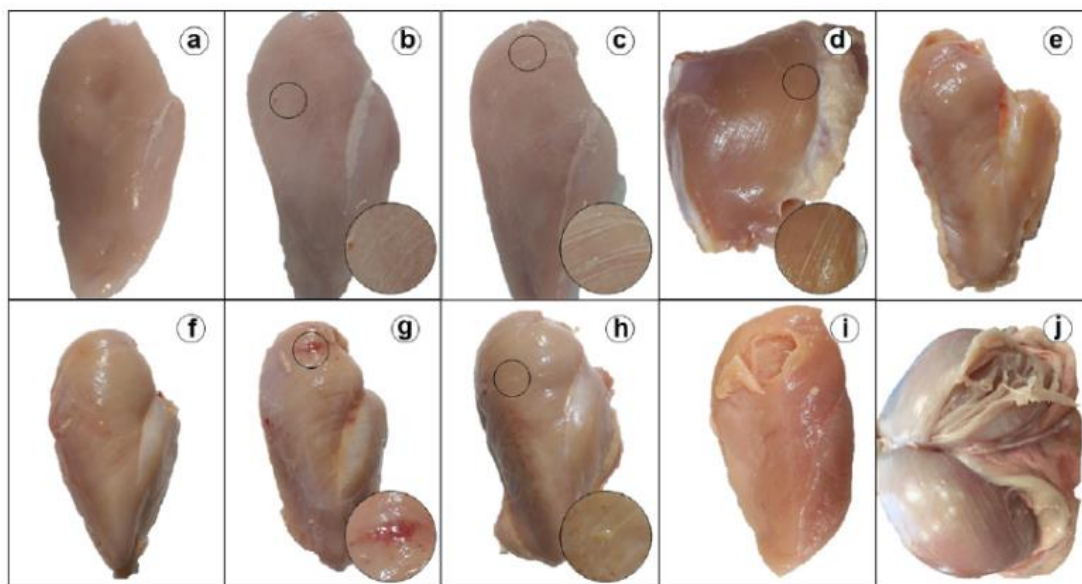
Figure 5: Classification of Broiler chicken Breast meat myopathies. (a) Normal breast (b) Moderate White Striping breast (c) Severe White Striping breast (d) Represents moderate White Striping thigh (e) Woody-breast (WB) with focal, hardened, and pale areas, without hemorrhages (f) Extremely severe WB case (g) Is the same case as figure f with hemorrhages (h) White

Striping plus WB (i) Extremely severe spaghetti meat breast (Image reproduced from Petracci., 2019).

Lameness in Broiler Chickens

The Issue

The poultry industry has been very successful in producing marketable products with reasonable turn-over times with less impact on the environment compared to other meat production systems. Unfortunately, these undertakings seem to be accompanied by several broiler health issues one of which is lameness. Lameness in birds is characterized by bone structure deformations or rigidity that culminates in partial or complete immobility of the birds. Lameness can be quantified using the gait scale (GS) scores of 0 for normal to 5 for complete immobility of the birds (Gocsik *et al*., 2017; Kestin *et al*., 1992). Some researchers, citing high GS and unusual nociceptor threshold, argue that lameness and its underlying pathologies are associated with pain (Caplen *et al*., 2014; Danbury *et al*., 2000; Hothersall *et al*., 2016; McGeown *et al*., 1999; Nääs *et al*., 2009; Gocsik *et al*., 2017). Some others counter the claims that there is a link between lameness and pain (McNamee *et al*., 1998; Sandilands *et al*., 2011 Siegel *et al*., 2011; Skinner-Noble *et al*., 2009). While the science of lameness and pain is inconclusive, all poultry researchers seem to agree that lameness in broiler chickens poses serious animal health and welfare issues in the USA and across the world (Bassler *et al*., 2013; Gocsik *et al*., 2017; Granquist *et al*., 2019; Knowles *et al*., 2008; Moura *et al*., 2006). Figure 6 depicts the different factors associated with the incidence of lameness. Factors that contribute to lameness include, but are not limited to genetics, weight, growth rate, exercise, husbandry practices, nutrition, long-day lighting pattern (1-hour darkness; 23-hour light), sex, age, and infectious agents (Brickett., 2007; Classen., 1989; Gocsik., 2017; Kestin., 1999; 2001; Moller., 1999; Muir & Aggrey, 2003; Reiter., 2001; 2006; Su., 1999). However, BCO is the leading cause of lameness in broiler chickens (Al-Rubaye *et*

al., 2015; 2017; Bradshaw *et al*., 2002; Dinev., 2009; Jiang *et al*., 2015; Thorp *et al*., 1993; 1994; 1997; Wideman., 2016; Wideman *et al*., 2012; 2013; 2015; Wideman and Prisby 2013).
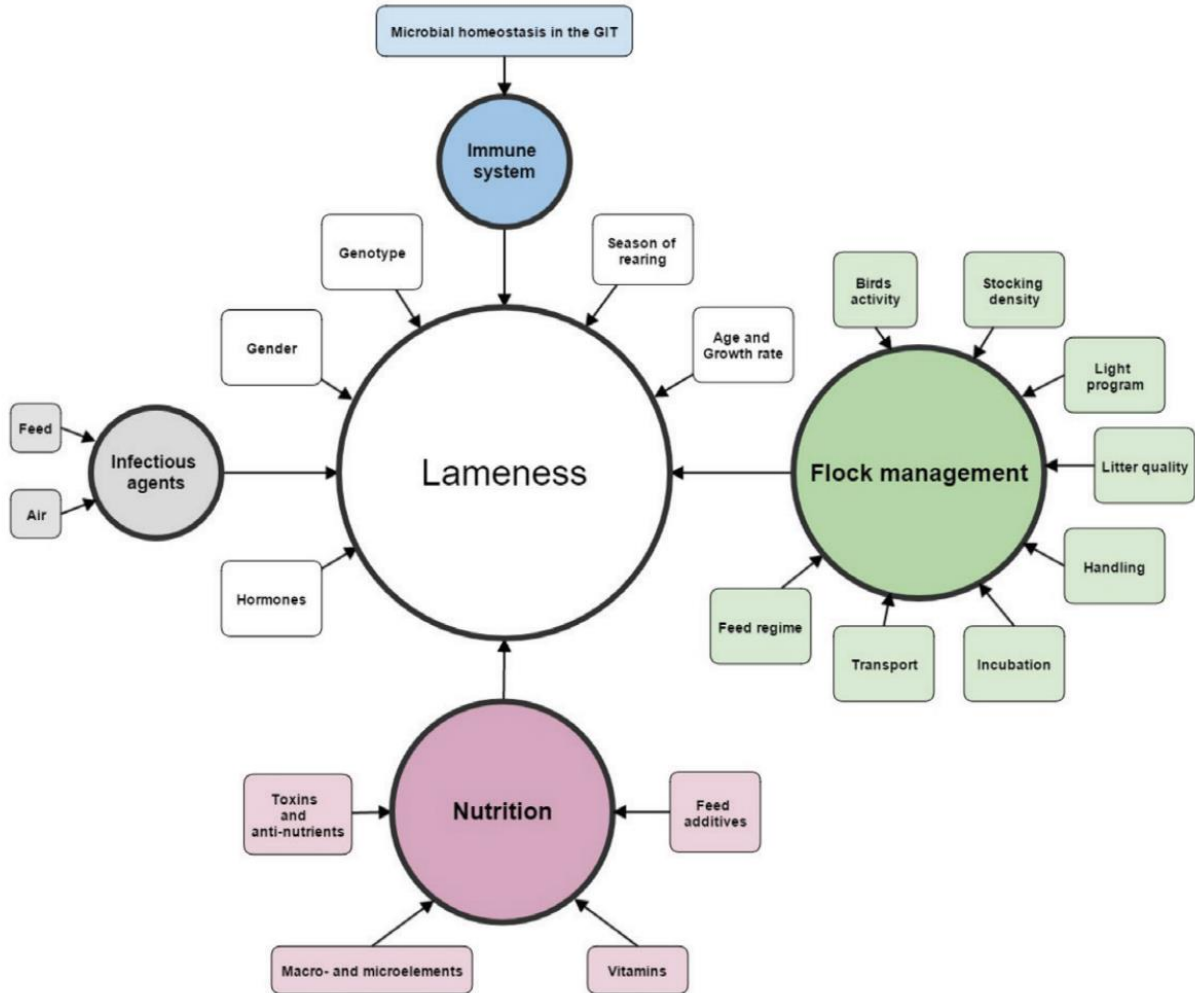
Figure 6: Factors that contribute to the incidence of lameness in broilers (Image reproduced from Kierończyk *et al*., 2017).

Prevalence of Bacterial Chondronecrosis with Osteomyelitis Lameness

BCO-lameness is important to the poultry industry for economic and animal welfare reasons.

Over 1.5 % meat-type chickens raised to processing weights at 5-8 weeks within the past 20

years in the USA may be affected with spontaneous BCO and lameness (Dinev, 2009; Stalker *et*

al., 2010; Wideman, 2016; Wideman *et al*., 2012; Wideman and Prisby, 2013). This number may

be even higher. According to Zinpro, a spontaneous outbreak of lameness can affect over 15% of

commercial broiler flocks (Rebello., 2019). A cross-sectional study of broiler flocks across

Britain, France, Italy, and the Netherlands indicated that there was a 16% prevalence of

lameness, with GS of at least 3 or more (Bassler *et al*., 2013; Gocsik *et al*., 2017). A similar

study in Sweden suggested a 14-26% prevalence with GS≥3 (Sanotra *et al*., 2003). A

longitudinal survey of 20 broiler flocks in Victoria, Australia revealed that BCO occurs

throughout the lifespan of broiler at a very high rate, with different lesions diagnosed in about

28% of the birds (Wijesurendra *et al*., 2017).

Economics of BCO Lameness

Over the past 70 years, the market of broiler production has seen a dramatic change from

smallholder chicken farms to a more intensive and integrated multibillion-dollar set-up operated

by a few corporations (Lowder *et al*., 2009). Lameness causes financial loss in poultry revenue.

This is due to increased mortality, culling of lame birds at different stages of production, and

condemning birds during processing. According to the *Farm Model,* the economic impact of

lameness is a function of the frequency of the incidence of lameness in birds with GS≥3 and its

impact on poultry productivity. Poultry production is expressed in terms of production costs,

gross margin (revenues - variable costs), and the net profit per kilogram of delivered broiler

(Gocsik *et al*; 2017). The *Farm model* considers increased mortality, higher feed conversion,

increased condemnation rate at slaughter, and lower weight gain in estimating the economic

burden of lameness (Gocsik *et al*; 2017). The damages due to the mortality of lame birds can be

estimated with Equation 1. And the damages incurred from condemning lame market-age birds

at processing are estimated with Equation 1 and Equation 2 (Gocsik *et al*., 2017; Nääs *et al*.,

2009). Considering a lot of factors, the poultry industry in the USA loses over $100 million per year which amounts to $.016 per broiler (Al-Rubaye *et al*., 2015; Aydin, 2018; Cook, 2000; Weaver, 1998). This affects production costs and thus the shelf-price of poultry products (Cook., 2000; Weaver., 1998).

$$\text{Cost of mortality} = \left(\text{price dayold chick} + \left(\frac{(\text{producer price} \times \text{weight at delivery}) - \text{price dayold chick}}{2} \right) - \text{cost of delivery} \right) \times \text{mortality rate}$$

(Equation 1 is reproduced from Gocsik *et al*.,2017).

$$\text{Cost of condemnation at slaughter} = (\text{producer price} \times \text{weight at delivery}) \times \text{condemnation rate}$$

(Equation 2 is reproduced from Gocsik *et al*., 2017).

Pathogenesis BCO-Lameness

BCO was formerly referred to as femoral head necrosis (FHN), proximal femoral degeneration, or bacterial chondronecrosis but the name was changed as researchers learned that proximal tibiotarsus and the fourth thoracic (T4) vertebra (with spondylitis) are also affected (Jiang *et al*., 2015; McNamee & Smyth., 2000). Broilers can grow to about 8 Lbs in 8 weeks (Wideman., 2016). This weight gain cannot be sustained without an equivalent increase in the size and strength of the skeletal frame of the bird. The mechanism of rapid bone growth is important to BCO and lameness. Growth of long bones in young broilers involves elongation of growth plates at both ends of the bone shaft/diaphysis, as well as an increase in the diameter as a result of the dynamic remodelling of the cortical bone (Wideman., 2016; Wideman & Prisby., 2013). Growing broiler birds see about four-times growth in length of femur and tibia, with a mid-shaft diameter increase that is three to five times the original width within the same time frame (Applegate & Lilburn., 2002; Bond *et al*., 1991; Wideman, 2016; Yair *et al*., 2012). Wideman (2016), notes that broilers are more susceptible to lameness than layers as the former has a

16

disproportionate weight gain ratio to skeletal structure maturation than it does cranial-caudal

redistribution of muscles mass (Wideman, 2016). Rapidly growing birds had a higher incidence

of lameness and efforts that reduce early growth lessens the disease in broilers (Wideman.,

2016). Dr. Wideman developed a wire-model flooring for inducing lameness in growing birds

(Wideman *et al*., 2012). This model creates shear stress in rapidly growing young birds, inducing

lameness with or without bacteria administration in water (Al-Rubaye *et al*., 2015; 2017;

Wideman *et al*., 2012, 2013, 2014; Wideman and Prisby, 2013; Wideman, 2016). Trials on the

wire-flooring system utilizing different broiler product lines revealed that they were all

susceptible to the incidence of BCO-lameness with some lines showing sire-effects (Al-Rubaye

et al., 2017; Wideman *et al*., 2013, 2014). The incidence of BCO lameness appears to begin with

mechanical micro-fracturing of poorly mineralized columns of cartilage cells (chondrocytes) in

the proximal growth plates of the femora and tibiae of early rapid-growing young broilers (Petry

et al., 2018; Wideman., 2016; Wideman & Prisby., 2013). The micro-fractures generate

osteochondrotic crypts that get colonized by hematogenously distributed opportunistic bacteria

(Al-Rubaye *et al*., 2015; Jiang *et al*., 2015; Mandal *et al*., 2016; Petry *et al*., 2018; Wideman.,

2016; Wideman & Prisby, 2013; Weimer *et al*., 2020). These bacteria come vertically from

broiler parent breeders to their chicks, or horizontally from a contaminated hatchery, and

eggshells (Stalker *et al*., 2010; Wideman., 2016). Bacteria may get translocated into the chick's

blood supply through the respiratory system, gastrointestinal tract, or integumentary system

(Figure 7; Al-Rubaye *et al*., 2015; 2017; Wideman *et al*., 2012, 2013, 2014; Wideman and

Prisby, 2013; Wideman, 2016). Translocated bacteria get hematogenously distributed to both

ends of the growth plate by the numerous terminal epiphyseal and physeal vascular plexuses

(Figure 8; Wideman, 2016; Wideman & Prisby, 2013). Since the blood supply of broilers is important to the incidence of lameness, it is crucial to study its anatomical composition.
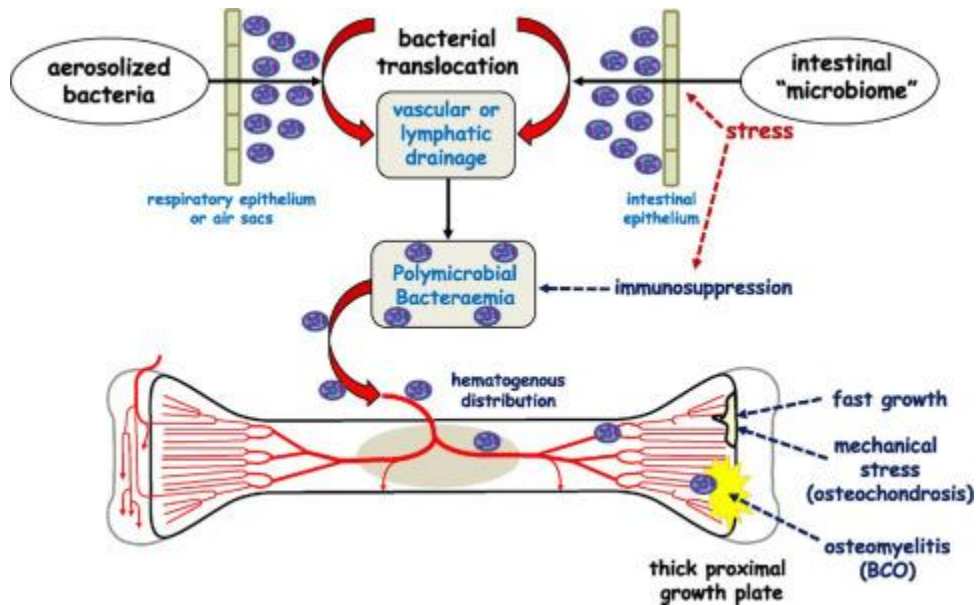
Figure 7. Routes of bacterial infections in rapidly growing birds. Bacteria transmitted to chicks from parent breeders, contaminated hatchery sources, eggshells, or bacteria that is translocated into bird's circulatory system via the integument, respiratory system or gastrointestinal tract gets distributed hematogenously and colonize the osteochondrotic crypts from microfractures resulting from mechanical stress (Image reproduced from Wideman, 2016; Wideman & Prisby, 2013).

Blood supply to proximal heads of rapidly growing broiler important to lameness.

There are three main structures in the blood supply of broiler long bones. These include (1) cartilaginous epiphysis (e), (2) the physis (p) also known as the growth plate (GP), and (3) the metaphysis (m). Cartilaginous epiphysis (e) is composed of articular cartilage (a) and hyaline cartilage (hy). The physis (p) or the growth plate (GP) comprises a cartilaginous matrix and long maturation columns of chondrocytes in consecutive layers with unique characteristics. The physis/gp spans the germinal chondrocytes (stem cells) of the resting zone (rz), to the highly mitotic proliferating zone (pz), the prehypertrophic zone (phz), and then the hypertrophic zone (hz). The metaphysis (m) is composed of the degenerative calcifying chondrocytes as well as the newly formed osteoid in the calcifying zone (cz). In the metaphysis, the spicules of trabecular

bone support the growth plate's scaffolding and the resorption zone (rez) wherein the trabecular bone thins out to form the medullary cavity (mc) of the diaphysis (d) (Figure 8 through 9; Wideman & Prisby, 2013; Wideman, 2012). In Figures 8 through 10, blood flows from the epiphyseal vascular supply (ev), travels either through epiphyseal vascular canals (ec) within the hy of the e or through the junctional canals (jc) moving down the growth plate. Branches of the ev can also terminate as epiphyseal vascular capillary complexes (evc) within the hz or they can become penetrating epiphyseal vessels (pev) that terminate as a penetrating vascular capillary plexus (pvp) and supplies blood to the rz, pz, and phz collectively called the maturing zone of the growth plate. The proximally traveling nutrient artery (ana) coming from mc divides severally inside the diaphysis (d) to form metaphyseal vessels (mv) within the m. The mv terminates as metaphyseal vascular capillary plexuses (mvp) and supplies the czi. The pvp or mvp does not usually cross the hz like the transphyseal vessels (tp) does. The pvp and mvp loop back around to form fenestrated capillaries that return as venules coursing through the same canal (Figures 8 – 10; Wideman., 2016; Wideman & Prisby., 2013).
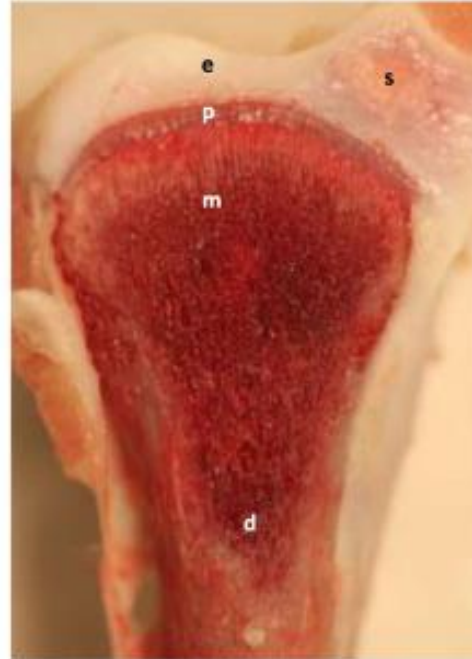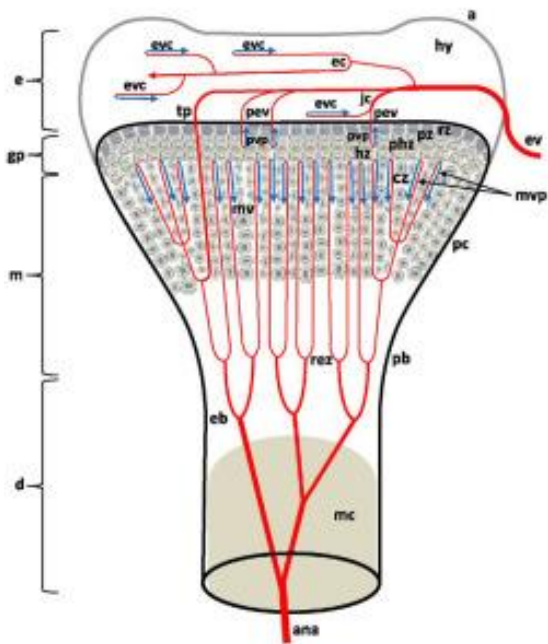
Figure 8. The diagram on the left pane and photograph on the right panel shows the blood supply and the anatomical structures of the long bone in growing broilers (Reproduced from Wideman & Prisby., 2013; Wideman., 2016).
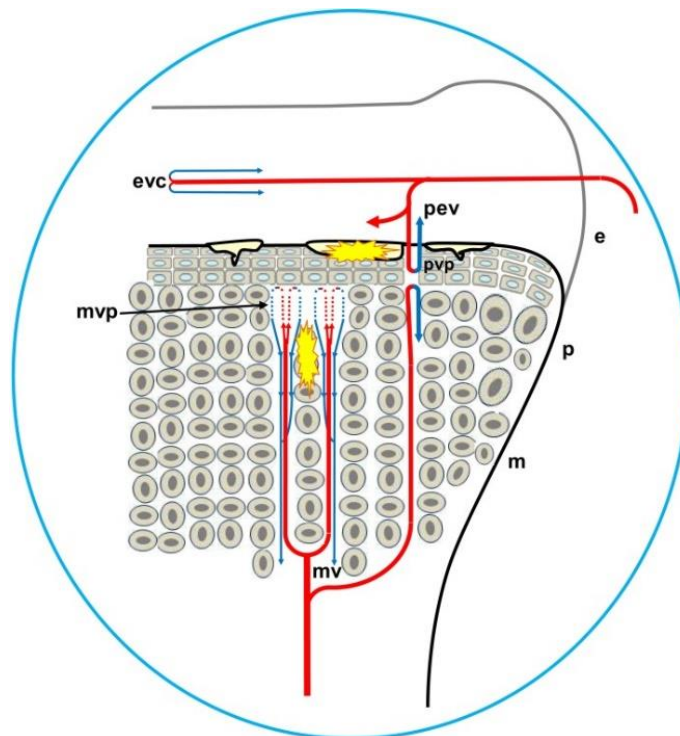
Figure 9. Diagram of the femoral proximal head illustrating the formation of osteochondrotic clefts/crypts at the boundary between the growth plate and the epiphysis (Wideman &Prisby., 2013).
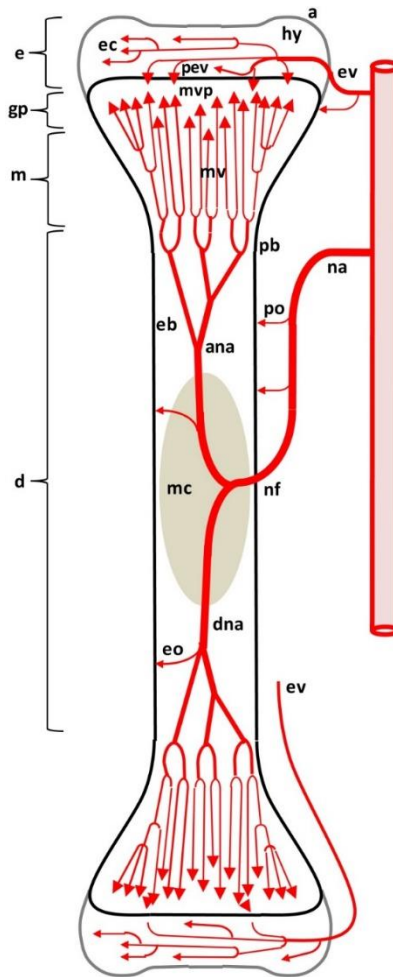
Figure 10. Arterial blood supply to the leg bone of a growing bird (Reproduced from Wideman, 2016).

Vertebral anatomy and blood supply in broilers important to BCO lameness

Of the five thoracic vertebrae in broilers, the fourth thoracic vertebra (T4) moves freely and

separates the notarium and synsacrum (Figure 11; Baumel *et al*., 1993; Wideman, 2016).

Wideman (2016), describes T4 be fused to the caudal surface of notarium and the cranial surface

of synsacrum (Wideman, 2016). He mentioned that the fusion of these bones is only partial until

the birds reach sexual maturity, perhaps to allow room for the continuing longitudinal growth of

the vertebral body in young birds (Wideman, 2016; Wideman and Prisby, 2013). The structure

and position than T4 with respect to the more rigid/inflexible cranial T3 and caudal T5

21

encourages the erosion of epiphysis and physis of T4 which is important to the incidence of

vertebral BCO (Wideman., 2016).
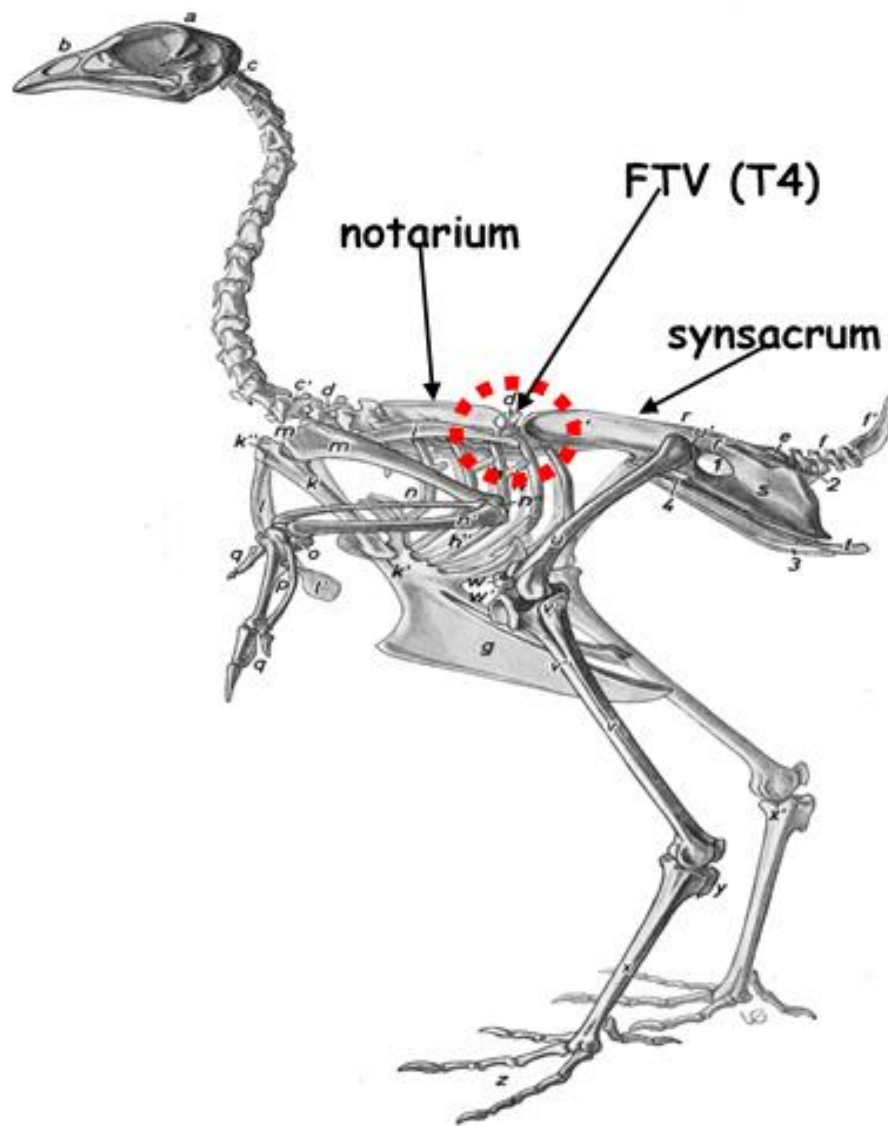
Figure 11. The skeleton of a bird. The image highlights the exposure of the very flexible thoracic vertebrae 4 or T4 (Image reproduced from Wideman, 2016; Wideman and Prisby, 2013).

The T4 vertebral body in rapidly growing broiler chickens is prone to various deformities and

non-inflammatory mechanical collapse. They are also susceptible to downward rotation

(subluxation), and scoliosis/lateral displacement (Figure 12; Wideman, 2016; Wideman and

Prisby, 2013). The clinical presentation of T4 subluxation is called spondylolisthesis or spine

slippage or "kinky back," a condition in which spinal cord compression leads to paraplegia, a

hock- or rumps sitting position, and permanent immobility (Figure 12; Wideman, 2016;

Wideman and Prisby, 2013).
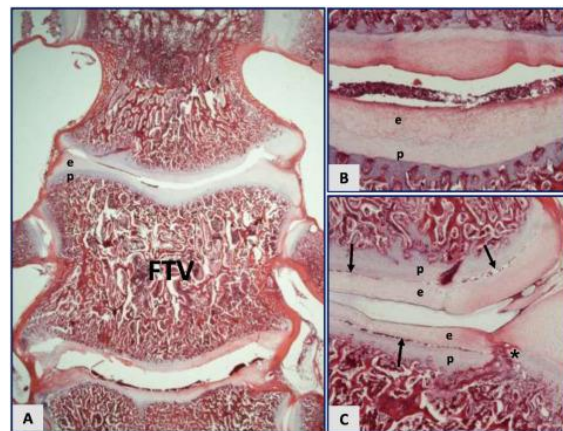
Figure 12. Broiler bone histopathology image for formalin-fixed 5 µM sections from 5-week
stained with hematoxylin and eosin. (A) Normal T4 (B) The boundary between the epiphysis (e)
and physis (p) normally should be seamless. (C) In broilers that appear to be clinically healthy
narrow osteochondrotic clefts or voids (arrows) containing cellular debris can be detected at the
boundary between the epiphysis (e) and the physis (p). Osteochondrotic clefts may interrupt the
local vasculature, cause distortions in the epiphyseal-physeal cartilage (*) and constitute wound
sites that are favourably colonized by opportunistic bacteria (modified from Figure 6 in
McCaskey *et al*., 1982)**.**

In Figure 12 A-C, T4 from apparently healthy broiler birds present uninfected minor

microfractures, osteochondrotic clefts, and subclinical deformations in their epiphyseal and

growth plate layers. At the time when these findings were reported, only a few healthy birds

were diagnosed as clinical spondylolisthesis or kinky back (McCaskey *et al*., 1982; Wideman.,

2016; Wideman & Prisby, 2013; Wise., 1970). Kinky back (KB) may have genetic backgrounds

as studies conducted on broiler lines deliberately selected for KB showed the incidence of

spondylolisthesis. In another study where birds with KB were nursed back to health and bred for

two generations, the offspring presented with KB (Khan., 1977; Wideman., 2016; Wideman & Prisby., 2013). In summary, the thoracic vertebra of rapidly growing meat-type chicken undergoes wear and tear that results in skewed vertebral bodies. This condition combines with numerous microfractures in the epiphysis and the physis of the vertebra and may stay non-clinical or may even progress to the non-infectious and heritable spondylolisthesis (Wideman., 2016; Wideman & Prisby., 2013). Non-infectious non-inflammatory osteochondrotic lesions are by themselves not considered the main initiator of lameness in broilers. The collection of crypts or clefts resulting from microfractures, packed with exposed collagen structures and fed by good vascularities, maybe good infection sites for opportunistic bacteria microbes that play a role in the onset of vertebral BCO (McNamee *et al*., 1998; Wideman, 2016; Wideman and Prisby, 2013).

Bacteria and Lameness

The etiology of BCO lameness is not fully understood, but bacteria are highly involved in the incidence of the disease. As aforementioned, the unsupported mass of rapidly growing birds causes microfractures and hence osteochondritic crevices. The crevices contain exposed collagen matrices that may favor inhabitation and colonization by hematogenously distributed opportunistic bacteria from various sources (Wideman & Prisby, 2013; Wideman, 2012; 2015; 2016). As discussed above, the vessels for blood supply to tibia, femur, and vertebra, narrows into capillaries. These capillaries are networks of fenestrated endothelium large enough to allow the translocation of some blood components, including bacteria, into the cartilaginous matrices (Wideman & Prisby, 2013, Wideman *et al*., 2012; 2013; 2015; 2016). Translocated bacteria adhere to exposed collagen complexes obstruct the epiphyseal and metaphyseal blood vessels (Wideman, 2016; Wideman & Prisby, 2013). Such obstruction permits bacterial foci formation

and occludes pathogens from the broiler's responses or antibiotics (Wideman, 2016; Wideman & Prisby, 2013). Multiple opportunistic microbes, including *Staphylococcus spp.*, *Escherichia coli*, *Enterococcus cecorum*, *Salmonella spp.*, have been isolated from BCO lesions (Al-Rubaye *et al.*, 2012; 2015; 2017; Dinev, 2009; Jiang *et al.*, 2015; Joiner *et al.*, 2005; Mandal *et al.*, 2016; Martin *et al.*, 2011; Stalker *et al.*, 2010; Thorp *et al.*, 1993; Wideman., 2016, Wideman and Pevzner, 2012, Wideman and Prisby., 2013; Wideman *et al.*, 2012; 2013; 2015; Wijesurendra *et al*; 2017). While we need to characterize BCO isolates to specify their role(s) in the incidence of lameness, we also must ascertain where they are coming from and how they are getting into the blood. In Figure 7, we proposed that bacteria important to be BCO may be translocated from the respiratory tract, the integument, or the gut microbiome (Wideman, 2016). It is therefore incumbent to analyze the microbial populations of the broiler chicken and their significance to the infection process of BCO.

Microbiota and Lameness

Chicken's natural microflora is associated with enrichment of intestinal villus and crypt morphology (Jiang *et al.*, 2016; Mandal, *et al.*, 2016; Yeoman, *et al.*, 2012). Analysis of villus length and macroscopic pathology in lameness revealed that villus length improved with probiotic treatments than the control group (Al-Rubaye, *et al.*, 2020). The gut microbiome promotes broiler growth by boosting energy-filled short-chain fatty acids. It is also involved in nutrient absorption, detoxification, polysaccharides metabolism, immune system regulation, and the general well-being of birds (Clavijo *et al.*, 2018; Yeoman, *et al.*, 2012). Microbiomes in organs other than gut are also important to animal health. Studies of organ microflora dysbiosis in humans, for example, are implicated in the pathogenesis of a host of diseases including colorectal cancers, inflammatory bowel diseases, and so on (Mandel *et al.*, 2016). Microbial

communities of the gut or other tissues important to lameness and other disorders are not fully characterized or understood (Jiang *et al*., 2015; Yeoman, *et al*., 2012). However, most chicken microbiome analysis seems to lean towards the gut compared to the blood, trachea, or feces, and least of them the bones (Jiang *et al*., 2015; Lim *et al*., 2015; Mandal *et al*., 2016; Sohail *et al*., 2015). Jiang *et al*. (2015) suspect that multiple bacterial species shuttle mainly from the gut communities into the bloodstream forming niches across tissues. They emphasized the importance of the gut microbes despite awareness about microbial communities present in yolk remnants, and respiratory tracts in apparently healthy birds (Jiang *et al*., 2015). There are still important questions to be answered: 1) Are all translocated bacteria commensals in BCO? 2) How do commensals with benefits become pathogenic? 3) Do some of these microbes come in as commensals evade immune effectors and then develop virulence? 4) Is translocation an acquired virulence factor? 5) Is translocation a synergistic property that drives further translocation? and 6) How and where can the immune system be boosted to better handle these invasions? In attempts to characterize tissue (including gut) microbiomes, Mandal *et al*. (2016) sampled the blood performing deep sequencing and analysing bacterial 16S rRNA sequences for bacterial communities in 240 healthy birds and 12 lame birds. They discovered that 97% of the phyla level communities in chicken blood was Proteobacteria (60%), Bacteroidetes (14%), Firmicutes (11%), Actinobacteria (10%), and Cyanobacteria (2%) (Mandal *et al*., 2016). These characterizations were determined from about 40 operational taxonomic units (OTUs) regardless of age, host physiology, or environmental conditions. Linear discriminant analysis effect size (LEfSe) showed significant population of *Staphylococcus, Microbacterium,* and *Granulicatella* in lame vs healthy bird's blood (Mandal *et al*., 2016). Wei *et al*. (2013) analyzed the intestinal microbiome of broilers using all available published and unpublished data. They identified 915

OTUs equivalent to species that delineated with a 3% phylogenetic distance. The species were grouped into 13 phyla comprising 70% Firmicutes, 12% are Bacteroidetes, and 9% proteobacteria. These data made up 90% of all phyla. They identified 117 genera, a majority of which include *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides*. The main representative at the genus level for Firmicutes was the ethanol metabolizing *Ethanoligenes* bacteria. While *Desulfohalobium* was the most represented Proteobacteria. Actinobacteria (with 1% of sequences revealing Bifidobacterium) was represented in minute quantities. Other phyla with small representations include Cyanobacteria, Spirochaetes, Synergisteles, Fusobacteria, Tenericutes, and Verrucomicrobia (Wei *et al*., 2013; Clavijo *et al*., 2018). Understanding the distribution of microbes may explain the routes and conditions necessary for bacterial colonization important to BCO and other dysbiosis associated diseases. Clavijo *et al*. (2018) analyzed the redistribution of microbial communities across the tissues of the gastrointestinal tract (GIT) and found that the taxonomic profiles described for different parts of this system vary. Factors include but are not confined to diet, sex, genetics, use of antimicrobials, and sampling techniques. The chicken crop and gizzard are mainly populated by the genus *Lactobacillus* and Clostridiaceae family. The crop environment promotes bacteria metabolization of starch and fermentation of lactate. In the gizzard, gastric juices, pepsin, and hydrochloric acid acidify this environment lowering the fermentation and general bacterial activity. The small intestine contains the highest bacterial cell count of mainly *Lactobacillus* (70%), *Enterococcus*, and Clostridiaceae (Clavijo *et al*., 2018). They also noted that the ceca are considered the richest in species diversity to some extent for its capacity to hold food for 12 to 20 hours as well as for its major water reabsorption role, the concentration of urea, and fermentation of undigested carbohydrates from the intestines. The ceca are rich in the phyla Firmicutes, Bacteroides, and

Proteobacteria, and Clostridiaceae. Further, the abundant microorganisms of unknown phylotypes belong to Firmicutes making this phylum to be of special interest. The gut microbiome of chickens contains taxa with *Campylobacter jejuni*, *Campylobacter coli, Salmonella enterica, Escherichia coli,* and *Clostridium perfringens* that may be harmful to birds and human (Clavijo *et al*., 2018; Oakley *et al*., 2014). *Campylobacter spp* is considered harmful to humans but not birds. *Salmonella enterica* may be deadly to birds and humans depending on the age of the bird, the serotype of the *Salmonella* spp, and the health condition of the bird. *Salmonella spp* is of lower prevalence. *Escherichia coli,* in chicken intestines, also has a low abundance thorough out the lifespan of apparently healthy birds. Avian pathogenic *Escherichia coli* (APEC) has virulence factors important to various diseases in birds (Clavijo *et al*., 2018; Oakley *et al*., 2014). Jiang *et al*. (2015) surveyed the microbial communities of 97 femoral or tibial heads from normal and lame broilers representing various ages, lines, lesion types, floor types, to understand the long bone's microbial importance to BCO. This study revealed a 91% prevalence of Proteobacteria, 6% Firmicutes, and ~2% Actinobacteria phyla. Several other phyla represented at lesser amounts, include Tenericutes, Bacteroidetes, Acidobacteria, Verrucomicrobia, Nitrospirae, and Cyanobacteria that accounted for less than 0.4% of the total phyla count. The overrepresented species were of *Staphylococcus spp* (Jiang *et al*., 2015). Table 3 accounts for differences between treatments recorded from these analyses (Jiang *et al*., 2015). We have isolated multiple Staph species from birds that develop BCO on our facilities as well as sick broilers from commercial farms (Al-Rubaye *et al*., 2012; 2015; 2017; Ekesi, 2020; Shwani *et al*., 2020). Jiang *et al*. concluded that diminished species diversity is associated with a higher degree of BCO lesions and lameness (Jiang *et al*., 2015). Further analysis of the BCO microbial communities is needed for understanding the etiology of lameness and potential remediation.

Table 3: Comparison of microbial communities in different groups (Table reproduced from Jiang *et al*., 2015)

Groups	R-value	P-value
Left vs. Right Leg	0.033512634	0.156
Femoral vs. Tibial Samples	0.216799541	0.001*
Clinically Healthy vs. Lame	-0.041557977	0.714
Individuals	0.626107125	0.001*
Age (7 through 49 days)	0.090237558	0.003*
Litter vs. Wire Flooring	0.02794451	0.248
Lesion Type (normal femur, normal tibia, FHS, FHN, THN, THNsc)	0.4393	0.001*
Line B vs. Line D	0.029131713	0.089

*significantly different ($p \leq 0.05$).

Stress, Immune responses, and BCO

In lameness literature, the role of stress and immune responses revolves around microbial proliferation important to the incidence of BCO that causes lameness in affected birds. The incidence levels of lameness, the weight-induced microfractures, and subsequent bacterial infection of physis and epiphysis of birds may initiate BCO (McNamee & Smyth, 2000; Wideman & Prisby, 2012; Wideman *et al*., 2012; Wijesurendra *et al*., 2017). Environmental stressors and septicemic pathogens, such as chicken anaemia virus (CAV) or infectious bursal disease virus (IBDV) can cause immunosuppression that furthers the proliferation of microbes that leads to the formation of BCO lameness (Wideman, 2016; Wideman & Prisby, 2013). The wire-flooring model for inducing lameness in broilers causes chronic stress that results in immunosuppression of broilers as shown by their elevated blood corticosterone levels (Wideman & Prisby, 2012; Wideman *et al*., 2012). Injection of glucocorticoids, specifically dexamethasone, resulted in femoral head necrosis (FHN) lesions, and the intravenous administration of prednisolone also causes epiphyseolysis; separation of the epiphysis from the physis. (Cui *et al*.,

1997; Durairaj *et al*., 2012; Wideman and Prisby., 2013). In many of the dexamethasone lameness trials, the responses were not typical of those recorded in spontaneous BCO cases. Particularly, the administration of dexamethasone shrunk growth rates in broiler birds even at the lowest dose that induced lameness in the birds (Wideman and Pevzner., 2012, Wideman and Prisby., 2013). In turkey osteomyelitis complex (TOC), environmental stressors were associated with the eruption of opportunistic pathogens harbored sub-clinically in the proximal tibia of turkeys (Huff *et al*., 1998-2000; 2006; Wideman and Pevzner., 2012, Wideman and Prisby, 2013). Rodgers JD *et al*. (2006) developed an ELISA with nuclease protein as an antigen to capture *S. aureus*-specific antibodies produced in response to bacteria administered to 500 broiler birds by aerosol. Bacteria were administered on Day 1 post-hatch with or without co-infectors to induce BCO lameness. Co-infectors were CAV and IBDV. They found 71% of serum samples from aerosolized *S. aureus*-treated birds had antibodies for nuclease protein. Only 35% of serum samples had antibodies for nuclease when there was no co-infection (Rodgers *et al*., 2006). The co-infection was reported to have resulted in profound effects until day 42 (Rodgers *et al*., 2006). These findings are important as they highlight adaptive immune/ humoral responses are activated in the birds infected by *S. aureus,* and co-infection with CAV and IBDV drives the development of nuclease-specific antibodies for up to 42 days in broilers. Further, because in our *Staphylococcus* BCO lameness model, we see an increasing amount of lameness around this period, this needs to be examined further. We are working on characterizing the pattern of innate immunity important to be BCO and lameness in our lab using phagocytosis assays for BCO bacteria and chicken macrophage in directed genome evolution trials (Zaki, 2020 Dissertation). Lowder *et al*. (2009) wrote that *S. aureus* isolates gained mobile genetic elements coincident with the jump from humans to birds to adapt to the avian ecosystem. Consequently,

they showed resistance to heterophil phagocytic killing in *in vitro* assays. A better understanding of this mechanism is important, as it may help explain how BCO isolates bypass the immune factors in the disease process of birds that get infected.

Microbiology of Common Pathogenic BCO isolates

Even though some of the findings described herein are not particular to chickens, the mechanisms of pathogenesis of each microbe described may be important in the incident of BCO and lameness.

Staphylococcus spp.

About 60 Staphylococcus species have been identified (Szafraniec *et al*., 2020). All *Staphylococcus spp* can cause diseases (Crossley *et al*., 2009). The staph genome is dynamic; the assortment of virulence factors varies by species and strains.

Staphylococcus agnetis

S. agnetis is a common cause of BCO lameness (Al-Rubaye *et al*., 2015; 2017). The species was named after Europe's first female veterinary surgeon, Agnes Sjöberg (1888–1964), who struggled her way into the profession despite resistance from her male colleagues. *S. agnetis* is a Gram-positive-staining, and a coagulase-variable bacterium (Adkins *et al*., 2017; Szafraniec *et al*., 2020; Taponen *et al*., 2012). It is generally coagulase-negative after 4 hours, but over 25 % of the isolates show coagulase-positivity after 24 hours. *S. agnetis* cells are facultatively anaerobic, non-spore-forming, non-motile cocci which grow either singly, or in pairs or small clusters. The bacteria colonies may grow to 3mm after 24 hours of incubation at 37 °C. The bacterium is catalase-positive and oxidase-negative. It is round, opaque, smooth, non-hemolytic, and light grey on bovine blood agar. *S. agnetis* is resistant to polymyxins, deferoxamine, and lysozyme. But it is susceptible to lysostaphin and novobiocin. This bacterium is negative for

clumping factors and hydrolyses DNA at 37 °C giving off a degradation halo hue. *S. agnetis* metabolizes and produces acids aerobically with D-glucose, D-fructose, D-mannose, lactose, sucrose, and D-ribose. Phylogenies based on 16S rRNA sequence analysis, two housekeeping genes (*rpoB* and *tuf*), or DNA fingerprinting with amplified fragment length polymorphism, show *S. agnetis* forms a separate branch within the *Staphylococcus* genus (Al-Rubaye *et al.,* 2015; Adkins *et al*., 2017; Taponen *et al*., 2012). The closest species that have also been recovered in BCO lameness include *S. hyicus* and *S. chromogenes*. *S. agnetis* are commonly isolated in milk samples in the incidence of bovine intramammary infections that results in subclinical or mild clinical mastitis in dairy cattle, and more recently BCO lameness lesions (Adkins *et al*., 2017; Al-Rubaye *et al*., 2015; 2017; Taponen *et al*., 2012). *S. agnetis* is the main BCO isolate on our farm and is induces lameness to statistically significant degrees using wire or litter- flooring. The role of *S. agnetis* in BCO and lameness in infected birds will be discussed further below.

Staphylococcus aureus

S. aureus is a coagulase-positive, Gram-positive bacterium. Although the production of coagulase differentiates *S. aureus* from other Staphylococcal species, the coagulase gene (*coa*) is not associated with virulence (Crossley *et al*., 2009). *S. aureus* is non-motile, non-spore-forming, catalase-positive, and oxidase-negative. *S. aureus* is a facultatively anaerobic bacterium. The bacterium has cell-bound clumping factors. It is slightly tolerant of sodium chloride. It ferments mannitol and produces hyaluronidase. The physical appearance may differ by media. The reason S. *aureus* causes more incidence of BCO and lameness in birds that develop the disease is not known than any know BCO isolate. This implies, however, that *S. aureus* does have an inherent capability to cause damage (McNamee *et al*., 2000). *S. aureus* infects several various hosts. Like

S. agnetis, S. aureus is also implicated in cattle mastitis. *S. aureus* also causes childhood

osteomyelitis, and hospital or community-acquired infections (Adkins *et al*., 2017; Al-Rubaye *et al*., 2015; McNamee *et al*., 2000). Almost all recovered *S. aureus* recovered from sick animals or

humans have virulence capsules that inhibit them from being phagocytized (McNamee *et al*.,

2000). It has been suggested that these capsules facilitate adherence to chicken cartilage, but the

actual role in bone and joint infection is not understood (McNamee *et al*., 2000). *S. aureus* is the

most predominant of all the disease-causing Staphylococci (Crossley *et al*. 2009). Compared to

S. epidermidis (another common BCO isolate), *S. aureus* contains 18 distinct genomic islands

that house virulence genes that disrupt host defenses (Foster, 2005; Gill *et al*., 2005). According

to Crossley *et al*. (2009), much of the work done to characterize *S. aureus* has been on a limited

number of strains derived from a primary strain NCTC 8325. This isolate was retrieved

originally in 1960 from a sepsis patient in the UK and is the reference genome for NCBI. There

are 11,870 *S. aureus* genome assembly entries (Crossley *et al*., 2009). Different strains of *S.

aureus* have been derived from NCTC 8325 for various purposes, where the derivative strains

still preserve the ancestral lineage (Crossley *et al*., 2009). Comparing derived isolates with

clinical *S. aureus,* Φ13 is integrated into the *att* site on *hlb* gene that normally expresses β-toxin

interrupts its function (Crossley *et al*., 2009). This may be observed in strain NCTC 8325-4

where the temperate Φ13 is excised, *hlb* gene production of β-toxin is restored. This is important

not only because of the β-toxin negative effect of Φ13 integration but also because phage that

utilizes the *att hlb* site alters functions for a combination of genes (*sea*, *sak*, *scn*, *chp*) with

virulence factors for disruption host immune functions (Crossley *et al*., 2009). More

discrepancies and connotations exist between derived and clinical *S. aureus* strains, but these

notions are not without controversy. Studies suggest that mutations exist in at least *tcaR* and

rsbU genes that regulate the expression of specific virulence factors in all NCTC 8325 derivatives making them references and models for studying the regulation of pathogenicity (Crossley *et al*., 2009; Sassi *et al*., 2014). Crossley *et al*. (2009) utilized genome-level transcriptional profile analysis of NCTC 8325-4 strain with clinical isolate UAMS-1 and argues that there are no such mutations in either *tcaR* or *rsbU*. This study revealed that over 300 genes were expressed in a strain-dependent manner comparing NCTC 8325-4 and UAMS-1 strain (without *tcaR* or *rsbU*) (Crossley *et al*., 2009). About half of these genes are under SigB regulon control (Cassat *et al*., 2005; Crossley *et al*., 2009). A correlation study was performed in over 400 *S. aureus* isolated from different infections and geolocations for variable genes and virulence with pulse-field gel electrophoresis (Booth *et al*., 2001; Crossley *et al*., 2009). Five of the 90 identified lineages were important to 65% of infections (Booth, *et al*., 2001; Crossley *et al*., 2009). Collagen-binding adhesin (*cna*) gene was in three of the five lineages. Booth *et al*. (2009) indicated, however, that no *cna*-containing lineages encoded the fibronectin-binding protein gene *fnbB*. Lack of *fnbB* does not eliminate the relevance of *S. aureus* fibronectin binding capability as they still possess a highly conserved *fnbA*. This does not make *fnbB* irrelevant either, as this gene is positioned in a location termed *region of difference* (RD5). A region that houses the surface-associated protein *SasG* and the regulatory elements *sarT* and *sarU* (Crossley *et al*., 2009). In a similar study of 334 *S. aureus* strains, 33 genes were determined important for virulence; *cna* was one of seven genes prevalent in invasive species (Crossley *et al*., 2009). Otsuka, *et al*. studied15 clonal clinical community-acquired methicillin-resistant *S. aureus* (CA-MRSA) particularly to osteomyelitis, and necrotizing pneumonia. They found *cna* in nine of them (Otsuka *et al*., 2006). The 15 CA-MRSA strains were either categorized as pandemic (Sequence type ST30) or continent-specific (ST1, ST8, OR ST80). Five of the *cna*-specific

strains were classified as pandemic CA-MRSA strains; they encoded gene *bbp* for adhesin that binds sialoprotein in bones and lacked *fnbB*. This absence, Otsuka *et al*. (2006) noted, implies the lack of RD5-linked genes (*sasG* and *sarT)* in the pandemic strain. The combination of *bbp* and *cna* has been considered for the potential to galvanize pathogenicity to the point of pandemicity (Crossley *et al*., 2009). Genome-level analysis suggests that certain *S. aureus* strains may exhibit higher virulence and that predominantly clonal populations possess the ability to cause disease. However, this is not always the case. Feil *et al*. (2003) performed a genome-scale microarray study that analysed core variable (CV) genes scattered throughout the chromosome and identified10 dominant lineages of *S. aureus* that lacked genes for diseases and were not linked to infectious processes. Crossley *et al*. (2009) added that multilocus sequence typing (MLST) failed to separate commensal versus invasive *S. aureus* based on clonal lineages. Feng *et al*. (2008) did a comparative genomic analysis of *S. aureus* pathogenic and non-pathogenic strains as well and argued that since no genotypic difference was recorded between the two groups, that observed differences between pathogenic and non-pathogenic *S. aureus* are a function of its state. One explanation for the divergent findings is that *S. aureus* is an opportunist; isolates recovered from a sick host may say more about the state of the host than it does the strain isolated. Conversely, *S. aureus* strains retrieved from healthy hosts may be important to invasive diseases (Crossley *et al*., 2009). Study and analysis of clinical isolates and NCTC 8325 derived strains in many *in vivo* and *in vitro* models show many structures, their interaction, and disease processes of *S. aureus*. The main classes of virulence factors in *S. aureus* are described below.

1) ***Surface Virulence Factors***: In *S. aureus*, cell surface-linked pathogenicity involves exposed cell wall structures like teichoic acid (WTA), lipoteichoic acid (LTA), lipoprotein, and

peptidoglycan (PG). The host immune systems may recognize such structures and express cytokines and chemokines in response. These structures collectively play important roles in helping *S. aureus* evade host defenses, and enhance colonization (Crossley *et al*., 2009). Studies suggest that WTA is important to nasal colonization, as this precedes many *S. aureus* infections (Baur *et al*., 2004; Crossley *et al*., 2009; Weidenmaier *et al*., 2004; van Dalen *et al*., 2020; Winstel *et al*., 2015). *S. aureus* strains without these structural factors had poor adherence to endothelial frame and were not virulent in rabbit endocarditis models (Weidenmaier, *et al*., 2004; 2005; Wertheim, *et al*., 2005). LTA and PG produce inflammatory responses that recruits neutrophils that trigger the onset of septic shock (Crossley *et al*., 2009; Fournier& Philpott., 2005; Ginsburg, 2002; Kengatharan *et al*., 1998). The involvement of TLR 2 in the host response to LTA and PG are inconclusive (Crossley *et al*., 2009). In *S. aureus* LTA (*ltaS* gene) controls autolysin activity, surface hydrophobicity, and therefore biofilm formation in a strain-dependent manner (Crossley *et al*., 2009; Fedtke *et al*., 2007).

2) ***Surface Adhesins:*** *S. aureus* cell surface adhesins include a variety of proteins. Bone sialoprotein-binding protein (*bbp*) that binds sialoproteins in bones and may result in *Staphylococcus*-related osteomyelitis and arthritis (Campoccia *et al*., 2009; Crossley *et al*., 2009; Persson *et al*., 2009; Tung *et al*., 2000). *S. aureus* expresses serine–aspartate repeat proteins *sdrC, sdrD, sdrE* that contiguous and have adhesive properties (Crossley *et al*., 2009). *SdrC* facilitates interactions with NRXN1 in the host extracellular matrix to promote bacterial adhesion and consequently pathogenesis (Crossley *et al*., 2009; Askarian *et al*., 2016; 2017). *sdrD* promotes the adhesion to host DSG1 decreasing bacterial clearance by posing resistance against neutrophil killing in the blood (Crossley *et al*., 2009; Askarian *et al*., 2016; 2017). *SdrE* interacts with host complement factor *H/CFAH* and improves the resistance to bacterial killing

by innate immune components in blood and therefore decreasing bacterial clearance (Crossley *et al*., 2009; Sharp *et al*., 2012). *SdrE* protein interacts with host complement regulator *C4BPA* inhibiting bacterial opsonization and killing by interrupting the activation of the host classical pathway (Crossley *et al*., 2009; Hair, *et al*., 2013). Protein A (*spa*) plays a role in the inhibition of the host innate and adaptive immune defenses, as they bind and capture the Fab and Fc arms of immunoglobulins (Ig) with their five Ig-binding domains. As a result, *S. aureus* is therefore protected from opsonophagocytosis (phagocyte killing of bacteria). Down the line, host B-cell response is averted as there is a decrease of Ig-secreting cell proliferation in the bone marrow and a decreased long-term antibody production (Crossley *et al*., 2009; Forsgren & Sjöquist., 1966; Graille *et al*., 2000; Moks *et al*., 1986). Protein A hinders osteogenesis by (i) preventing osteoblast proliferation, (ii) expression of alkaline phosphatase, (iii) expression of type I collagen, as well as (iv) expression of osteopontin and osteocalcin. Protein A is a pro-inflammatory factor in the lung. It binds and activates tumor necrosis factor-alpha receptor 1*TNFRSF1A* (Crossley *et al*., 2009; Forsgren & Sjöquist., 1966; Graille *et al*., 2000; Moks *et al*., 1986; Widaa *et al*., 2012). *S. aureus* produces surface-associated molecules and secreted proteins that bind fibronectin (Crossley *et al*., 2009). The two main classes of these proteins are fibronectin-binding proteins A (*fnbA*) and B (*fnbB*). In the chromosome of *S. aureus,* the genes for *fnbA* and *fnbB* are contiguous and either of them are is sufficient for proper binding of fibronectin; however, the only *fnbA* can mediate platelet clumping (Crossley *et al*., 2009; Heilmann, *et al* 2004; Peacook *et al*., 2000). The *fnbA* binds fibrinogen and elastin while *fnbB* binds elastin but they both are associated with invasive diseases (Peacook, *et al*., 2000). Collagen adhesin (*cna*) introduced earlier facilitates *S. aureus* adhesion to collagenous tissues such as cartilages that may be important to bone disorders (Crossley *et al*., 2009). The *cna* protein is an

important virulence agent in many different animal models of *Staphylococcal* infections including arthritis, endocarditis, and keratitis. It stops the activation of the classical complement pathway by interacting and interfering with host C1q by forming a link between C1r with C1q (Kang *et al*., 2013; Patti *et al*., 1993; 2002; Rhem *et al*., 2000; Xu *et al*., 2004). Fibrinogen binding factors or clumping factors A (*clfA*) and B (*clfB*) are expressed on the cell surface of *S. aureus* and are important to infections. Clumping factors A *(clfA)* enables the specific bacterial binding to the gamma-chain of human fibrinogen. This stimulates aggregation of bacteria which inhibits phospholipase A2 from bacterial phospholipid hydrolysis and hence inhibited the killing and clearance of bacteria (Crossley *et al*., 2009). *ClfA* plays a role in the pathogenesis of sepsis and septic arthritis (Crossley *et al*., 2009; Dominiecki & Weiss, 1999; O'Brien *et al*., 2002; Palmqvist *et al*., 2004; Siboo *et al*., 2001). Fibrinogen-binding protein B *clfB* facilitates bacterial attachment to the alpha- and beta-chains of human fibrinogen, thereby causing aggregation of bacterial in clumps. It interacts with cytokeratin k10 and is partly implicated in the binding of highly keratinized squamous epithelial cells from the nasal cavity. *clfB* binds the mice model cytokeratin. And it causes platelet aggregation in humans (Crossley *et al*., 2009; Dominiecki & Weiss, 1999; O'Brien, *et al*., 2002; Palmqvist, *et al*., 2004; Siboo, *et al*., 2001). *S. aureus* also expresses *sas* genes; *sasA* is involved in platelet binding, and *SasG* is important for biofilm formation (Crossley *et al*., 2009).

3) **Secreted Adhesins:** *S. aureus* possesses a group of secretable adhesive proteins collectively termed SERAM (secretable expanded repertoire adhesive molecules). Extracellular fibrinogen-binding protein (*Efb*) is a SERAM that is important to virulence (Crossley *et al*., 2009; Posner *et al*., 2016; Pickering *et al*., 2019). *Efb* protein interacts with the alpha chain of fibrinogen and fibrin product promoting interrupting normal platelets-fibrinogen functions that

result in the inhibition of platelet aggregation (Crossley *et al*., 2009; Posner *et al*., 2016). *Efb* also forms a fibrinogen protective shield around the bacteria and inhibits phagocytic clearance by the host. It does this by binding the C3b complement placed on its surface for opsonization with the C-terminal end and then attaches to fibrinogen via its N-terminal (Bodén & Flock,1994; Crossley *et al*., 2009). *S. aureus* also secretes *Atl* and *Aaa* proteins. *Atl* protein is the major autolysin of *S. aureus*, which has been implicated in cell separation of newly divided *S. aureus* (Crossley *et al*., 2009; Singh, 2014; Sugai, *et al*., 1995). Even though *Atl* participates in cell wall turnover, this does not contribute to virulence (Takahashi *et al*., 2002). *Aaa* (Alias Sle1), binds host proteins like fibrinogen, fibronectin, and vitronectin resulting in pathogenicity in mouse models (Kajimura, *et al*., 2005; Crossley *et al*., 2009).

4) **Surface Polysaccharides:** *S. aureus* expresses capsular polysaccharides (*cap* genes), and polysaccharide intercellular adhesin (*icaADBC*) (Crossley *et al.,* 2009; Visansirikul *et al*., 2020). Polysaccharides capsules coat C3b decreasing opsonization and phagocytosis of *S. aureus* by phagocytic cells (Crossley *et al*., 2009; Visansirikul *et al*., 2020). Polysaccharide intercellular adhesin is important to *S. aureus* biofilm formation *in vitro* (Crossley *et al*., 2009).

5) **Extracellular Virulence Factors/ Exotoxins**: *S. aureus* makes several extracellular toxins important for diseases. These toxins are group as exfoliative toxins, enterotoxins, and TSST-1 (Bukowski *et al*., 2010; Crossley *et al*., 2009). *S. aureus* encodes toxins in a strain-dependent manner. A number of the predominant *S. aureus* diseases produce specific toxins. Exfoliative toxins are common to *Staphylococcal* scalded skin syndrome (SSSS), bullous impetigo is due to enterotoxins in staph food poisoning, and TSST-1 is associated with toxic shock syndrome (Bukowski *et al*.,2010; Crossley *et al*., 2009). Many exotoxins cause superantigenicity (a strong activation of the immune system) that causes disease (Thomas, *et al*;

2007). All *S. aureus* isolates encode α- and β-toxin (*hla* and *hlb* respectively) which are linked to increased virulence in animal models of *Staphylococcal* diseases (Crossley *et al*., 2009; Walker, *et al*., 1992; Wilke, *et al*., 2010). Alpha-toxin (*hla*) in staph binds the membrane of various eukaryotic cells (particularly erythrocytes) drilling pores in them that results in ion imbalance and consequently lysis (Crossley *et al*., 2009; Walker, *et al*., 1992; Wilke, *et al*., 2010). α-Toxin is dermonecrotic and neurotoxic. Animal studies reveal that β-Toxin (Beta-hemolysins) encoded on *hlb* causes cell lysis. These exotoxins also interact with blood cell membranes and cause them to lyse (Crossley *et al*., 2009). While it is suggested that α-toxin is a factor in biofilm formation, strains UAMS-1 and Sanger-252 that have a missense mutation in *hla*, and insertion of lysogenic prophage in *hlb* do not produce alpha and beta-toxins (Crossley *et al*., 2009). The implication of this is that α- and β-toxin are not necessary for virulence, but when made they cause infections. The δ-toxin (a small, helical, amphipathic peptide) lysis mammalian cells, especially RBCs. γ-Toxin and PVL (*lukS-PV, lukF-PV*) are bicomponent toxins made by interaction with *hlgA* and *hlgC* (Crossley *et al*., 2009). A signature of several *S. aureus* diseases, including toxic shock syndrome, is secretions of exfoliative toxins. Staph diseases with exfoliative toxins are associated with strains that produce one of *etA*, *etB*, *etC*, and *etD* genes. Exfoliative toxin A possesses serine protease-like properties; it binds profilaggrin on the skin and cleaves substrates after acidic residues (Bailey, *et al*., 1990; Crossley *et al*., 2009; Dancer, *et al*., 1990). *Staphylococcus aureus* produces many enterotoxins and exotoxins one of which is TSST-1 that has superantigenic properties and has heat-stability crucial to food poisoning (Hamad, *et al*., 1997; Shupp *et al*., 2002). TSST-1 can cross epithelial surfaces. This is an ability lacking in most enterotoxins. Enterotoxins can penetrate the intestinal lining and initiate a local immune response that includes activation of mast cells to produce histamine with frequent triggers of

vomiting/emesis (Hamad, *et al*., 1997; Shupp, *et al*., 2002). Toxic shock syndrome toxin-1(TSST-1) is associated with toxic shock syndrome disease presented by high fever, rash, hypotension, peeling of the skin, and multi-organ failure that often results in death (Crossley *et al*., 2009). Though TSST-1 is mostly linked with toxic shock syndrome, enterotoxins like *SEA*, *SEB,* and *SEC*, have superantigenicity and cause the disease if they get introduced via other means than food (Crossley *et al*., 2009). TSST-1 is unique in its ability to cross the vaginal mucosa though, hence the link with menstruation-associated toxic shock syndrome (Crossley *et al*., 2009).

6) **Enzymes:** *S. aureus* produces and employs exoenzymes for metabolism and tissue invasion important in pathogenesis. Enzyme classes include proteases, lipases, hyaluronidases, and nucleases (Crossley *et al*., 2009; Drapeau *et al*., 1972; Prokesová, *et al*., 1992). Enzyme *sspA* breaks the carboxyl-terminal of aspartate and glutamate of the peptide bond and along with extracellular proteases triggers pathogenesis in human tissues. *SspA* engages in proteolytic maturation of thiol protease *sspB* and inactivation of *SspC* (an inhibitor of *SspB*). *SspA* is important to the degradation of the fibronectin-binding protein (*FnBP*) and surface protein A crucial for the adherence to host cells. It also shields bacteria against host defenses by cleaving IgG, IgA, and IgM (Crossley *et al*., 2009; Drapeau, *et al*., 1972; Prokesová, *et al*., 1992). Cysteine protease *SspB* is involved in the inhibition of host innate mechanisms. It breaks down host elastin, fibrinogen, fibronectin, and kininogen (Massimi, *et al*., 2001; Rice *et al*., 1992; Shaw *et al*., 2004; 2005). *SspB* stops the phagocytosis of opsonized *S. aureus* and decreases the surface expression of CD31 on neutrophils (Massimi, *et al*., 2001; Rice, *et al*., 1992; Shaw, *et al*., 2004; 2005). It degrades host galectin-3/LGALS3, thereby preventing the neutrophil activation by lectin (Massimi, *et al*., 2001; Rice, *et al*., 1992; Shaw, *et al*., 2004; 2005). Some

protease enzymes in *S. aureus* play regulatory roles in virulence (Crossley *et al*., 2009). The *Clp*

proteolytic complexes (*clpX* and *clpP*) are ATP-dependent and they direct protease to specific

substrates. A mutation in *clpX* reduced *S. aureus* biofilm formation, while a mutation in *ClpP*

increased biofilm formation. In the absence of *clpP*, *clpX* can serve as chaperone (Crossley *et al*.,

2009; Frees *et al*., 2004; Gersch *et al*., 2015).

7) **Iron acquisition:** *S. aureus* requires iron, which is limited in the host, for its infection

process (Crossley *et al*., 2009). *S. aureus* meets its iron needs through (1) siderophore which

consists of heme and high-affinity chelators, and (2) direct reuptake with surface proteins. Four

well-studied siderophores include staphyloferrin A, staphyloferrin B, aureochelin, and

staphylobactin. Stapyloferin A and B are encoded respectively on *SfaA* and *SfaB* genes. The

enzymes required for making staphylobactin are encoded on a nine-gene *sbnABCDEFGHI*

operon (Conroy *et al*., 2019; Crossley *et al*., 2009). Mutation of *sbnE* resulted in stunted growth

in iron-restricted media and lowered pathogenicity in the murine kidney model (Crossley *et al*.,

2009). Siderophores attach to host iron and passage them into the bacterial cell via iron-regulated

ABC transporter, such as the ferric hydroxamate uptake *Fhu* system. *Fhu* system has an iron-

binding lipoprotein ligand, integral membrane proteins, and ATPase (Crossley *et al*., 2009;

Hannauer *et al*., 2015). *S. aureus* has two other systems for the extraction of iron from Heme.

The first system designated *isd* includes five transcriptional units *isdA*, *isdB*, *isdCDEFsrtBisdG*,

isdH, and *isdI* (Crossley *et al*., 2009). The second is designated *HtsABC* for the heme transport

system, so named as they were identified from the search for proteins like ABC transporters

(Crossley *et al*., 2009). These systems are important to *S. aureus* pathogenesis, as 95% of the

iron in the human host is bound to heme proteins (Crossley *et al*., 2009). *IsdB* protein pulls heme

from oxidized methemoglobin (metHb) and transports it to *IsdA* or *IsdC* proteins, and on to

membrane transporter/*IsdEF* for internalization. The *isdB* protein induces resistance to hydrogen peroxide and neutrophils killing (Bowden *et al*., 2014; 2018; Crossley *et al*., 2009; Gaudin *et al*., 2011).

8) **Regulation of *S. aureus* virulence:** like every other process involved in bacterial physiological processes, *S. aureus* produces virulence molecules in a regulated manner to boost pathogenicity. It produces the accessory gene regulator, *agr* that is involved in the regulation of several virulence *S. aureus* important to disease processes (Bibalan *et al*., 2014; Crossley *et al*., 2009). The *agr* system is a two-part system composed of RNAII and RNAIII transcriptional units which are respectively controlled by P2 and P3 promoters (Bibalan *et al*., 2014; Crossley *et al*., 2009; Novick *et al*., 1995). The RNAII transcript traverses operon *agrACDB* that houses *agrA* (regulator) and *agrC* (membrane sensor). *AgrD* encodes the extracellular-bound autoinducing peptide (AIP) that is important to quorum sensing and is processed by *agrB* molecule (Abdelnour *et al*., 1993; Booth *et al*., 1995; Cheung *et al*., 1994)*. AgrA* plays a role in post-exponential phase expression of a string of secreted proteins. (Bibalan *et al*., 2014; Crossley *et al*., 2009) (Abdelnour *et al*., 1993; Booth *et al*., 1995; Cheung *et al*., 1994). *Sar* genes in *S. aureus* regulates *Agr* gene functions (Crossley *et al*., 2009). *SarA* controls the production of virulence factors and drives the biofilm formation process in a cell density-dependent manner (Balamurugan *et al*., 2017; Bibalan *et al*., 2014; Crossley *et al*., 2009). *SarA* is also important to multi-drug resistance mechanisms (Valle, *et al*., 2003). *SarA* is on a locus with three overlapping transcripts *sarA*, *sarC*, and *sarB* with a similar terminus and promoters P1, P2, and P3 (Crossley *et al*., 2009). *SarA* is regulates RNAII and RNAIII transcripts on agr locus and serves as a transcriptional activator for *fnbA*, *fnbB*, hemolysins (*hla, hld, hlgB,* and *hlgC*), serine proteases (*splA, splB, splD and splF*), *bap* that are strain-dependently important to biofilm formation in

diseases (Crossley *et al*., 2009; Valle *et al*., 2003). *SarA* down-regulates protein A (*spa*), lipase (*lip*), thermonuclease (*nuc*), immunodominant staphylococcal antigen B (*isaB*), staphylococcal serine and cysteine proteases (*sspA* and *sspB*), staphostatin B (*sspC*), metalloprotease aureolysin (*aur*) and collagen adhesin (*cna*) (Crossley *et al*., 2009). Homologs of *SarA* include *SarR, SarV, SarX, SarZ, Rot, and MgrA* whose interactions are not fully understood, but sarA and SarR regulates agr RNAII expression. SarA activates and SarR represses the P2 in transcription (Crossley *et al*., 2009; Reyes *et al*., 2011). In one study, *sarV (rat)* and *mgrA(norR)* are looped in a feedback mechanism; mutation of *mgrA* results in increased autolysis (Crossley *et al*., 2009; Ingavale *et al*., 2003). *MgrA* is responds to reactive oxidative changes that result in resistance to certain antibiotics (Williams *et al*., 2006; 2015). There are more factors of regulation employed in *S. aureus* virulence. The *S. aureus* exoprotein expression gene (*saeRS*) regulates exotoxins (Crossley *et al*., 2009; Feng *et al*., 2008). Staphylococcal respiratory response gene (*srrAB*) responds to changes in oxygen levels (Crossley *et al*., 2009; Ulrich *et al*., 2007). The two-component response regulator gene (*vraSR*) that regulates agr and responds to cell wall-directed antibiotics and increases resistance to β-lactams and vancomycin (Crossley *et al*., 2009; Taglialegna *et al*., 2019). The two-component response regulator gene (*graRS*) that promotes resistance to vancomycin and antimicrobial peptides (Crossley *et al*., 2009; Meehl *et al*., 2007; Hu *et al*., 2016). The two-component response regulator gene (*apsRS*) that promotes resistance to antimicrobial peptides (Crossley *et al*., 2009). The two-component response regulator gene (*alsSD*) for Cell wall integrity and biofilm formation (Crossley *et al*., 2009). The metabolic pathway regulation gene (*Spx*) for Stress response, as well as biofilm formation (Crossley *et al*., 2009). The modulator of the *sarA* gene (*Msa*) which increases the expression of *sarA*, and the virulence factors under its control (Crossley *et al*., 2009). The peptidoglycan synthesis gene

(*murF*) which is involved in the expression of cell-associated or extracellular virulence factors (Crossley *et al*., 2009; Sobral *et al*., 2006).The concepts discussed in this unit encapsulates broad association of structures, , patterns, , and regulatory mechanisms important to the pathogenesis of *S. aureus* in many different diseases in different organisms. We find that *S. aureus* disease mechanism per disease and organism seem to be resultant of the combination of factors utilized. In chapter two we will discuss further how some of these processes may be involved in the pathogenesis of a clonal population of S. *aureus* isolated from BCO lame birds.

Escherichia coli

E. coli is a rod-shaped, Gram-negative, facultatively anaerobic bacterium that possesses both respiratory and fermentative metabolism (coliform). It is a member of the Enterobacteriaceae family of the phylum γ-Proteobacteria. *E. coli* occurs in straight rod stacks which are 1.0–1.5-μm wide, and about 2–6-μm-long. It does not produce oxidase. *E. coli* can be motile (with lateral flagella) or non-motile (with polar flagella). *E. coli* is methyl red-positive, citrate-negative, and Voges–Proskauer-negative. This bacterium is phylogenetically closest to *Shigella spp* (Desmarchelier & Fegan., 2016; Percival *et al*., 2014). *E. coli* is a physiologically and metabolically versatile organism classified as pathogenic or non-pathogenic. The non-pathogenic or commensal strains are part of the normal gut microflora of endotherms (Mellata, 2013; Kaper 2005; Köhler& Dobrindt., 2011). Pathogenic strains have also been identified and associated with various types of intestinal (IPEC- intestinal pathogenic *E. coli*) and extraintestinal (ExPEC) ailments. IPEC are obligate pathogens and ExPEC are facultative. IPEC is phylogenetically and epidemiological distant from ExPEC and commensal *E. coli*. This is because ExPECs and non-pathogenic *E. coli* share large genomic segments (Köhler & Dobrindt., 2011). Commensal *E. coli* strains are not prevalent in disease cases expect in immunocompromised hosts, or in situations

where the gut integrity is compromised (Kaper, 2005; Kaper *et al*., 2004). The natural niche in humans is the mucosal layer of the colon, where *E. coli* is an effective competitor. Sweeney *et al*. (1996) reported that *gntP* (mapped immediately downstream of the *fim* gene cluster) encodes a high-affinity gluconate permease observed with the abundance of gluconate in the mice model large intestine. The hypothesis is that the metabolism of gluconate may be the reason why commensal *E. coli* are more successful than other resident species (Sweeney *et al*., 1996). Some *E. coli* have acquired virulence capabilities for colonizing and adapting to niches and unleashing a variety of diseases. These virulence factors are often on genetic elements passaged from one strain to another resulting in a combination of new sets of pathogenic material in many cases. So that a once mobile element can become immobile in its new genomic environment. (Kaper *et al*., 2004). Pathogenically successful combinations of virulent materials have resulted in the formation of *E. coli* pathotypes that can cause diseases in an organism or a range of hosts (Kaper, 2005; Kaper *et al*., 2004; Palaniappan, *et al*., 2006). Infection with *E. coli* pathotypes generally results in (1) diarrhoeal or gut (enteric) disease, (2) sepsis and meningitis, or urinary tract infections (UTIs). There are six characterized gut *E. coli* pathotypes: (I) enteropathogenic *E. coli* (EPEC), (II) enterohaemorrhagic *E. coli* (EHEC), (III) enterotoxigenic *E. coli* (ETEC), (IV) enteroaggregative *E. coli* (EAEC), (V) enteroinvasive *E. coli* (EIEC), and (VI) diffuse adherent *E. coli DAEC* (Kaper *et al*., 2004; Mansan-Almeida, *et al*., 2013). Infection of the urinary tracts are caused by the pathotype uropathogenic *E. coli* (UPEC) and are the most common extraintestinal *E. coli* infections. Meningitis and sepsis causing pathotype is meningitis-associated *E. coli* (MNEC) and are becoming increasingly prevalent in non-gut infections. Two of the newest *E. coli* pathotypes include the endometrial pathogenic *E. coli* (EnPEC) and the mammary pathogenic *E. coli MPEC* (Johnson & Russo, 2002; Russo & Johnson, 2000). EPEC,

ETEC, and EHEC have been observed in animals to employ similar pathogenesis molecules and patterns seen in human diseases (Kaper *et al*., 2004). About BCO lameness, ExPEC also causes significant economic losses in animal production, especially the poultry industry. Avian pathogenic *E. coli* (APEC) behave like human pathogenic ExPEC strains. A recent examination of virulence genes in human and avian ExPEC suggest that poultry products are important to ExPEC that causes sepsis infection in human. A conclusion was drawn since poultry contaminated with higher levels of *E. coli* has more multidrug resistance (MDR) compared to other meat sources (Manges *et al*., 2007). Such finding implies that increased consumption of poultry worldwide could be a contributing factor to antibiotic resistance in human ExPEC, and hence the emergence of ExPEC diseases in humans (Pitout *et al*., 2012). A globalized market coupled with ease of travel plays a role in the spread of the infection as well (van der Bij & Pitout, 2012). Preventing and eradicating *E. coli* diseases in both humans and animals requires full classification of virulence factors per pathotype and understanding their individual and combined characteristics. There are four main phylogenetic groups of *Escherichia coli* A, B1, B2, and D, of which B2 is the most abundant and pathogenic strains in ExPEC infections (Table 4; Mellata, 2013). *E. coli* is classified into 150 to 200 serotypes or serogroups based on somatic (O), capsular (K), fimbrial (F) and flagellar (H) antigens. At least 53 H antigens, 188 O liposaccharides antigens, and numerous different capsular K polysaccharide antigens.

Table 4. Virulence Traits in Pathotypes of Extraintestinal Pathogenic *E. coli* determined to be associated with pathogenicity (Reproduced from Köhler & Dobrindt, 2011).

| ExPEC | Serogroups | Phylo-genetic group | Virulence factors[a] | | | | |
			Adhesins/ fimbriae	Iron uptake	Toxins	Protectins/ invasins	Others
APEC	O1, O2, O78, O18	A, B1, B2, D	Type 1 P AC/1 Stg curli	Aerobactin Salmochelin	VAT-PAI ECVF	K1 capsule LPS O78 TraT Iss IbeA, IbeB	ColV plasmid Tsh
UPEC	O1, O2, O4, O6, O18, O75	B2, D	Type 1 P Dr Afa S F1C Iha	Aerobactin Salmochelin Hma ChuA IreA	CNF1 HlyA Sat CDT Sat	K 1 capsule TraT OmpT	Sat Flagellin Usp
NMEC	O18, O7, O16, O1, O45	B2, D	Type 1 S fimbriae	Salmochelin Enterobactin	CNF1	K1 capsule O-LPS Ibe proteins AslA OmpA	NlpI flagellin
SEPEC[a]	O1, O2, O4, O6, O18, O45, O83	B2, D	Type 1 P S Curli	Salmochelin Yersiniabactin Hma ChuA	HlyA	K1 capsule Iss TraT O-LPS	ColV plasmid NanA

Although many ExPEC and their roles in the disease mechanism have been mostly characterized, there are many unique factors between pathogenic and non-pathogenic *E. coli* (Kaper *et al*., 2004; Köhler& Dobrindt., 2011). With ExPEC, a model has been postulated for the site of isolation combined with two detected associated virulence factors per pathotype and is described (Köhler& Dobrindt., 2011). ExPEC is diverse with only a few shared pathogenic factors, but there is still no methodology to clearly define commensals apart from ExPECs. The classical serotyping method is useful for IPEC classification because a good number of virulent strains in this category are established well within the O: H serotypes (Köhler& Dobrindt., 2011). To some degree, highly pathogenic ExPEC strains seem to be confined within a few O serogroups and pathogenic factor combinations (See Table 5; Köhler& Dobrindt., 2011).

Table 5. Virulence factors in ExPEC (Reproduced from Köhler & Dobrindt, 2011)

Functional category	Virulence factor
Adhesin	Type 1 fimbriae (Fim)
	P fimbriae (Pap/Prf)
	S/F1C fimbriae (Sfa/Foc)
	N-acetyl D-glucosamine-specific fimbriae (Gaf)
	M-agglutinin (Bma)
	Bifunctional enterobactin receptor/adhesin (Iha)
	Afimbrial adhesin (Afa)
	Temperature sensitive hemagglutinin (Tsh)
Invasin	Invasion of brain endothelium (IbeA)
Iron acquisition	Siderophore receptor IreA
	Aerobactin (Iuc)
	Yersiniabactin (Ybt)
	Salmochelin (Iro)
	Periplasmic iron binding protein (SitA)
Toxins	alpha-Hemolysin (HlyA)
	Cytolethal distending toxin IV (CDT 1)
	Cytotoxic necrotizing factor 1 (CNF-1)
	Putative hemolysin (HlyF)
	Colibactin (Clb)
	Serine protease autotransporters Sat, Pic
Protectins	Group II capsule incl. K1 capsule
	Conjugal transfer surface exclusion protein (TraT)
	Outer membrane protease T (OmpT)
	Increased serum survival (Iss)
	Colicin V (Cva)
Others	D-Serine deaminase (DsdA)
	Maltose and glucose-specific PTS transporter subunit IICB (MalX)
	Flagella

Multilocus enzyme electrophoresis *MLEE* analysis reported that only a few *E. coli* genotypes

exist despite its diversity and forms the A-E phylogroups (Köhler& Dobrindt., 2011; Mellata,

2013). This was valuable as it helped distinguish many ExPEC from IPEC or non-pathogenic.

Many ExPECs belonged to B2 and to some degree D phylogroups. IPEC or non-pathogenic

strains are mainly A1 and B1 isolates. Multilocus VNTR analysis (MLVA) a relatively fast DNA

Sequence typing technique has been applied in the characterization of IPEC particularly EHEC

and is used for epidemiological inspection (Köhler& Dobrindt., 2011). Multilocus sequence

typing (MLST) employed for its worldwide comparability and usability, reveal *E. coli* strains to

be of comparable delineation (Köhler& Dobrindt., 2011; Martin, *et al*., 1998). Because

sequencing and analysis of various chromosomal regions do not characterize the phylogenetic

composition of *E. coli* strains, MLST is useful still for phylotype determination (Köhler&

Dobrindt., 2011). MLST-based molecular epidemiology reveals that phylogroup B2 (already

described to contain most ExPEC) is the evolutionarily oldest strains within the group (Köhler&

Dobrindt., 2011; Tenaillon, *et al*., 2010). Next-generation and large-scale sequencing give a

better in-depth phylogenomic analysis, as well as strain classification (Köhler& Dobrindt.,

2011). ExPECs and APECs were initially treated separately as initial studies did not connect

both to human and animal diseases in terms of virulence and hosts (Mellata, 2013). The

importance of APEC pathogenicity was initially minimized, as they were designated

opportunistic pathogens effectively reliant on external stress conditions (Mellata, 2013). This

notion has since been reversed, by studies that reveal significant variances in the distribution of

virulence elements in *E. coli* retrieved from chickens with colibacillosis and feces of healthy

ones (Johnson, *et al*., 2012; Mellata, 2013; Schouler, *et al*., 2012). Comparison of human

pathogenic *E. coli,* strains from chicken colibacillosis, with strains isolated from feces identified

virulence factors that may categorize APECs. Examination of these factors has shown them

important to APEC infection processes (Mellata, 2013). Also, APEC that was once thought of as

a distinct population, is now divided into several subtypes due to pathogenicity factors that are

important to diverse chicken diseases. Although the genetic traits that define APECs are not fully

categorized, the subsets do contain a combination of unique virulence stet of genes. These factors

include (I) *iss, tsh, iucC, cvi, iutA, hylA, iss, iroN, and ompT* genes carried on colicin V plasmids,

(II) toxin genes (*astA*, *vat*), (III) iron acquisition system genes (*irp2* and *iucD*), (IV) adhesin genes (*papC* and *tsh*), and (IV) the ColV genes *cva-cvi* (Mellata, 2013). Schouler *et al*. suggested that the following four pathogenic factors can be used for strain determination as they can detect over 70% APECs: (I) *iutA and sitA* for iron acquisition, (II) fimbriae *P (F11)*, (III) *frz_{orf4}* for sugar breakdown, and (IV) O-antigen O78, and T6SS *aec26*, *aec4* (Schouler, *et al*., 2012). APEC poultry diseases are generally regarded as colibacillosis. The presentation of colibacillosis depends on the strain involved, the route of entry, and external factors. Like many microbes, APEC harnesses host weaknesses to advance its pathogenesis. APEC causes local infections such as cellulitis, salpingitis, synovitis, and omphalitis. They also cause septicemia, fibrinous lesions of internal organs, and death of infected birds (Mellata, 2013). APEC is said to pose a zoonotic risk as they share pathogenic factors and phylogenic features with human ExPEC. Various epidemiological studies suggest poultry as a conduit for Human ExPEC, as avian ExPEC (that was comparable genetically) has been isolated from the gut of healthy birds and poultry meat (Manges & Johnson, 2012). Experimental evidence suggests that human ExPECs can cause disease in chickens models, and Avian ExPECs alike to human animal-models (Mellata, 2013). Other reports suggest that some ExPECs from ST95 and ST23 clonal groups may be endemic to a range of hosts with the capability to cause many infections (Mellata, 2013). This was a major concern because poultry colibacillosis was initially treated with antibiotics that were important for AMR resistance in human ExPECs. Many countries have curbed the use of antibiotics for livestock rearing, perhaps it might help mitigate the acquisition of antimicrobial resistance in human and poultry pathogens. How do we now control colibacillosis outbreaks in poultry? Not Long Ago, after a 3-year consecutive microbiologically sampling of 650,000 chickens from 38 broiler flocks in two large farms, Dinev reported the isolation of *E. coli* in over

90% of FHN associated with osteomyelitis. Urging that *E. coli* had a primary role in the etiology of BCO in commercial birds particularly FHN (Dinev, 2009; Wijesurendra *et al*., 2017) Dinev, noted that in contrast to *E. coli, S. aureus* seemed endemic to the parent stock lines (Dinev., 2009). Wijesurendra *et al*. also examined chickens from 20 broiler farms in Australia and reported that *E. coli* plays a crucial role in the bacteremia and hematological spread crucial to BCO formation (Wijesurendra *et al*., 2017). Based on the amount of APEC isolated, Wijesurendra *et al*. proposed that a remedy for BCO should be targeted at the species (Wijesurendra *et al*., 2017). In Chapter 2, we report on *E. coli* isolated from BCO birds in three different farms in the Arkansas area.

   Enterococcus cecorum

Enterococcus cecorum, in the past 15 years, has become an important emerging pathogen to the incidence of BCO lameness, even though it was initially considered a commensal in the mammalian gut microflora when it was first described in 1983 (Dolka *et al*., 2017; Jung *et al*., 2018; Kierończyk *et al*., 2017). *E. cecorum* is Gram-positive cocci, that often occur in pairs (diplococci) or short chains. *E. cecorum* is a non-spore-forming, facultatively anaerobic bacteria of the phylum Firmicutes. They are tolerant of diverse environments; they thrive in under varying oxygen contents, temperature conditions, high sodium chloride, and pH. There was an initial outbreak of *E. cecorum* in 2001 in Scotland, and multiple outbreaks have since followed across Europe, United States, Iran, and Southern Africa (Jung *et al*., 2018). Pathogenic *E. cecorum* has a signature inflammatory mass that occurs in the spinal cavities of the flexible thoracic vertebrae. The infections of pathogenic *E. cecorum* goes by Vertebral BCO, vertebral osteomyelitis, vertebral enterococcal osteomyelitis and arthritis, enterococcal spondylitis, and 'kinky-back.' *E. cecorum* has also been isolated from tibial and femoral head lesions (Jung *et al*.,

2018; Personal observation). Kinky back (KB) incidentally is the also colloquial name for the developmental spinal anomaly, *spondylolisthesis* (Jung *et al*., 2018; Muir & Aggrey, 2003). Broilers with KB experience higher morbidity and mortality as a result of a combination of sepsis at the early growing days, starvation, and dehydration due to paralysis of infected birds in the late growth stage (Jung *et al*., 2018). Embryo lethality assay has been used to determine the pathogenicity and lethal dosage of pathogenic *E. cecorum* (Borst *et al*., 2015; Jung *et al*., 2018). Some *E. cecorum* are inherently resistant to β-lactam-based antibiotics (like carbapenems, cephalosporins, and penicillin), various aminoglycosides, and more recently vancomycin (Fisher &Philips., 2009; Ryan & Ray., 2004). In chapter 3, we discuss our attempt to characterize the pathogenicity of *E. cecorum* recovered from a commercial farm.

Clinical Diagnosis of Terminal BCO

Significant progress has been made in the study of BCO lameness since the first report of the condition. Bacterial chondronecrosis with osteomyelitis, formerly known as femoral head necrosis, is a complex disease with etiology that is not fully characterized. There is some consistency, however, in the manifestation of terminal BCO in spontaneous cases or Staphylococcal-induced models. Terminal BCO presents varying necrotic degradation and bacterial infection of (1) the proximal ends and growth plate of long bones (tibiae and femora), and (2) the flexible thoracic vertebrae T4 (Figures 13-15; Applegate *et al*., 2002; 2017; Al-Rubaye *et al*., 2012; 2015; 2017; Dinev, 2009; Jiang *et al*., 2015; Joiner *et al*., 2005; Mandal *et al*., 2016; Martin *et al*., 2011; McNamee & Smyth., 2000; Stalker *et al*., 2010; Thorp *et al*., 1993; Wideman., 2016, Wideman and Pevzner., 2012, Wideman and Prisby., 2013; Wideman *et al*., 2012; 2013; 2015; Wijesurendra *et al*; 2017). Common clinical presentations of BCO on long

bones include (I) femoral head transition, FHT (Figure 13B), (II) femoral head necrosis, FHN (Figures 13 C-D, and 14-3), (IV) tibial head necrosis with caseous, THNC (Figures 13-F-H; 14-6), (V) femoral head separation, FHS (Figure 14-2), (VI) tibial head necrosis, THN (Figure 14-5), (VII) tibial head necrosis severe, THNS (Figure 14-6), (VIII) Tibial Dyschondroplasia, TD (Figure 15), (IX) twisted leg, and (X) BCO diagnosis of unknown origins. The distal portions of the long bones are less affected that the proximal heads. The presentations can be one-sided, contralateral, or bilateral (Applegate *et al*., 2002; 2017; Al-Rubaye *et al*., 2012; 2015; 2017; Al-Rubaye, personal observation; Dinev, 2009; Ekesi, personal observation, Jiang *et al*., 2015; Joiner *et al*., 2005; Mandal *et al*., 2016; Martin *et al*., 2011; McNamee & Smyth., 2000; Rhoads, personal observation; Stalker *et al*., 2010; Thorp *et al*., 1993; Wideman., 2016, Wideman and Pevzner., 2012, Wideman and Prisby., 2013; Wideman *et al*., 2012; 2013; 2015; Wideman, personal observation, Wijesurendra *et al*; 2017). Lameness associated with the T4 thoracic vertebrae is generally termed spondylopathy or "Kinky back" for non-bacterial deformities, and vertebral or enterococcal spondylitis if an infection is involved (Figure 16; Dinev *et al*., 2012). The levels of inflammation in the flexible vertebra vary between cases (Dinev *et al*., 2012).
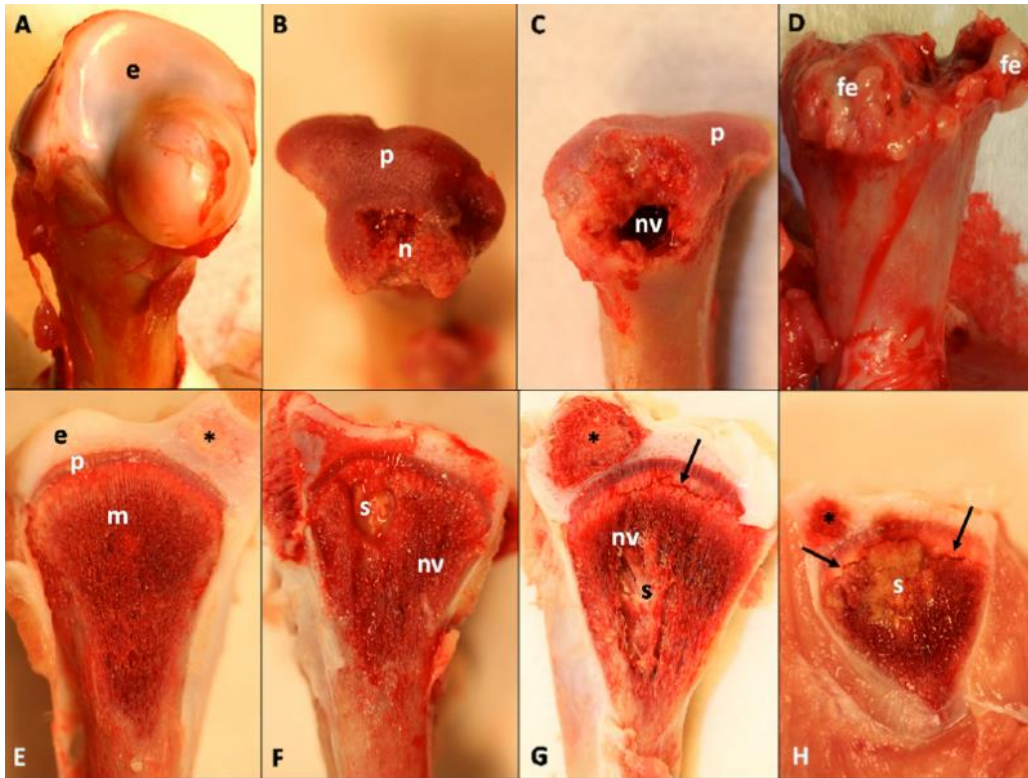
Figure 13. BCO progression on femoral (A-D) and tibial (E-H) proximal heads that result in lameness in broiler chickens. (A) Normal femoral head with a white cap of epiphyseal cartilage (e). (B) Epiphyseolysis or femoral head separation (FHS) epiphysis. Early necrosis (n) has begun on the exposed underlying surface of the growth plate or physis (p). (C) Fractured growth plate revealing necrotic void (nv) within the metaphysis (m). (D) The necrosis weakened diaphysis is fractured during the disarticulation of the femoral head from acetabulum. The femoral epiphysis, physis, and a great part of the metaphysis remain in the acetabulum. Abundant fibrinonecrotic exudate (fe) oozes out in the terminal or most severe femoral head necrosis (FHN). (E) Normal tibia comprising e, p, m with a center of ossification (designated *); the growth plate of the healthy tibia is structurally in place with struts of trabecular bone in the metaphysis. (F-H) Levels of bacterial permeation and sequestrate(s), arrows pointing to microfractures, along with necrotic voids (nv) below the physis (Image Reproduced from Applegate *et al.*, 2017).

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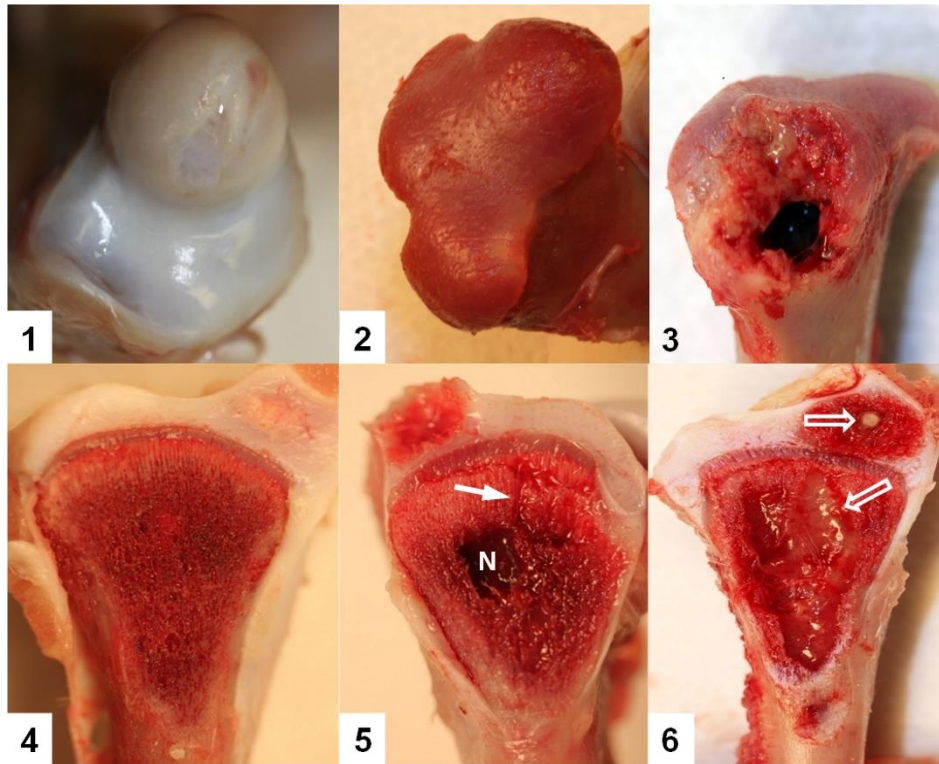
Figure 14. Common clinical presentations of proximal femoral and tibial BCO lesions. (1) Normal proximal femoral head; (2) FHS: epiphyseolysis; (3) FHN; (4)Normal proximal tibial head with struts of trabecular bone in the metaphyseal zone fully supporting the growth plate; (5) Tibial head necrosis (THN); (6) THNs with caseous (Reproduced from Mandal *et al*., 2016).

Figure 15. Tibial dyschondroplasia is characterized by abnormal masses of proliferated, avascular, prehypertrophic cartilage in the proximal metaphyseal region of the proximal tibiotarsus. This is attributable to the formation and retention of a large cartilage mass filling in the entire metaphysis (Image reproduced from Dinev *et al*., 2012).
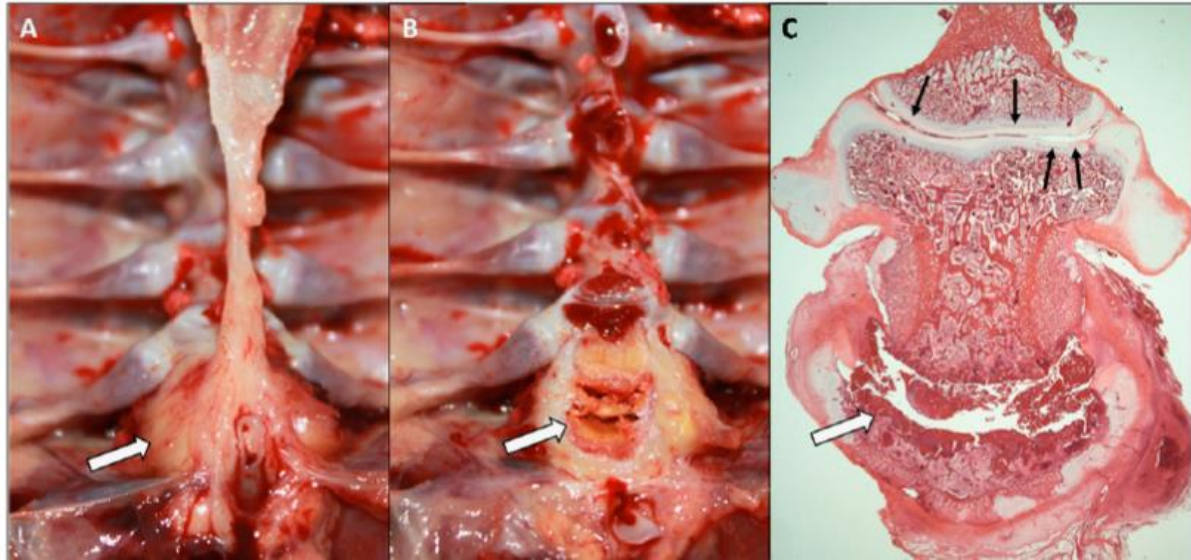
Figure 16. Vertebral BCO in broiler that exhibited paraplegic hock-resting posture, and spinal compression due to build-up of abscess in vertebral bodies (follow white arrows). (A) Externally visible nodal swelling of the flexible thoracic vertebral body with yellow discoloration of the translucent remnants of the vertebral body. (B) The caudal portion of the vertebral body filled with caseous abscess. (C) The epiphysis and growth plate have been replaced with necrotic abscess. Osteochondrosis (follow black arrows) in the cranial articular cartilage (Image reproduced from Applegate *et al*., 2017).

The S. agnetis induced BCO Lameness model

Our *S. agnetis* models for studying BCO lameness use different flooring systems: litter, wire, or a combination of both. Ever since Dr. Wideman invented the elevated wire-flooring structure for inducing lameness in broilers, we have induced lameness at high incidence (Al-Rubaye., 2015; 2017; Wideman *et al*, 2012, 2013, 2014; Wideman and Prisby, 2013; Wideman, 2016). This model is primarily directed towards tibial and femoral BCO and has proven effective (Al-Rubaye., 2015; 2017; Wideman *et al*., 2012, 2013, 2014; Wideman and Prisby, 2013; Wideman, 2016). Utilizing wire-floor, we found that *S. agnetis* is the prevalent isolate on our research farm recovered from BCO lesions irrespective of site sampled or lesion type (Tables 4-5; Al-Rubaye., 2015; 2017). In this model, we found administering *S. agnetis* in drinking water increases the incidence of BCO lameness (Al-Rubaye et al., 2015; 2017). The finished genome sequence for a

S. agnetis BCO isolate has been published (Al-Rubaye et al., 2015). Our team also reported that BCO is sometimes connected with significant bacteraemia (Al-Rubaye et al., 2015; 2017). The broiler lines tested for susceptibility to lameness showed that all lines are predisposed, but line differences and sire-effects may exist between birds (Wideman *et al*., 2013; 2014). In either the wire or litter floor model, rapidly growing broilers that seem healthy (or have normal gait) can over 24 hours, exhibit early symptoms of lameness. Depending on the project goals, we generally place 60-day-old broilers per pen and then cull on day 15 to 50 of the clinically healthiest birds (Al-Rubaye et al., 2015; 2017; Wideman *et al*., 2012, 2014). Pen configuration, bird densities, food, and water (ad libitum), and environmental conditions (as light, and temperature) are as described (Al-Rubaye *et al*., 2015; 2017; Wideman *et al*., 2012, 2013, 2014). The bacterial administration in the broiler's drinking water supply is also described (Al-Rubaye *et al*., 2015; 2017; 2020 A & B). From a series of experiments, the minimum effective dose of *S. agnetis* isolate 908 in drinking water starting on day 20 for two days is 10^5 CFU/mL on wire-flooring (Figure 17).
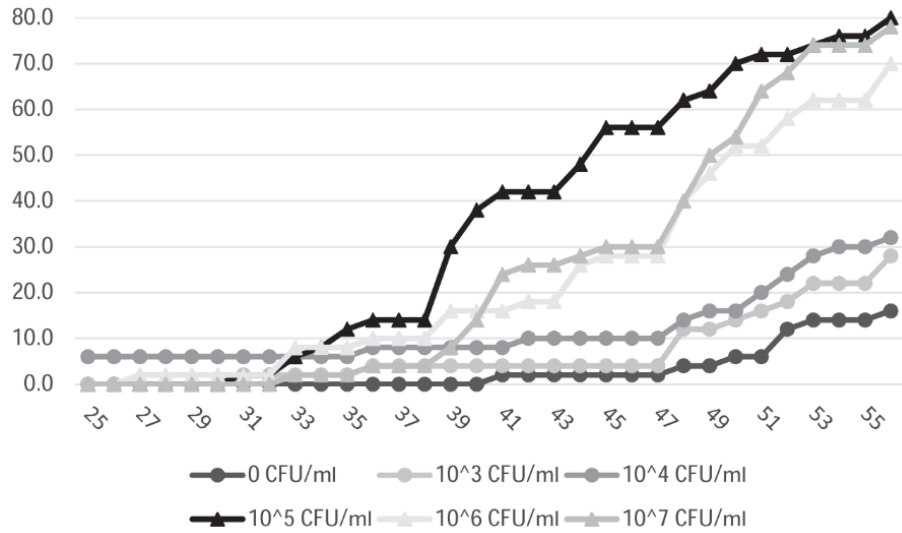
Figure 17. The minimum effective dosage of *S. agnetis* in drinking water for the induction of lameness. The cumulative percentage lameness is plotted for broilers reared on wire flooring and administered the indicated concentrations of *S. agnetis* in the drinking water. The birds were provided with tap water throughout (0 CFU/mL) or with *S. agnetis* in the drinking water on days 5 and 6 and again on days 15 and 16. Each treatment was for one pen of 50 birds. Values are calculated as the percentage of the total number of birds in the treatment group. Final lameness counts for challenges ≥105 CFU/mL were significantly different ($P \leq 10^{-7}$) from the challenges with ≤10^4 CFU/mL (Image reproduced from Al-Rubaye *et al*., 2017).
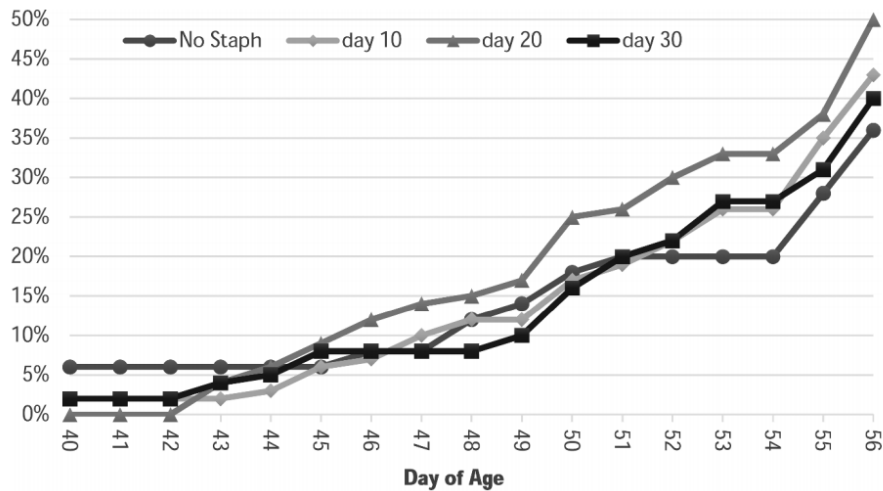
Figure 18: Most effective days for the administration of *S. agnetis* in drinking water for induction of lameness for broilers reared on wire flooring. Cumulative percent lameness (y-axis) is plotted for broilers reared on wire flooring with tap water throughout (No Staph; 1 pen; 50 birds), or administered *S. agnetis* at 10^5 CFU/mL (per treatment 2 pens, 50 birds/pen) for 2 days beginning at 10, 20, or 30 days of age. Values are calculated as the cumulative lameness percentage for days 40 through 56 based on the number of birds in the treatment group (Image reproduced from Al-Rubaye *et al*., 2017).

Early symptoms of lameness will include, a poor gait (Score of 3), hesitancy to stand, eagerness to sit, and slight wing-tip dipping (Al-Rubaye *et al*., 2015). The birds are encouraged to walk by gentle prodding or herding with a common kitchen broom from Day 15 through Day 56. This is done to minimize birds' distress and diagnose the ailment in a timely fashion (Al-Rubaye *et al*., 2015; Wideman, 2016; Wideman *et al*., 2012; Wideman and Prisby, 2013). Once birds present the earliest sign of lameness, they are humanely euthanized and necropsied to grade or score BCO, if any, on the femora, tibiae, and sometimes the vertebrae. In this model, we only categorize birds presenting with specific lesions as lame. The percent (%) cumulative lameness per pen/treatment is calculated with the formulas below:

$$\% \ cumulative \ lameness \ per \ pen \ = \frac{\# \ of \ lame \ in \ pen}{\# \ of \ birds \ in \ pen \ on \ Day \ 15} \ X \ \ 100$$

$$\% \ cumulative \ lameness \ per \ treatment = \frac{\# \ of \ lame \ for \ Treatment}{\# \ of \ birds \ in \ Treatment \ on \ Day \ 15} \ X \ \ 100$$

In this model, treatment effects are evaluated for significant differences ($P < 0.05$) mainly by logistic regression (binomial distribution) using the GLM procedure implemented in the R Foundation for Statistical Computing. Blood and BCO lesions are usually assessed for bacteremia and bacterial species utilizing protocols described (Al-Rubaye *et al*., 2015; 2017). We find that for broilers raised on (a) litter, (b) litter for 35 days and then transferred to wire, or (c) wire, the incidence of lameness was highest in birds raised on the wire-floor with the administration of *S. agnetis* in water for two days on Day 20 (Figure 19). The incidence of lameness on the wire using the *S. agnetis* model is ≥71%. Birds initially raised on litter and transferred to wire have a lameness incidence of ~52%, compared to the birds raised on only

litter floor with ~3%. The more recent incidences of lameness on the wire-floor are higher, over 80%, and those of broilers raised on wood shaving litter floor has risen to 50% (Unpublished). We believe that the repeated experiments in the same building on our research facility may have facilitated the increase in amount, as well as, mutation of *S. agnetis* and made it hypervirulent (Alrubaye *et al*., 2020a, 2020b; Shwani *et al*., 2020).
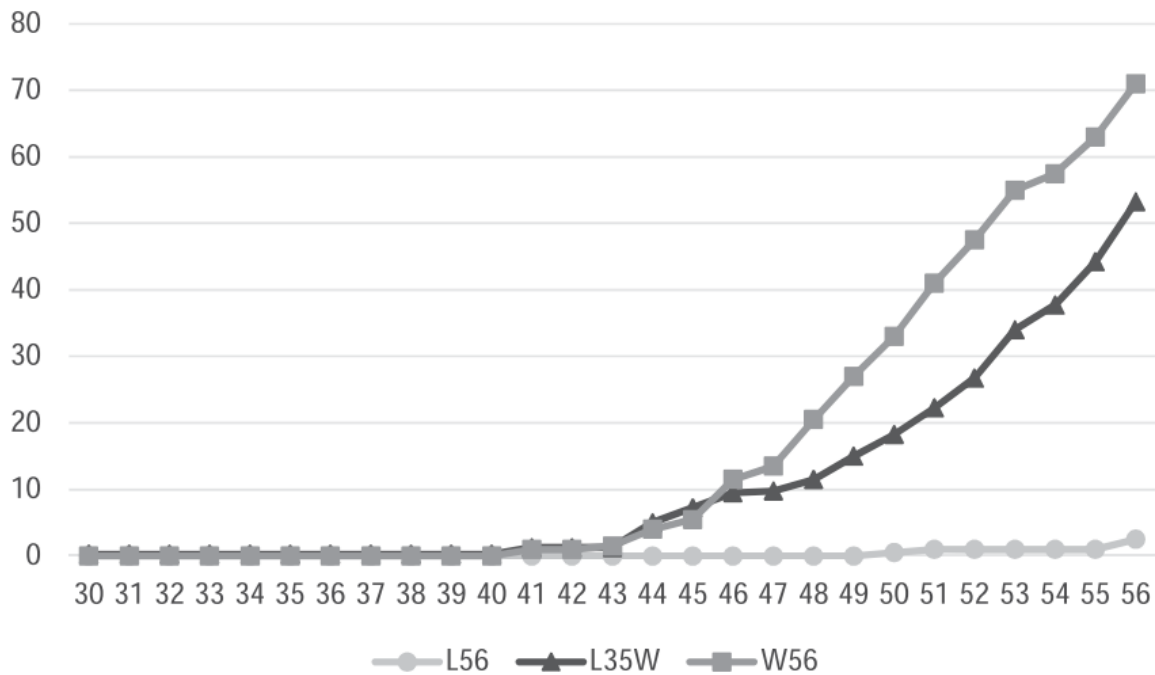
Figure 19. Cumulative percent lameness for birds raised on litter for 56 days (L56; 4 pens; 200 birds), litter for 35 days then transferred to wire till 56 (L35W; 6 pens; 300 birds), or wire for 56 days (W56; 4 pens; 200 birds). Lameness % (y-axis) was calculated based on the number of birds in the treatment group. The figure includes an average % lameness and ±S.D. The % Lameness per pen was 2.5 ± 2.5 for L56, 52.5 ± 15.1 for L35W, and 71 ±10.4 for W56. The P-values for L56 v L35W, L56 v W56, and L35W v W56 are all significant (P<0.05) Image reproduced from Al-Rubaye *et al*., 2017.

Table 6: BCO isolates from lame birds based on-site sampled (Reproduced from Al-Rubaye *et al.*, 2015).

Bacterial Species	Right Femur	Left Femur	Right Tibia	Left Tibia	Blood	Total
Enterococcus faecalis	0	0	1	0	0	1
Staphylococcus agnetis	13	12	21	20	15	81
Staphylococcus aureus	1	1	0	0	1	3
Staphylococcus epidirmidis	1	1	0	0	0	2
Staphylococcus hominis	1	1	0	1	0	3
Staphylococcus saprophyticus	0	1	0	0	0	1
Staphylococcus xylosus	1	1	0	0	0	2
Total number of infections diagnosed	17	17	22	21	16	93

A total of 24 lame birds were sampled from all five locations and bacterial colonies diagnosed by PCR-sequencing of a portion of the 16S rDNA. The number of infection sites diagnosed excludes sampled that either did not show bacterial growth, failed in the PCR, or yielded poor sequence data.

Table 7: BCO isolates from lame birds based on the bone lesion (Reproduced from Al-Rubaye *et al.*, 2015).

Bacteria	Normal	FHS	FHT	FHN	THN	THNs	THNc	Total
E. faecalis	0	0	0	0	0	0	1	1
S. agnetis	3	1	12	10	9	16	15	66
S. aureus	0	0	2	0	0	0	0	2
S. epidirmidis	0	1	1	0	0	0	0	2
S. hominis	0	0	1	1	0	1	0	3
S. saprophyticus	1	0	0	0	0	0	0	1
S. xylosus	0	0	2	0	0	0	0	2
Total number of infections diagnosed	4	2	18	11	9	17	16	77

The *S. agnetis* model has been used to study bacterial translocation into the blood, and colonization of the growth plates of the proximal femora and tibiae in rapidly growing birds. This was done by tracing and quantifying administered *S. agnetis* in the blood at different times for birds raised for 56 days on litter, wire, or initially of litter and then moved to the wire. Table 6 shows that only a few bacteria were picked up from the blood of apparently healthy birds through Day 41 in birds raised on litter and wire (Al-Rubaye *et al.*, 2017). The colony count in broiler blood increased by Day 49. The colony count in blood of birds raised on the wire-floor, and those initially raised on litter and transferred to wire had approximately 10 times more than those in the blood of those raised on litter. Of the bacteria recovered on Day 49 from healthy birds raised on litter, 70% were not *S. agnetis*. Approximately 50% of apparently healthy birds

raised on wire-floor, or initially on litter and transferred to wire-floor after 35 days were *S. agnetis.* Birds raised on wire-floor that were positive for *S. agnetis* had a higher colony count (Unpublished). Some of the sampled lame birds raised on litter that were positive for *S. agnetis* had about 120 CFUs/mL. Lame birds raised on wire had a higher average colony count per mL of blood. In trials including other BCO isolates, *S. agnetis* induced the highest amount of lameness (Figure 20). Because non-agnetis isolates (*S. saprophyticus* and *S. epidermidis*) had a lower incidence of lameness in birds that developed lameness, we studied them to see if they can confer probiotic protection against BCO lameness. The one-dose treatment of non-agnetis isolates in the drinking water of broilers was not sufficient to protect lame growing birds from BCO lameness. We did not follow up on the continual dosage experiment, but studies like these are promising for BCO remedies.

Table 8. BCO Microbiological sampling from the blood of healthy or lame birds raised on litter (L56), litter for 35 days then transferred to wire (L35W), or wire (W56) at various times. For lame vs healthy birds tested, the table includes average colony count per 0.1 mL of blood plated on CHROMagar Orientation, the percentage of birds positive for *S. agnetis* or non-agnetis samples Diagnoses as *S. agnetis* or non-*S. agnetis* was based on colony color on CHROMagar Orientation and qPCR-HRM of 16S V2 region (Reproduced from Al-Rubaye *et al*., 2017).

	Day	L56	L35W	W56
Healthy birds				
Birds tested (n)	14	20	41	19
	30	20	40	20
	41	20	40	20
	49	20	39	20
Avg. colonies per 0.1 mL blood	14	0.0	0.1	0.1
	30	0.7	2.3	0.5
	41	0.3	3.5	0.7
	49	4.7	31.0	42.5
% birds positive for *S. agnetis*	14	0	2	0
	30	0	0	0
	41	0	0	5
	49	0	36	30
% birds positive other	14	0	12	11
	30	30	33	35
	41	25	20	30
	49	70	26	35
Lame birds				
Birds tested	41	0	4	1
	49	1	7	8
	56	6	33	16
Avg. colonies per 0.1 mL blood	41		38.8	3.0
	49	0.0	117.1	94.4
	56	11.7	57.3	94.5
% birds positive for *S. agnetis*	41		0	100
	49	0	71	88
	56	50	82	100
% birds positive other	41		75	0
	49	0	14	0
	56	17	3	6
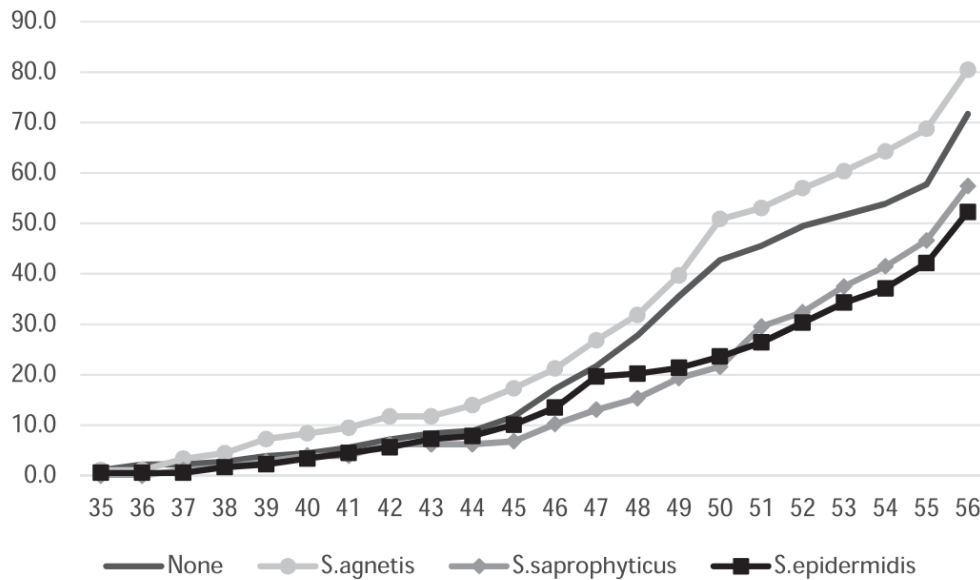
Figure 20. Incidence of lameness with *S. agnetis*, *S. saprophyticus*, and *S. epidermidis*. *S. agnetis* in drinking water. Cumulative percent lameness (y-axis) is plotted for broilers reared on wire-flooring with three different *Staphylococcus spp* administered at 10^5 CFU/mL in drinking water on days 20 and 21 (3 pens per treatment; 60 birds/pen). Values are calculated as the cumulative lameness percentage for days 35 through 56 based on the number of birds in that treatment group. Only the *S. agnetis* treatment was statistically different from None (P = 0.021). Average (±S.D.) % Lameness per pen was None 71.7 (±10); *S. agnetis* 80.5 (±1.8); *S. saprophyticus* 57.4 (±5.2); *S. epidermidis* 52.3 (±3.5). (Reproduced from Al-Rubaye *et al*., 2017).

An important question in the incidence of lameness whether in the lab or on a commercial farm for birds that develop the illness is the mode of transfer of transmission if it is contagious. We observed that some pens in our BCO trials showed lameness faster than others, so we performed a typhoid Mary experiment to study the potential intra-pen transmission of BCO. This work is discussed further in chapter five. Also, owing to a publication that *S. agnetis* has been identified in the guts of sheep scab mites (Hogg *et al*., 1999), we requested an entomologist look for mites on our poultry research facility. They did not find any (unpublished). In a recent project, we had an infestation of domestic flies (*Musca domestica*) in our facility, we retrieved some flies and preserved in the -80C freezer. In the future, we will be evaluating their microbiota to determine if houseflies are potential propagators of *S. agnetis* or other non-*agnetis* BCO agents that are

important to the incidence of BCO lameness. We also used the *S. agnetis* induced lameness model to evaluate the effectiveness of various probiotics and prebiotics for the ability to reduce lameness results are published (Alrubaye *et al*., 2020). In some of the trials to evaluate probiotic effects on the incidence of BCO, we utilized transepithelial electrical resistance (TEER) and Local Short Circuit (LSC) to evaluate the integrity of fresh gut (illeal) samples using the Ussing chamber. The results are published (Alrubaye *et al*., 2020). We evaluated the adherent illeal microbiome for control treatment vs two probiotic treatment groups with or with *S. agnetis* at Day14 or 59. We found that the age of birds was important for shaping the gut bacterial population associated with ileal mucosa of broiler chickens. Significant differences were observed in the abundance of phyla and genera taxonomic groups. Alpha diversity and beta diversity between the two different age groups were significant. Some commercial products may confer protections against BCO. This is important because our model is effective for evaluating the formulations proposed for remediation of BCO lameness (Unpublished). Using the *S. agnetis* model, we performed bone histopathology analysis that has been published, and some of which will be discussed in chapter four (Alrubaye *et al*., 2020a; 2020b). We graded the villi integrity and measured the length of villi per treatment of CHR Hansen probiotics. We found that illeal villus lengths correlated with reduced lameness for probiotic treatment (Al-Rubaye *et al*., 2020a, 2020b). Zinpro OTM micronutrients given to birds in feed at different levels significantly reduced the incidence of lameness (Alrubaye *et al*., 2020a). The evaluation of Zinpro micronutrients as a potential treatment of the BCO lameness manuscript is published and is discussed herein in chapter four. Paired ileal and jejunal samples from broilers utilized for TEER and LSC were stored in RNA later and used for expression analysis for multiple gut genes. We investigated the expression levels of three chicken mucin genes, *Muc4, Muc13,* and *Muc16*,

predicted to contain transmembrane domains (Lang *et al*., 2006; Forder *et al*., 2012). Our

expression analyses of lung tissues as a control revealed that *Muc4* is not significantly expressed,

but *Muc13* and *Muc16* appear to be expressed in the lung. Using the Reverse Transcriptase

quantitative PCR (RT-qPCR), we found that only *Muc13* (not *Muc4* or *Muc16*) is expressed at

detectable levels in either small intestinal tissue with respect to the TATA Binding Protein (*TBP*)

as a reference (Livak *et al*., 2001; Radonic *et al*., 2004). The differential expression for Occludin

1 (*OCLN1*), a gap junction protein alpha 1 (GJA1), Claudin 1 (*CLDN1*), and Catenin beta 1

(*CTNNB1*) with administrations of different levels of Zinpro micronutrients is described further

in Chapter four (Alrubaye *et al*., 2020 [A]). Recently, we utilized the *S. agnetis* lameness model to

perform whole-genome resequencing to identify genetic determinants of resistance to BCO that

cause lameness. We looked for SNP regions that distinguish birds that develop BCO lameness

early in the infection cycle versus those that survive to the end of the trial (56 days). Even though

this work is on-going, we believe this technique is sufficiently cost-effective for locating genetic

markers for many different quantitative traits in the incidence of lameness in broiler flocks. The

S. agnetis model for inducing BCO that causes lameness has been utilized to quantify the effects

of dietary mycotoxins in the incidence of BCO lameness. Dietary mycotoxins have been linked

to the onset of tibial dyschondroplasia (Wijesurendra *et al*., 2017). Mycotoxins can exacerbate

the incidence of lameness by contributing to the compromise of gut integrity and increased

translocation of pathogens into the bloodstream (BCO Lameness, 2019; Wijesurendra *et al*.,

2017). Even though *S. agnetis* was not administered in water in our recent trial, we induced

lameness by exposing birds to mycotoxins in feed and raising them on wire flooring. We found

that mycotoxins in broiler feed (simulating "spoiled" feed) did not significantly increase the

incidence of BCO lameness in broilers raised on litter- or wire-flooring systems (Unpublished).

S. agnetis BCO lameness model is efficient in testing various factors important to the disease. In

other aspects of studying BCO lameness, colleagues of mine have been working to perfect

techniques for typing *Staphylococcus* species important to the disorder (Zaki, Unpublished

Dissertation). This technique is important because it is less culture-dependent as it involves

targeting and amplifying conserved sequence regions of the bacterial 16S rRNA gene using

specific primer pairs for at least 5 of the 9 hypervariable regions (V1-V9) region (Chakravorty *et

al.*, 2007). This technique includes the high-resolution melting curve (HRM) in the real-time

PCR amplification of different hypervariable regions. This technique has the potential to be cost-

effective as a primer-based fluorescent-marked DNA fragment analsysis for reliable species

typing. This would be crucial to BCO lameness and other microbial infections (Steer *et al.*,

2009; Tong *et al.*, 2012). In addition to perfecting the PCR-HRM species determination

techniques, my colleague is working on chicken macrophage functions in the clearance of BCO

bacteria during the infection process *in vitro* (Zaki, Unpublished Dissertation). The team has

been uncovering the ability of pathogenic *S. agnetis* (isolated from chicken and/or cattle milk) to

evade the killing mechanism performed by macrophages (Zaki, Unpublished Dissertation). Since

Lowder *et al.* (2009) found that *S. aureus* has virulence properties that ensure reduced

phagocytic killing of the bacteria, we performed heterophil/neutrophil assay in-vitro with *S.

agnetis* and other BCO isolates using isolated WBC from leghorns and Arkansas random-bred

(ARB) chicken blood. Even though our results were inconclusive, we believe the protocol could

be perfected, and it has the potential to reveal the innate chicken immune responses to BCO

species that will be important for solving lameness. Overall, the multiple methodologies relevant

in the *S. agnetis* model for inducing BCO lameness are very efficient and cost-effective for

studying the mechanisms of pathogenesis of the disease and may be applied to other infectious disorders.

Human Osteomyelitis

One in 10, 000 children (more in boys than girls) are prone to acute osteomyelitis in western countries, and even higher in other countries (Heikki & Pääkkönen, 2014). Adults can also develop osteomyelitis, especially smokers, or people suffering from kidney disease, and diabetes. Bacteria may travel from injuries, spread from neighboring tissues with septic arthritis or cellulitis or hematogenous seeding during bacteremia. Most cases of childhood osteomyelitis are from seeding through the blood supply. And like many diseases, if osteomyelitis is not caught early and treated it can be devastating and have a long-term effect especially in children with lower access to resources. *S. aureus,* among other bacteria, is the leading of childhood osteomyelitis (Heikki & Pääkkönen, 2014; Maleb *et al*., 2017). Clinical symptoms in childhood osteomyelitis include limping, limited mobility, pain, fatigue, fever, focal tenderness, visible redness, and swelling around long bones more in the leg than the arm (Heikki & Pääkkönen, 2014; Bhowmik *et al*., 2018). In the pathogenesis of osteomyelitis when the bone is infected WBCs enter the area. As WBCs attempt to phagocytize bacteria, chemicals are secreted that degrade bone structures (Bhowmik *et al*., 2018). Vaso-occlusion occurs as pus clogs blood vessels in bones impairing blood flow and creating a bacterial foci. The pathogenicity of human osteomyelitis rivals that of BCO in broilers, there have not been any chicken models utilized to study human osteomyelitis. Rabbit, rat, Ovine/sheep, canine, goat, porcine, guinea pig, and hamster models have been utilized to study human cases (Reizner *et al*., 2014). Rabbit models involved either drilling holes or directly injecting various concentrations of *S. aureus* inoculum into the site of implantation, as most of these studies are of orthopaedic background. This action

resulted in a range of effects from chronic staphylococcal osteomyelitis in 88 % rabbits that got 2

x 10^8 CFUs after tibial fracture & rod insertion to no significant difference in infection rate

between treatment and control groups (Reizner *et al*., 2014). Injection of *S. aureus* into femoral

arteries of New Zealand white rabbits at 10^8 CFUs resulted in septic shock and death with 72

hours (Poultsides *et al*., 2008; Reizner *et al*., 2014). The same experiment with 10^8 CFUs

resulted in 80% osteomyelitis. Although there are successes recorded with some models more

than others, Reizner, *et al*. writes that there is a need for some new clinically relevant models to

reference prosthetic joint infection and hence the staphylococcal osteomyelitis (Reizner *et al*.,

2014). Judging from similarities in pathogenesis between human and chicken osteomyelitis, we

propose that broiler chickens might be suitable model to study staphylococcal osteomyelitis.

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CHAPTER 2

Analysis of Genomes of Bacterial Isolates from Lameness Outbreaks in Broilers

Analysis of Genomes of Bacterial Isolates from Lameness Outbreaks in Broilers

Abstract:

We investigated lameness outbreaks at commercial broiler farms in Arkansas. From Bacterial Chondronecrosis with Osteomyelitis (BCO) lesions, we isolated distinct bacterial species. Genomes assembled from *Escherichia coli* isolates were quite different between farms, and more similar to genomes from very different geographical locations. Genomes for *Staphylococcus aureus* were highly related to chicken isolates from Europe, but present in the Arkansas area for at least a decade. Phylogenomics suggest that this *S. aureus* is restricted to poultry, while the *E. coli* phylogenomics suggests broader host transfers. The results show that BCO-lameness pathogens on particular farms can differ significantly. Isolate-specific genome characterizations will help further our understanding of the disease mechanisms of BCO-lameness, a significant animal welfare issue.

Running Title: Broiler Lameness Bacterial Genomes

Introduction

Lameness poses animal health welfare issues which results in significant losses in poultry production. Modern broilers selectively bred for rapid growth are particularly prone to leg problems (1). Bacterial chondronecrosis with osteomyelitis (BCO) is the leading cause of lameness in broiler and broiler breeder flocks (1-3). In birds that develop lameness, bacteria translocate into the bloodstream via the integument, respiratory system or gastrointestinal tract (1, 4, 5). Bacteria may have come from the immediate environment, or vertical transfer though the egg (6). Bacteria that survive in the blood may colonize the proximal growth plate of the rapidly growing leg bones inducing BCO (1, 5, 7). Stressors, or other factors contributing to immunosuppression, can facilitate bacterial colonization and BCO spread in commercial poultry flocks (1, 8-13). In our research facility, *Staphylococcus agnetis* is the primary bacterial isolate from lame broilers induced by growth on raised-wire-flooring (2, 5). Multiple bacterial isolates have been identified from surveys in various BCO studies (1, 2, 11, 14-21). Recent surveys of 20 broiler flock farms in Australia suggested that avian pathogenic *E. coli* was the main BCO isolate (22). Genetic analysis by multilocus sequence type, pulsed field gel electrophoresis and PCR phylogenetic grouping, of 15 *E. coli* isolates from 8 flocks in Brazil indicated significant diversity for vertebral osteomyelitis and arthritis isolates, even in the same flock (23). The aim of this study was to characterize the genomes of BCO isolates from three different commercial broiler farms in Arkansas.

Methods

Microbiological Sampling and Bacterial Species Identification

Diagnosis of and sampling of BCO lesions and blood have been described (1, 2, 7, 24). Air sampling was by waving open CHROMagar Orientation (CO; DRG International, Springfield, NJ) plates within the building. CO and CHROMagar Staphylococcus (CS; DRG International) plates,

were used for chromogenic identification of species diversity. Bacterial species identification by 16S rDNA has been described (2, 5).

Genomic DNA Isolation and Sequencing

Cultures were preserved in 40% glycerol at -80°C. Working stocks were maintained on tryptic soy agar slants at 4°C. For DNA extraction, staphylococci were grown in tryptic soy broth to log phase and DNA was isolated using as described (25). DNA isolation from *E. coli* used lysozyme treatment, followed by organic extractions (26). DNA was quantified using a GloMax® Multi Jr Detection System (Promega Biosystems Sunnyvale Inc., CA, USA) and purity evaluated with a Nanovue spectrophotometer (Healthcare Biosciences AB Uppsala, Sweden). DNA size was verified by agarose gel (1.5%) electrophoresis.

Library construction and Illumina MiSeq 2 x 250 sequencing were at the Michigan State University Genomics Core Facility. Libraries for Illumina HiSeqX 2 x 125 sequencing were prepared using a RipTide kit (iGenomX, Carlsbad, CA) and sequenced by Admera Health (South Plainfield, NJ). Long reads were generated using Oxford Nanopore-MinION bar-code kit, as described (25).

Genome Assembly and Analysis

Hybrid and *de novo* genome assemblies were generated as described (25). Unicycler hybrid assembly graphs were further analyzed for contiguity in Bandage 0.8.1 (27) to discern replicons. The PATRIC (Pathosystems Resource Integration Center) webserver (28) was used to identify the most similar genomes. Average Nucleotide Identity (ANI) values were determined using pyANI 0.2.9 (29). ANI values were subtracted from 1 to generate distance matrices which were submitted to FastME 2.0 (30) to generate Newick trees. Archaeoptryx 0.9928 beta (31) was used to transform

Newick trees into graphic representations. Assemblies were annotated and compared using the Rapid Annotation using Subsystem Technologies (RAST) and SEED viewer (32, 33). Serotype prediction was using the ECTyper module at GalaxyTrakr.org.

Results and Discussion

Diagnosis and Microbiological Sampling

In June of 2016, we surveyed two commercial broiler houses on separate farms experiencing outbreaks of BCO-lameness. Both houses had experienced a loss of cooling a week earlier, causing heat stress for several hours. The farms were in rural, western Yell County (Arkansas) separated by 6.3 km, operated by the same integrator, and stocked from the same hatchery. The company veterinarian reported that samples from lame birds had been routinely submitted to a poultry health diagnostics laboratory and were primarily diagnosed as *E. coli*. Lame birds were randomly collected for necropsy for BCO lesions. Blood and BCO lesions were collected from these birds, and house air was sampled, for bacterial species surveys.

In Farm 1 the birds were 31 days old. We diagnosed and necropsied six lame birds (Table 1). KB1 and KB2 were symptomatic of spondylolisthesis/kinky-back (KB). KB1 had BCO lesions in T4, left tibia, and both femora. We obtained thousands (TNTC; too numerous to count) of small green colonies from the T4 sample that were determined to be *Enterococcus cecorum*. KB2 had BCO of only the left tibia, but no colonies were recovered from sampling from this site. We did recover approximately 50 green colonies from what appeared to be a normal T4 that was *E. cecorum*. Lame3 and Lame4 both had bilateral BCO of the femora and tibiae. Lame3 had TNTC white colonies from the microbiological sampling of T4 that were *S. agnetis*. Lame5 had bilateral FHN, bilateral tibial dyschondroplasia (TD), and pericarditis. We recovered green colonies (20 from left

100

and TNTC from right) from the TD lesions that were *E. cecorum*. Due to limited supplies, there was no microbiological sampling for Lame4 and Lame6.

Table 9. Microbiological sampling of bone and blood samples from two commercial broiler farms experiencing BCO outbreaks. BCO diagnoses are listed along with necropsy comments; RF- right femur, LF- left femur, RT-right tibia, LT- the left tibia, T4- vertebral joint, N- normal, THN- tibial head necrosis, THNS- THN severe, FHN- femoral head necrosis, KB- kinky back, TD- tibial dyschondroplasia, FHS- femoral head separation.

		BCO Diagnoses					High Colony Counts	
Bird	Farm	LT	RT	LF	RF	T4	Species	Site
KB1	1	THN	N	FHN	FHN	KB	*E. cecorum*	T4
KB2	1	THN	N	N	N	N	*E. cecorum*	T4
Lame3	1	THN	THN	FHN	FHN	KB	*E. coli*	RT
							S. agnetis	T4
Lame4	1	THN	THN	FHN	FHN	KB	-	-
Lame5	1	TD	TD	FHN	FHN		*E. cecorum*	LT RT
Lame6	1	THN	THN	FHN	FHN	KB	-	-
Lame7	2	N	THN	FHN	N	N	*E. coli*	blood
Lame8	2	THN	THN	FHS	FHN	N	-	
Lame9	2	THN	THN	FHN	N	KB	*E. coli*	blood LT LF
Lame10	2	THN	THN	N	FHN	KB	*S. enterica*	LT RF
Lame11	2	THNS	THNC	FHS	FHN	N	*E. coli*	blood LT LF
Lame12	2	THN	THN	FHT	FHT	N	*E. coli*	blood LT LF
Lame13	2	-	-	FHN	FHN	-	-	-
Lame14	3	THNS	THNS	FHN	N	-	*S. aureus*	LT LF RT
Lame15	3	THN	THNS	FHN	FHN	-	*S. aureus*	LT LF RT RF
Lame16	3	THNS	THNS	FHS	FHN	-	*S. aureus*	LT
Lame17	3	THNS	THNS	N	FHN	-	*S. aureus*	LT RT RF
Lame18	3	THNS	THNS	FHN	FHN	-	*E. coli*	RF LF
Lame19	3	THNS	THNS	FHN	FHS	-	*S. epidermidis*	LT RF
Lame20	3	THNS	THNS	N	N	-	*S. cohnii*	LT RT
Lame21	3	THNS	THNS	FHN	FHN	-	*S. aureus*	LT LF RT RF
Lame22	3	THNS	THNS	FHN	FHS	-	*S. simulans*	RF
Lame23	3	THNS	THN	FHN	FHN	-	*S. aureus*	LT LF RT RF
Lame24	3	THNS	THN	FHT	FHT	-	*S. aureus*	LT LF RT RF

For Farm 2 the birds were 41 days of age. We diagnosed and necropsied seven lame birds (Table 7). Lame7 was diagnosed with BCO lesions of the left femur, and right tibia. We obtained 30 purple colonies that were *E. coli* from the blood sample. We diagnosed Lame8 with bilateral BCO of tibia and femur. No colonies were obtained from sampling this bird. Lame9 was diagnosed with BCO of bilateral tibia, and the left femur, with evident pericarditis. Approximately 100 purple colonies were produced from sampling of the left femur that were determined to be *E. coli*. Lame10 was diagnosed with BCO of both tibia and right femur. Lame10 also had pericarditis. We got 40 white colonies from the left tibia, and 70 white colonies from the right femur that were *Salmonella enterica*. Lame11 had BCO lesions on both tibiae and femora. We got TNTC purple colonies from the left tibia, and 15 from blood that were determined to be *E. coli.* Lame12 was diagnosed with bilateral BCO of tibia and femur. We recovered approximately 500 purple colonies from blood and TNTC purple colonies from the left femur that were *E. coli*. Due to limited supplies there was no microbiological sampling for Lame13.

Average plate counts for air sampling were 80 and 125 for Farm 1 and Farm 2, respectively. The predominant species was *Staphylococcus cohnii* (~95%) with 3-4% *Staphylococcus lentus* and 1-2% *E. coli*.

In July 2019, we sampled a third commercial broiler farm (Farm 3) near Lincoln, Arkansas, more than 88 km from Farms 1 & 2 and operated by a different integrator supplied from a different hatchery. We sampled 11 lame birds at 35 days of age (Table 7). Lame14, was diagnosed with BCO of bilateral tibiae and right femur. We recovered numerous white colonies from the left femur, right tibia, and left tibia that were determined to be *Staphylococcus aureus*. Lame15 had BCO of all femorae and tibiae. Culture plates had numerous white colonies from all four sampled sites that were *S. aureus*. Lame16 had BCO of all tibiae and femorae. We recovered 10 green

colonies from the right femur that were not analyzed, and 10 white colonies from the left tibia that were determined to be *S. aureus*. We diagnosed Lame17 with BCO of the right femur and both tibiae. We recovered numerous white colonies from all three sites that were *S. aureus*. Lame18 was diagnosed with bilateral BCO of the femorae and the tibiae. We got TNTC purple colonies from both femorae, and a few purple colonies from both tibiae, that were *E. coli*. Lame19 had BCO of all femorae and tibiae. Swabs gave only a few colonies of *S. epidermidis* that we assumed were contaminants during sampling. Lame20 had bilateral BCO of the tibiae. We recovered a few green colonies of *Staphylococcus cohnii* which were presumed contaminants during sampling. Lame21 had BCO of all femorae and tibiae. We isolated TNTC white colonies from all four sites that were *S. aureus*. Lame22 was diagnosed with bilateral BCO of femorae and tibiae. Microbial sampling only yielded only 10 green colonies from the right femur that were determined to be *Staphylococcus simulans*. Lame23 was diagnosed with bilateral BCO of femorae and tibiae. Culture plates had only white colonies, TNTC from both femora and tibiae, that were *S. aureus*. Lame24 had BCO of all femorae and tibiae. We recovered more than 100 white colonies from all four BCO lesions that were *S. aureus*.

BCO Genome Assemblies

We chose to characterize genomes for representative *E. coli* isolates: 1409 for Farm 1, 1413 from Farm 2, with 1512 and 1527 from one bird on Farm 3 (Table 8). A hybrid assembly for 1409 produced 5.05 Mbp in 23 contigs that organized into 4 DNA assembly graphs. We resolved the replicons using the long reads for contiguity analysis of the assembly graphs using the Bandage software. The resolved genome appears to contain a 4.15 Mbp chromosome, with episomes of 643.5, 113.6, 108.7, 41.6, 2.3. There was also a 181 bp circle predicted based on the contiguity analyses. The predicted serotype is O16. The hybrid assembly for 1413 produced 5.37 Mbp in 58

contigs and 3 DNA assembly graphs. Unfortunately, the Nanopore reads were not of sufficient quality or length to complete a contiguity analysis of the entire genome, but does identify at least two episomes of 98.8 kbp and 2257 bp. The predicted serotype is O78. Draft assemblies were generated for *E. coli* 1512 and 1527. The assembly of 1512 contained 4.96 Mbp in 152 contigs with a N50 of 150 kbp. The assembly of 1527 was 4.90 Mbp in 179 contigs. The N50 was 97 Kbp with the largest contig of 258 Kbp. Both 1512 and 1527 are predicted to be serotype O78, like 1413. We generated draft assemblies for 14 *S. aureus* isolates from Farm 3 to examine genome diversity within a farm and within individual birds (Table 8). Two separate colonies from lame bird were used for draft genome assembly (1510 & 1511, 1513 & 1514, 1515 & 1516, 1517 & 1518, 1519 & 1520, 1521 & 1522, 1523 & 1524). The assemblies ranged from 2.79 to 2.82 Mbp in 60 to 96 contigs (excluding contigs < 300 bp). The largest contigs were between 279 and 284 Kbp. N50 values ranged from 58 to 113 kbp. The L50 values ranged from 7 to 14 contigs. Each of the *S. aureus* assemblies had at least 3 circular contigs (episomes). Table 3 summarizes all BCO isolate assemblies analyzed.

Table 10. Bacterial genome assemblies used or produced in these analyses are listed by species, Isolate designation, host source, assembly Genome Status, NCBI Accession number, and citation. Abbreviations are as in Table 1.

Isolate	Source	Genome Status	Biosample/Accession ID	Citation
E. coli				
1409	RT Lame3	Finished	SAMN12285857	This work
1413	Blood Lame12	Finished	SAMN12285859	This work
1512	LF Lame18	Draft	SAMN13245724	This work
1527	RF Lame18	Draft	SAMN13245725	This work
S. aureus				
1510	LT Lame14	Draft	SAMN13245722	This work
1511	RT Lame14	Draft	SAMN15589960	This work
1513	LF Lame15	Draft	SAMN15589961	This work
1514	RF Lame15	Draft	SAMN15589962	This work
1515	RF Lame16	Draft	SAMN15589963	This work
1516	LT Lame16	Draft	SAMN13245723	This work
1517	LT Lame17	Draft	SAMN15589964	This work
1518	RF Lame17	Draft	SAMN15589965	This work
1519	LT Lame21	Draft	SAMN15589966	This work
1520	RF Lame21	Draft	SAMN15589967	This work
1521	RF Lame23	Draft	SAMN15589968	This work
1522	RT Lame23	Draft	SAMN15589969	This work
1523	RF Lame24	Draft	SAMN15589970	This work
1524	LT Lame24	Draft	SAMN15589971	This work

Phylogenetic Comparison

To examine the phylogenetic relationships between *E. coli* isolates from the three farms, we identified the most closely related genomes according to PATRIC for 1409 (Farm 1), 1413 (Farm 2), 1512, and 1527 (Farm 3). We then identified the clades to which the closest related genomes are assigned in the NCBI dendrogram for all *E. coli* genomes (Figure 21). The *E. coli* from each

farm clearly map to very different clades. We downloaded 57 genomes representing the most closely related genomes identified by PATRIC for our four new *E. coli* genomes. We generated a phylogenetic tree for all 61 genomes using Average Nucleotide Identity (ANI), with isolates identified by region and host/source (Figure 22). Isolate 1409 grouped with one chicken isolate from Pakistan and 4 isolates from chickens in China. The other closely related isolates in that same branch were from cows, pigs, dogs, and humans from either the USA, China, France, or Mexico. Isolate 1413 clustered with one *E. coli* isolate from layer peritonitis in the United States, four Denmark chickens, and one US ground turkey meat. Other closely related isolates in the same branch came from US chicken breast, Bolivia human feces, UK chicken feces, US chicken thigh, US chicken brain, Latvia human clinical sample, US chicken pericardium, and two US chicken feces. Isolate 1512 and 1527 are virtually identical, with an ANI of 0.99995, which is not surprising since they were isolated from the same bird. Isolates 1512 and 1527 grouped with a pig isolate from China, a human isolate from France, and one chicken isolate from the UK, with ANI of 0.9996. The other closely related isolates came from human in Japan, sick domestic chickens in Poland, pigs from South Dakota, a FDA water contamination project in Arizona, US chicken eggs, human clinical samples, a coliseptic turkey in Israel, US citizens afflicted with haemolytic uraemic syndrome during a 2011 outbreak in Germany, Switzerland chicken meat, and US deer feces. The ANI for 1512/5127 relative to 1409 and 1413, is 0.98, while the ANI between 1409 and 1413 is 0.97, so the three genomes are equally distant from each other.

We generated another phylogenetic tree for our 14 *S. aureus* genomes and the 35 closest known genomes in PATRIC (Figure 23). Our 14 BCO *S. aureus* genomes grouped together with an average ANI of 0.999914, indicating a clonally-derived population. The 14 BCO *S. aureus* clustered with genomes for four isolates from retail chicken meat from Tulsa, Oklahoma, in 2010

(ANI=0.9998). The other closely related chicken isolates in this clade came from Poland in 2008, a US chicken hock in 1999, an infected chicken in Ireland in 1986-1987, and Belgium in 1976 (Table 9). The closest human isolates are from US sputum samples in 2012 (ANI=0.9994). The isolate designated UK_GgBroiler was the type strain (ED98) for a United Kingdom 1980s outbreak in chicken of *S. aureus* (34) which was attributed to a recent jump from humans to chickens (35). However, the phylogenomics indicate that this clade of *S. aureus* was infecting chickens in Belgium as early as 1976 (Figure 23: BE_GgBroiler and BE_GgBroiler1). We used the RAST SEED Viewer proteome comparison tool to analyze the evolution of this *S. aureus* chicken clade since 1987 in the UK (Table S2). We selected our assembly for 1519 as it was the largest assembly with the fewest contigs to represent the 2019 isolates from Farm 3. ED98 represents a 1986-1987 isolate, Ch21 (PO_GgBroiler1) is from Poland in 2008, and B4-59C is from 2010 in Tulsa poultry meat. We used the SEED Viewer compare function at rast.nmpdr.org with ED98 as the reference relative to the other proteomes to identify predicted proteins lacking (<50% identity) in one or more of the other three proteomes (Table 10). The analysis suggests that 32 proteins (31 phage and hypothetical proteins, and an efflux pump for Tetracycline resistance) were lost between 1996 and 2008. Eight phage and hypothetical proteins in ED98 and Ch21, were lost by 2010 in Tulsa, and only 4 hypothetical proteins in ED98, Ch21 and B4-59C, and 2019 in Lincoln Arkansas. We then reversed the analysis with 1519 as the reference to identify new proteins that appeared in the lineage to 1519 from ED98, through Ch21 and then B4-59C. The analysis identified 35 proteins present in 1519 for which the other 3 genomes lack a protein with 50% or greater identity. Twenty-eight are phage, hypothetical or plasmid-maintenance related. The remaining seven include a partial coding sequence for phosphoglycerate kinase, an aminoglycoside N6'-acetyltransferase, a DUF1541 domain-containing protein, and a

108

lead/cadmium/zinc/mercury/copper transporting ATPase. Two open reading frames (genes 1916 and 1917) are also novel to the 1519 genome that have overlapping open reading frames (ORFs) and may represent a frame shifted LPXTG cell wall anchor protein with a SdrC adhesin of unknown specificity. The adjacent gene (1915) is for a SdrD adhesin of unknown specificity. We reexamined this particular 4512 bp contig for assembly errors and could not find any based on templated alignments of the Illumina reads. BLASTn searches at NCBI found identical regions in a few other *S. aureus* genomes from human isolates. Therefore, the assembly appears correct, but that does not preclude ribosomal frame-shifting. The Unicycler assembly predicted this contig to be circular, so this may be a plasmid encoding adhesin functions. DUF1541 proteins of similar size are found in a wide range of different bacterial species. The divalent cation transporter is found in many different *Staphylococcus* species. The aminoglycoside-N6'-acetyltransferase (gene 1919) has no significant BLASTp homologs in any *S. aureus* genome in NCBI, and the best homologs are 70% identical in isolates of *Staphylococcus sciuri*, *Staphylococcus lentus,* and *Staphylococcus fleurettii.* This gene is present in a 4357 bp contig that Unicycler could not circularize. However, the contig termini each contain portions of a plasmid recombination MobE mobilization protein that likely could be fused into one ORF with long reads. The other genes in this contig are two hypothetical proteins, a tetracycline resistance predicted region, and an ArsR-family transcriptional regulator. However, this contig appears to possibly contain a mobile element affecting antibiotic resistance with the aminoglycoside transferase and the tetracycline resistance marker.

Further evolution of this genome is evidenced by proteins highly conserved (>80%) in 1519 and B4-59C, but not (<50%) in ED98 and Ch21. The Tulsa 2010 and Arkansas 2019 isolate genomes contain 16 proteins not found in ED98 and Ch21; including a toxic shock syndrome toxin 1 (gene

327), a phage associated exotoxin superantigen (gene 329) and a cluster (genes 2179, 2180, and 2181) of homologs to hypothetical proteins in superantigen-encoding pathogenicity islands. Genes 327 and 329 are in a 97,219 bp contig predicted as circular with a number of genes for exotoxins and pathogenicity islands. The contig only contains two phage predicted proteins, so it may be a large plasmid containing many virulence determinants.

There were only 3 proteins identified in 1519, B4-59C and Ch21, but not in ED98; two are hypothetical and the other a secretory antigen SsaA-like protein. This secretory antigen has been associated with transposons and also annotates as a CHAP domain protein, or putative cell wall lysis protein.

Our conclusions on the evolution of this clade of *S. aureus* that infects chickens is that the genome continues to evolve as it adapts from 1986 to 2020, with the acquisition of additional adhesins and virulence determinants. As such, this clade seems to be restricted, or specialized, for infecting chickens. The clade appears to have been in the Oklahoma, Arkansas region for more than a decade, but how it is transmitted to different farms or flocks is not clear. It could be vertically transmitted from hen to chicks. Alternatively, chicks could be exposed at the hatchery, or workers could spread the bacterium to farms through breakdowns in biosecurity.

In contrast the four *E. coli* genomes we characterized from three different farms show a very different pattern. Isolates 1512 and 1527 are highly related as they came from different BCO lesions in the same lame bird. For most lame birds we have reported that there is a predominant species that can be isolated from different BCO lesions, and often from the blood (2, 5). Our surveys of the three farms also demonstrate that there may be a predominant BCO pathogen within each farm, while in some broiler houses multiple species can be causing BCO lameness. *E. coli* 1409, 1413, and 1512/1527 genomes are very distinct and come from very different clades, which

is very different from the patterns for *S. aureus,* or as we have reported for *S. agnetis* infecting chickens (25). Reports from Brazil using virulence genes or MLST reported distinct *E. coli* genotypes within a flock (23). However, their data could not place the *E. coli* relative to those from non-chicken sources. The pattern we report from *E. coli* phylogenomics is most consistent with a generalist pathogen that easily jumps from host to host. Remarkably, two neighboring farms (Farm1 and Farm2) supplied by the same hatchery and operated by the same integrator, had very different *E. coli* (1409 and 1413) involved in BCO lameness outbreaks. This is more consistent with the *E. coli* on each farm originating from other hosts (zoonoses) or each farm could have "evolved" its own *E. coli* BCO pathogen over many flocks and years.

Conclusions

Overall, the *E. coli* isolates from BCO lesions in Arkansas appear to be highly diverse, as they derive from different clades that contain *E. coli* from non-chicken hosts. Conversely, the *S. aureus* isolates appear to come from a clade of chicken-specific isolates that date back five decades. Thus, the phylogenomics suggest that *E. coli* appears to be a generalist and can switch hosts much more easily than can *S. aureus*, which appears to be more of a specialist. This distinction may likely derive from a difference in genome size as the *E. coli* genomes are roughly twice the size of the *S. aureus* genomes.

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Appendix

Figure 21. Dendrogram based on genomic BLAST for 17,824 *E. coli* genomes from NCBI (https://www.ncbi.nlm.nih.gov/genome/167). The locations of the nearest genomes for the *E. coli* isolates presented in this work are indicated on the dendrogram.
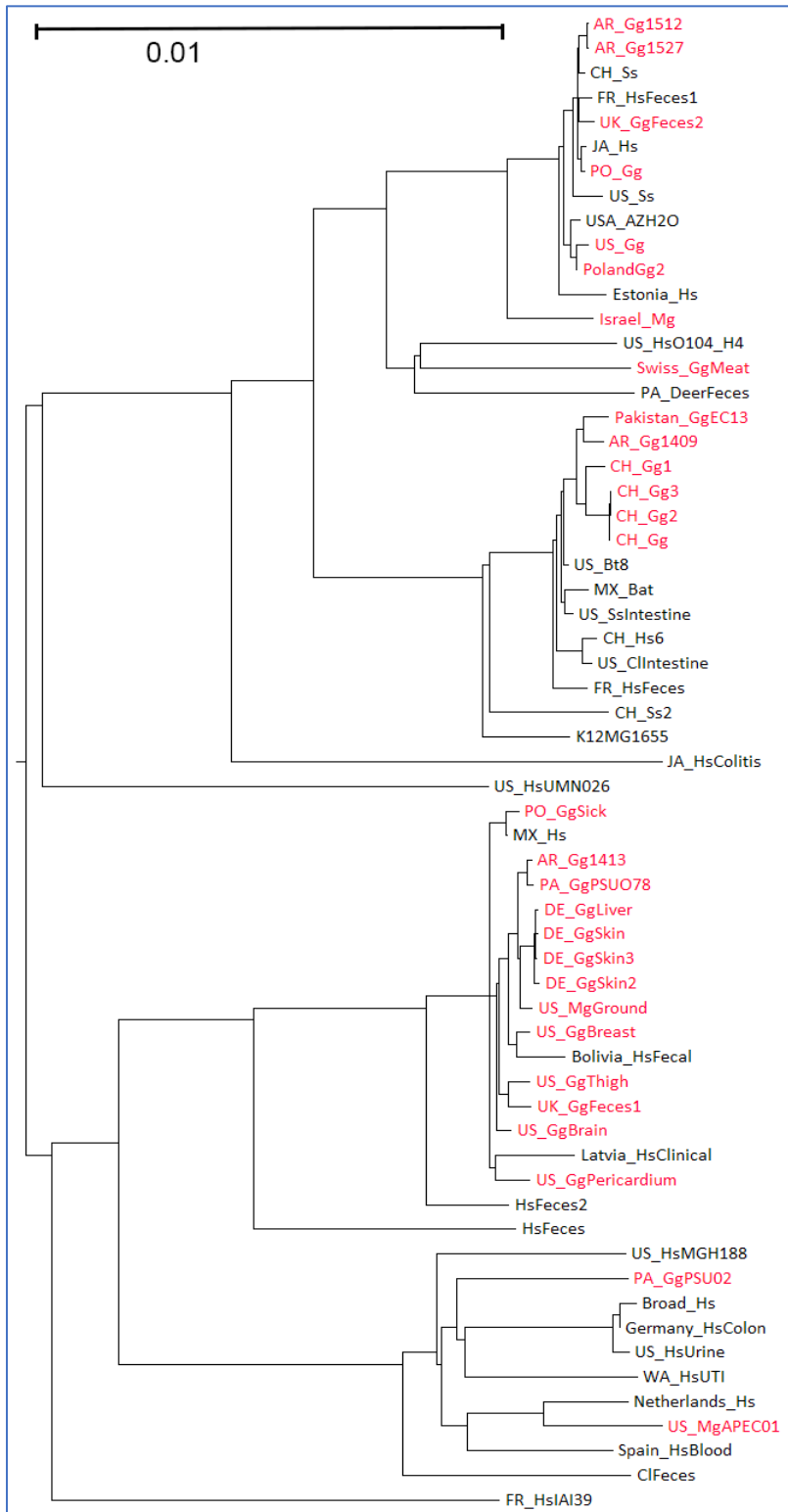
Figure 22. Phylogenetic tree for 61 *E. coli* genomes based on Average Nucleotide Identity. Key for isolate genomes is in Table S1. In brief, first two characters indicate location, first two characters after the underline indicate host, and remaining characters indicate source or isolate. Isolates in red are from poultry or poultry products.
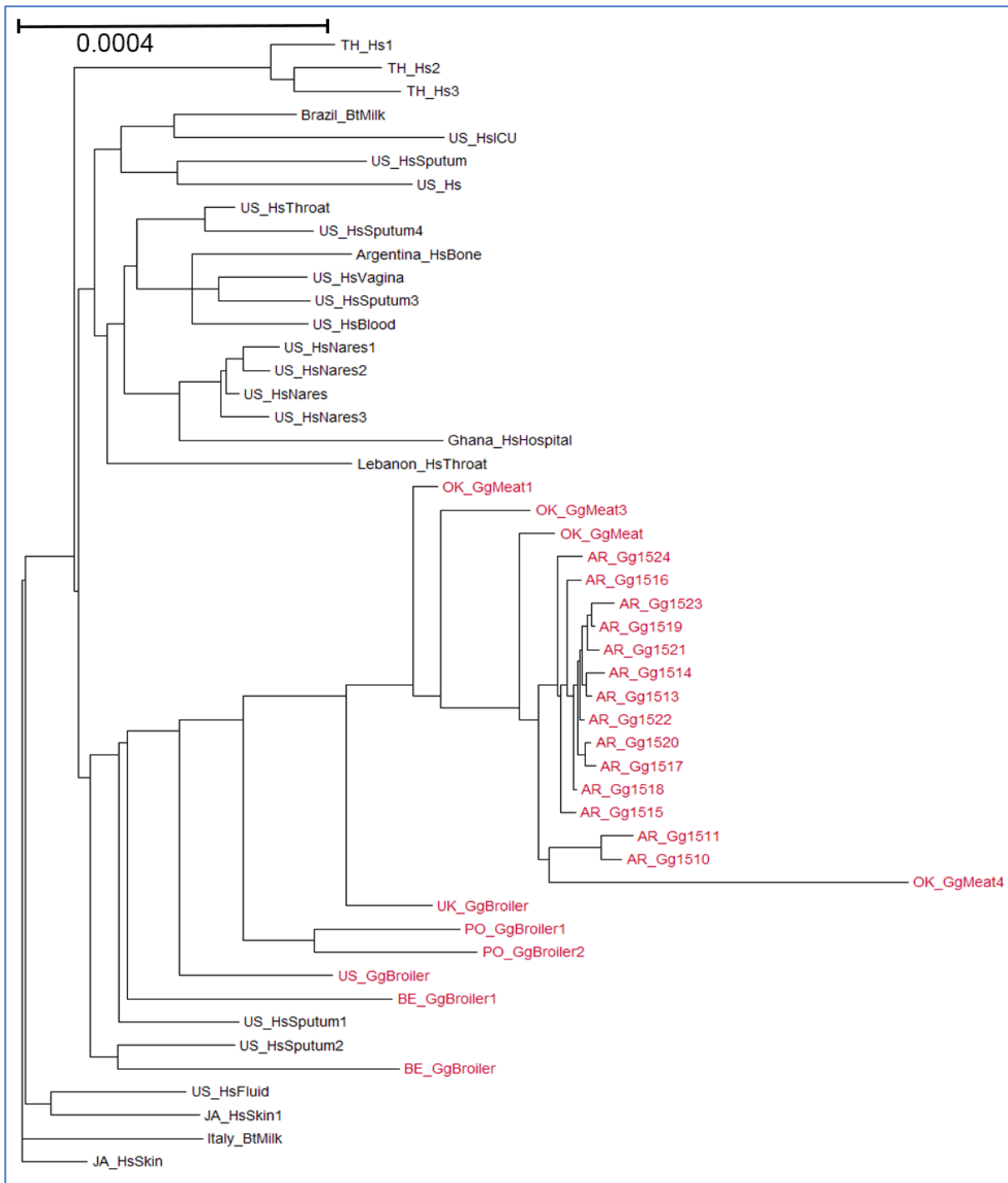
Figure 23. Phylogenetic tree for49 *S. aureus* genomes based on Average Nucleotide Identity. See legend to Figure 1 and Table 9 for isolate designations

Table 11. Genomes used for phylogenomic analyses. Designation is the coding used in the trees, Assembly is the NCBI accession, Country is the source of the isolate (if known), State is indicated for some USA isolates, Host is genus species from which the bacterium was isolated.

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
S. aureus							
OK_GgMeat	GCA_007726565.1	USA	OK	*Gallus gallus*	Meat	B3-17D	PRJNA555718
OK_GgMeat1	GCA_007726495.1	USA	OK	*G. gallus*	Meat	B2-15A	PRJNA555718
PO_GgBroiler	GCA_003336545.1	Poland		*G. gallus*	Broiler	ch23	PRJNA344860
OK_GgMeat3	GCA_007726545.1	USA	OK	*G. gallus*	Meat	B8-13D	PRJNA555718
PO_GgBroiler1	GCA_003343155.1			*G. gallus*	Broiler	ch21	PRJNA344860
PO_GgBroiler2	GCA_003350605.1			*G. gallus*	Broiler	ch22	PRJNA344860
OK_GgMeat4	GCA_007726525.1	USA	OK	*G. gallus*	Meat	B4-59C	PRJNA555718
UK_GgBroiler	GCA_000024585.1	United Kingdom		*G. gallus*	Broiler	ED98	PRJNA39547
US_GgBroiler	GCA_003336495.1	USA		*G. gallus*	Broiler	ch9	PRJNA344860
BE_GgBroiler	GCA_003336635.1	Belgium		*G. gallus*	Broiler	ch5	PRJNA344860
BE_GgBroiler1	GCA_003336625.1			*G. gallus*	Broiler	ch3	PRJNA344860
JA_HsSkin	GCA_003421965.1	Japan		*Homo sapiens*	Skin	M6K089	PRJDB5246
US_HsSputum	GCA_003720355.1	USA		*H. sapiens*	Sputum	CFBR-122	PRJNA480016
US_HsNares	GCA_000562505.1	USA		*H. sapiens*	Nares	W76127	PRJNA224506
US_Hs	GCA_900081525.1	USA		*H. sapiens*		MRSA	PRJEB1915
TH_Hs1	GCA_900126025.1	Thailand		*H. sapiens*		3688STDY6124906	PRJEB9575
US_HsThroat	GCA_003720885.1	USA		*H. sapiens*	Throat	BCH-SA-12	PRJNA480016
US_HsNares1	GCA_000559345.1	USA		*H. sapiens*	Nares	F41882	PRJNA224323
US_HsNares2	GCA_000571715.1	USA		*H. sapiens*	Nares	F29982	PRJNA225050
US_HsFluid	GCA_000609945.1	USA		*H. sapiens*	Fluid	DAR1890	PRJNA228339
US_HsNares3	GCA_000561365.1	USA		*H. sapiens*	Nares	H27777	PRJNA224442

Table 11 (Cont'd)

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
Argentina_HsBone	GCA_000610685.1	Argentina		*H. sapiens*	Bone	DAR3178	PRJNA228381
Lebanon_HsThroat	GCA_003038745.1	Lebanon		*H. sapiens*	Throat	SAM-7	PRJNA437720
US_HsSputum1	GCA_003719905.1	USA		*H. sapiens*	Sputum	CFBR-171	PRJNA480016
US_HsSputum2	GCA_003720135.1	USA		*H. sapiens*	Sputum	CFBR-149	PRJNA480016
Italy_BtMilk	GCA_006511605.1	Italy		*Bos taurus*	Milk	Lodi13K	PRJNA531079
Brazil_BtMilk	GCA_001676955.1	Brazil		*B. taurus*	Milk	A53	PRJNA315778
TH_Hs2	GCA_900127725.1	Thailand		*H. sapiens*		3688STDY6125002	PRJEB9575
TH_Hs3	GCA_900124915.1	Thailand		*H. sapiens*		3688STDY6125000	PRJEB9575
US_HsICU	GCA_000361205.1	USA		*H. sapiens*	ICU	M0455	PRJNA173479
US_HsBlood	GCA_000609765.1	USA		*H. sapiens*	Blood	DAR1813	PRJNA228330
US_HsVagina	GCA_001019375.1	USA		*H. sapiens*	Vagina	NRS156	PRJNA231221
US_HsSputum3	GCA_002123885.1	USA		*H. sapiens*	Sputum	CFSA134	PRJNA380429
JA_HsSkin1	GCA_003422345.1	Japan		*H. sapiens*	Skin	M6K136	PRJDB5246
US_HsSputum4	GCA_003720115.1	USA		*H. sapiens*	Sputum	CFBR-102	PRJNA480016
Ghana_HsHospital	GCA_008630855.1	Ghana		*H. sapiens*	Hospital	GHA2	PRJNA564764
AR_Gg1510	JACEHY000000000	USA	AR	*G. gallus*	Bone	1510	PRJNA554887
AR_Gg1511	JACEHW000000000	USA	AR	*G. gallus*	Bone	1511	PRJNA554887
AR_Gg1513	JACEHV000000000	USA	AR	*G. gallus*	Bone	1513	PRJNA554887
AR_Gg1514	JACEHU000000000	USA	AR	*G. gallus*	Bone	1514	PRJNA554887
AR_Gg1515	JACEHT000000000	USA	AR	*G. gallus*	Bone	1515	PRJNA554887
AR_Gg1516	JACEHX000000000	USA	AR	*G. gallus*	Bone	1516	PRJNA554887
AR_Gg1517	JACEHS000000000	USA	AR	*G. gallus*	Bone	1517	PRJNA554887
AR_Gg1518	JACEHR000000000	USA	AR	*G. gallus*	Bone	1518	PRJNA554887

Table 11 (Cont'd)

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
AR_Gg1519	JACEHQ000000000	USA	AR	*G. gallus*	Bone	1519	PRJNA554887
AR_Gg1520	JACEHP000000000	USA	AR	*G. gallus*	Bone	1520	PRJNA554887
AR_Gg1521	JACEHO000000000	USA	AR	*G. gallus*	Bone	1521	PRJNA554887
AR_Gg1522	JACEHN000000000	USA	AR	*G. gallus*	Bone	1522	PRJNA554887
AR_Gg1523	JACEHM000000000	USA	AR	*G. gallus*	Bone	1523	PRJNA554887
AR_Gg1524	JACEHL000000000	USA	AR	*G. gallus*	Bone	1524	PRJNA554887
E. coli							
Africa_Bt	GCA_001419785.1	Tanzania		*B. taurus*		33	PRJNA293513
AR_Gg1409	JACGTG000000000	USA	AR	*G. gallus*	Bone	1409	PRJNA554886
AR_Gg1413	JACGTF000000000	USA	AR	*G. gallus*	Bone	1413	PRJNA554886
AR_Gg1512	JACGTE000000000	USA	AR	*G. gallus*	Bone	1512	PRJNA554886
AR_Gg1527	JACGTD000000000	USA	AR	*G. gallus*	Bone	1527	PRJNA554886
Bolivia_HsFecal	GCA_003850735.1	Bolivia		*H. sapiens*	feces	286A	PRJNA427943
Broad_Hs	GCA_002244745.1	USA	WA	*H. sapiens*	Urine	136-1758	PRJNA269984
CH_Gg	GCA_003009015.1	China		*G. gallus*		12c7	PRJNA417344
CH_Gg1	GCA_002959165.1	China		*G. gallus*		YH17134	PRJNA434044
CH_Gg2	GCA_003008775.1	China		*G. gallus*		12c8	PRJNA417344
CH_Gg3	GCA_003009715.1	China		*G. gallus*		12c5	PRJNA417344
CH_Hs6	GCA_003302635.1	China		*H. sapiens*	feces	A61	PRJNA400107
CH_Ss	GCA_003328175.1	China		*Sus scrofa*		E565	PRJNA450836
CH_Ss2	GCA_000987875.1	China		*S. scrofa*	Feces	SEC470	PRJNA244370
ClFeces	GCA_003043915.1			*Canis lupus*	Feces	RM14723	PRJNA341281
DE_GgLiver	GCA_001652345.1	Denmark		*G. gallus*	liver	E44	PRJNA321591
DE_GgSkin	GCA_003015065.1	Denmark		*G. gallus*	skin	L7S7	PRJNA438734

Table 11 (Cont'd)

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
DE_GgSkin2	GCA_003013555.1	Denmark		*G. gallus*	skin	L3S3	PRJNA438662
DE_GgSkin3	GCA_003015075.1	Denmark		*G. gallus*	skin	L5S5	PRJNA438735
Estonia_Hs	GCA_006238365.1	Estonia		*H. sapiens*	clinical sample	EEIVKB55	PRJNA528606
FR_HsFeces	GCA_900536595.1	France		*H. sapiens*	feces	CEREMI_E32	PRJEB28341
FR_HsFeces1	GCA_900499885.1	France		*H. sapiens*	feces	884A	PRJEB28020
FR_HsIAI39	GCA_000026345.1	France		*H. sapiens*		IAI39	PRJNA33411
Germany_HsColon	GCA_000183345.1	Germany		*H. sapiens*	Ileum	NRC857c	PRJNA41221
HsFeces	GCA_003018255.1			*H. sapiens*	Feces	2012C-4502	PRJNA218110
HsFeces2	GCA_003018095.1			*H. sapiens*		2014C-3338	PRJNA218110
Israel_Mg	GCA_000819645.1	Israel		*Meleagris gallopavo*	Blood	789	PRJNA262513
JA_Hs	GCA_006535915.1	Japan		*H. sapiens*		SMEc189	PRJDB8148
JA_HsColitis	GCA_000008875.1	Japan		*H. sapiens*	Hemorrhagic colitis	SAKAI (EHEC)	PRJNA226
K12MG1655	GCA_000005845.2					MG1655	PRJNA603343
Latvia_HsClinical	GCA_006236595.1	Latvia		*H. sapiens*	Clinical sample	LVSTRB103	PRJNA528606
MX_Bat	GCA_002456375.1	Mexico		*Tadarida brasiliensis*	Feces	MOD1-EC908	PRJNA230969
MX_Hs	GCA_002485345.1	Mexico		*H. sapiens*	Feces	MOD1-EC6621	PRJNA230969
Netherlands_Hs	GCA_002888415.1	Netherlands		*H. sapiens*	Meninges	SP-16	PRJNA429029
PA_DeerFeces	GCA_002215155.1	USA	PA	*Odocoileus virginianus*	Feces	PSUO103	PRJNA314794
PA_GgPSU02	GCA_002215095.1	USA	PA	*G. gallus*	Peritoneum	PSUO2	PRJNA287563
PA_GgPSUO2	GCA_002215095.1	USA	PA	*G. gallus*	Peritoneum	PSUO2	PRJNA287563
PA_GgPSUO78	GCA_002215115.1	USA		*G. gallus*	Peritoneum	PSUO78	PRJNA287566

Table 11 (Cont'd)

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
Pakistan_GgEC13	GCA_004284075.1	Pakistan		*G. gallus*	infection	EC_13	PRJNA522294
PO_Gg	GCA_001709145.1	Poland		*G. gallus*		019PP2015	PRJNA319144
PO_GgSick	GCA_001696335.1	Poland		*G. gallus*	sick domestic birds	012PP2015	PRJNA319144
PolandGg2	GCA_001758245.1	Poland		*G. gallus*		022PP2016	PRJNA319144
Spain_HsBlood	GCA_000500875.1	Spain		*H. sapiens*	Blood	GSK2024	PRJNA221787
Swiss_GgMeat	GCA_001660565.1	Switzerland		*G. gallus*	Meat	S51	PRJNA323827
UK_GgFeces1	GCA_900490165.1	United Kingdom		*G. gallus*	feces	VREC0540	PRJEB8774
UK_GgFeces2	GCA_900482085.1	United Kingdom		*G. gallus*	feces	VREC0637	PRJEB8774
US_Bt8	GCA_004792865.1	USA		*B. taurus*	feces	KCJK8229	PRJNA420036
Germany_BtMastitis	GCA_000829985.1	Germany		*B. taurus*	Mastitis	1303	PRJNA46529
US_ClIntestine	GCA_002232435.1	USA	NY	*C. lupus*	Intestine	MOD1-EC5097	PRJNA230969
US_Gg	GCA_002512585.1	USA	AL	*G. gallus*	Egg	MOD1-EC6339	PRJNA230969
US_GgBrain	GCA_002537555.1	USA		*G. gallus*	brain	MOD1-EC6094	PRJNA230969
US_GgBreast	GCA_003793955.1	USA		*G. gallus*	Chicken Breasts	CVM N17EC0744	PRJNA292663
US_GgPericardium	GCA_002231405.1	USA	PA	*G. gallus*	Pericardial Sac	MOD1-EC5115	PRJNA230969
US_GgThigh	GCA_003794735.1	USA		*G. gallus*	Chicken Thighs	CVM N17EC0412	PRJNA292663
US_HsMGH188	GCA_002152225.1	USA	MA	*H. sapiens*		MGH188	PRJNA271899
US_HsO104_H4	GCA_000299455.1	USA		*H. sapiens*	Feces	2011C-3493	PRJNA81095
US_HsUMN026	GCA_000026325.2	USA		*H. sapiens*		UMN026	PRJNA33415
US_HsUrine	GCA_002245075.1	USA	WA	*H. sapiens*	Urine	225-2935	PRJNA269984

Table 11 (Cont'd)

Designation	Assembly	Country	State	Host	Source	Strain	BioProject
US_MgAPEC01	GCA_000014845.1	USA	IA	*M. gallopavo*	Lung	APEC 01	PRJNA16718
US_MgGround	GCA_003774815.1	USA		*M. gallopavo*	ground meat	CVM N17EC1100	PRJNA292663
US_Ss	GCA_002474525.1	USA	SD	*S. scrofa*	Ileum	MOD1-EC5757	PRJNA230969
US_SsIntestine	GCA_002464015.1	USA		*S. scrofa*	Jejunum	MOD1-EC6458	PRJNA230969
US_Ss	GCA_002474525.1	USA	SD	*S. scrofa*	Ileum	MOD1-EC5757	PRJNA230969
US_SsIntestine	GCA_002464015.1	USA		*S. scrofa*	Jejunum	MOD1-EC6458	PRJNA230969
USA_AZH2O	GCA_002534895.1	USA	AZ		water	MOD1-EC5915	PRJNA230969
WA_HsUTI	GCA_000778565.1	USA	WA	*H. sapiens*	Urine	UPEC-208	PRJNA248737

Table 12. Proteome differences in four genomes of *S. aureus* infecting chickens. The SEED viewer was used to identify genes present in 1519 where the predicted polypeptide had a % identity less than 50% in one or more of the genomes for the indicated isolates. Gene is the RAST 1519 annotation gene number, Length is for the 1519 polypeptide, and function is the annotation from RAST.

B4-59C	Ch21	ED98	1519 Gene	Length	function
0	100	100	6	148	hypothetical protein
0	100	100	7	83	hypothetical protein
0	100	100	16	112	hypothetical protein
0	0	0	32	182	DUF1541 domain-containing protein
36.39	35.91	36.39	33	688	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)
0	0	0	117	43	hypothetical protein
0	0	0	153	38	FIG01108408: hypothetical protein
100	0	0	296	97	hypothetical protein
100	34.45	0	327	235	Toxic shock syndrome toxin 1 (TSST-1)
100	0	0	328	55	hypothetical protein

Table 12 (Cont'd)

B4-59C	Ch21	ED98	1519 Gene	Length	function
100	0	0	331	140	Phage protein
100	0	0	332	390	Phage protein
0	0	0	421	40	Phosphoglycerate kinase (EC 2.7.2.3)
100	45.9	45.9	500	48	hypothetical protein
100	100	0	562	49	Secretory antigen SsaA-like protein transposon-related
0	0	0	595	44	hypothetical protein
100	36.02	36.02	729	222	hypothetical protein
100	26.09	26.09	732	242	hypothetical protein
100	0	0	733	71	Phage protein
0	100	100	1021	128	hypothetical protein
100	100	0	1197	45	hypothetical protein
0	100	100	1210	39	hypothetical protein
0	0	0	1289	64	hypothetical protein
100	100	0	1421	44	hypothetical protein
0	98.63	100	1553	74	Hypothetical protein, phi-ETA orf24 homolog [SA bacteriophages 11, Mu50B]
0	0	0	1554	72	Phage protein
37.38	36.1	37.38	1555	414	Phage DNA helicase
0	0	0	1556	119	Phage protein
0	0	0	1557	255	Phage replication initiation protein
0	99.53	100	1560	213	Phage-associated recombinase
0	100	100	1561	160	ORF027
39.53	97.67	98.84	1563	87	Hypothetical protein, PVL orf39 homolog [SA bacteriophages 11, Mu50B]
0	98.63	97.26	1565	74	Hypothetical protein, PV83 orf12 homolog [SA bacteriophages 11, Mu50B]
0	98.21	98.21	1566	57	Uncharacterized protein pCM2_0059
0	100	100	1567	97	phage protein
0	81.35	100	1568	251	Phage antirepressor protein
0	100	100	1569	62	hypothetical protein

Table 12 (Cont'd)

B4-59C	Ch21	ED98	1519 Gene	Length	function
0	100	100	1570	201	Phage protein
0	0	0	1572	65	Phage protein
0	0	0	1573	257	Phage antirepressor protein
0	0	0	1574	79	hypothetical protein
0	93.44	100	1576	110	Phage protein
0	100	99.69	1577	553	DNA adenine methylase (EC 2.1.1.72)
25.71	100	100	1578	350	Phage integrase
0	0	0	1719	41	hypothetical protein
100	0	0	1760	52	hypothetical protein transposon-related
0	0	0	1862	158	hypothetical protein
0	0	0	1863	76	hypothetical protein
0	0	0	1864	47	hypothetical protein
0	0	0	1865	65	hypothetical protein
0	0	0	1866	56	hypothetical protein
0	0	0	1867	88	hypothetical protein
0	0	0	1868	77	hypothetical protein
0	0	0	1869	157	hypothetical protein
0	0	0	1870	99	hypothetical protein
0	0	0	1871	186	hypothetical protein
0	0	0	1872	54	hypothetical protein
0	0	0	1916	52	Adhesin of unknown specificity SdrC
0	0	0	1917	205	Type I restriction-modification system, DNA-methyltransferase subunit M (EC 2.1.1.72) / Type I restriction-modification system, specificity subunit S
24.35	24.35	24.35	1919	178	Aminoglycoside N6'-acetyltransferase (EC 2.3.1.82)
0	0	0	1921	144	hypothetical protein
31.82	31.82	31.82	1922	110	Transcriptional regulator, ArsR family
0	0	45	1923	173	Plasmid recombination, MobE mobilization protein

Table 12 (Cont'd)

B4-59C	Ch21	ED98	1519 Gene	Length	function
100	0	0	2179	95	Hypothetical SAV0793 homolog in superantigen-encoding pathogenicity islands SaPI
100	30.08	30.08	2180	121	Hypothetical SAV0792 homolog in superantigen-encoding pathogenicity islands SaPI
100	0	0	2181	486	Hypothetical SAV0791 homolog in superantigen-encoding pathogenicity islands SaPI
100	0	0	2189	45	hypothetical protein
100	29.38	29.38	2191	193	hypothetical protein
100	0	0	2192	59	hypothetical protein
0	0	0	2358	280	hypothetical protein
0	0	0	2369	205	hypothetical protein
0	0	0	2384	146	Phage holin
0	100	100	2688	39	hypothetical protein

CHAPTER 3

Investigation of Embryo Lethality Assay for Assessing Virulence of Isolates from Bacterial

Chondronecrosis with Osteomyelitis in Lame Broilers.

Investigation of Embryo Lethality Assay for Assessing Virulence of Isolates from Bacterial Chondronecrosis with Osteomyelitis in Lame Broilers.

Abstract

We used an embryo lethality assay (ELA) to assess virulence for different isolates from cases of bacterial chondronecrosis with osteomyelitis (BCO) in broilers. ELA has been previously used to measure virulence and lethal dosage of *Enterococcus faecalis* and *Enterococcus cecorum*. We hypothesized that ELA could substitute for more laborious and costly assessments of BCO isolate pathogenicity using live birds. We evaluated two different levels of bacteria injected into eggs from layer and commercial broiler embryos. Significant findings include a) *Escherichia coli* from neighboring farms operated by the same integrator had very different embryo lethality, b) isolate *Staphylococcus agnetis* 908 had low virulence in ELA, even though this isolate can induce more than 50% BCO lameness, c) *Enterococcus cecorum* 1415 also had low pathogenicity; even though it was recovered from severe bilateral tibial dyschondroplasia, and d) human and chicken isolates of *S. aureus* had significant pathogenicity. Therefore, ELA may not be an effective measure for assessing virulence with respect to BCO.

Keywords**:** Embryo lethality; Lameness; Virulence; Bacterial chondronecrosis with osteomyelitis

Introduction

Bacterial chondronecrosis with osteomyelitis (BCO) is the leading cause of lameness in rapidly growing broilers (1-4). Lameness in broilers is significant as an animal welfare issue, and as a financial cost, in the poultry industry (2). Our research group isolated and characterized an isolate of *S. agnetis,* designated 908, from lame broilers on our research farm (1). *S. agnetis* 908 can induce greater than 50% BCO lameness by 56 days of age when administered in a single dose in drinking water at 10^4 to 10^5 CFU/mL on day 20 (1, 3, 5, 6). Our current model for lameness etiology is that stress can lead to increased leakage (translocation) of bacteria across the gut and pulmonary epithelia into the blood system (1-3, 7-9). Particular species are then able to colonize the growth plate, a vulnerable niche in the blood system of the rapidly growing leg bones of fast-growing broilers (2, 3, 10). Therefore, significant lameness can be induced by specific bacterial pathogens. Distinct bacterial species have been isolated from lame birds including *Staphylococcus aureus*, *Enterococcus cecorum*, and *Escherichia coli* (11-31). However, there are few comparisons of different BCO-associated species, or isolates, for pathogenicity (17, 32). In this study, we investigated the pathogenicity of *S. agnetis*, *Staphylococcus chromogens, E. coli*, *E. cecorum,* and *S. aureus* isolates using an embryo lethality assay (ELA). The isolates were obtained from BCO lesions on our research farm or commercial broiler farms in Arkansas. ELA has been used to correlate the expression frequency of nine virulence-associated *E. coli* genes with embryo mortality (33). Borst *et al*. (32) used this technique to compare the virulence of *E. cecorum* isolated from broiler spinal lesions (kinky back) to non-pathogenic *E. cecorum* strains isolated from ceca of unaffected birds. Blanco *et al*. (34) used ELA to determine the virulence and the lethal dose of *Enterococcus faecalis*.

Methods

Microbiology

Isolation and handling of the isolates has been described (1, 35). Media included: CHROMagar Orientation (CO; DRG International, Springfield Township, NJ), tryptic soy broth (Difco brand, Becton, Dickinson and Company, Franklin Lakes, NJ); and Luria broth (LB; per liter 10 g tryptone, 5 g yeast extract, 5 g NaCl).

ELA

Fertilized eggs were obtained from leghorns (LCL) and cobb700 commercial broilers (BCL) on the University of Arkansas research farm. The eggs were washed with warm soapy water containing a small amount of household bleach. Eggs were incubated (NatureForm™ Hatchery Systems, Jacksonville, FL, USA) at 37.5°C, relative humidity of 56%, on autorotate. On day 12, stationary-phase bacterial cultures were washed with 1xphosphate buffer saline (1xPBS; 150mM NaCl, 10 mM KHPO4 pH 7.2), CFU concentration estimated by spectrophotometry (A650) using pre-calibrated standard curves for each isolate, and then diluted in 1xPBS to the required concentration. eggs were candled, and fertile eggs were injected using a tuberculin syringe and 20G needle (Becton, Dickinson, and Company) with 100µL of the appropriate bacterial suspension, or vehicle control, into the allantois cavity. The opening was sealed with transparent cello tape. Inoculated embryos were scored for mortality every day for 4 days after bacteria administration (32, 34).

Electro-transformation

Overnight cultures of *E. coli* in LB were diluted 20-fold with warm LB and incubated for 4 hours at 37 °C with good aeration. The cells were pelleted (5000 x g; 5 min; 4°C) then resuspended in an equal volume of ice-cold sterile deionized H_2O, then pelleted. The pellet was again

resuspended in 0.5 volumes of ice-cold sterile deionized H2O, then pelleted. The pellet was resuspended in 0.01 volumes of ice-cold sterile 10% glycerol. Then 100 ul of cells were mixed with DNA and transferred to a 1 mm gap electroporation cuvette. The cuvette was precooled on ice, then electroporated in a BIO-RAD Gene Pulser™ set to 200 Ohms, 25 µFD, and 1.75 kvolts. The cells were diluted into 1 ml pre-warmed LB and incubated for 45 minutes with shaking at 37 °C. Then 10^6 CFUs were injected into the allantois cavity of a 12-day old fertilized LCL embryo, as described above. After 5 days, a live embryo was sampled after surface sterilization to collect 100 µl of yolk with a tuberculin syringe through the same opening as for injection. The sample was added to X ml of LB and grown overnight with shaking at 37 °C. An aliquot was spread on CHROMagar Orientation for chromogenic verification as pure *E. coli*. The injection and recovery were sequentially repeated for a total of three times.

Statistical Analysis

The results of the ELA were analyzed with either Pearson's Chi-squared ($\chi 2$) or Fisher's Exact (FE) analysis using SAS and R software (SAS Institute. 2011; RStudio Team. 2016). Significant differences between Phosphate saline vehicle and treatments were accepted at $P < 0.05$.

Results

Embryo Lethality Assay with BCO isolates

To establish a suitable assay for comparing different isolates, we first injected *E. coli* 1413 at a range of amounts ($10^3 – 10^8$ CFUs) in sterile 1xPBS to estimate the lethal dosage for Leghorn Chicken Line (LCL) embryos (Figure 24). For doses above 10^5 CFUs of 1413 had significant embryo lethality with doses 10^5, 10^{7-8} CFUs compared with 1XPBS control. We, therefore, assessed different BCO isolates at 10^5 and 10^6 CFUs (Table 11). We included *S. agnetis* 908 recovered from a femoral BCO lesion on our research facility as this isolate can induce lameness

≥50% by day 56 when administered in drinking water for two days to 20-day old broilers (1, 3, 5, 6). Surprisingly, 908 injections of even 10^6 CFUs resulted in only 14% embryo lethality, a level not statistically different from phosphate saline control treatment (Figure 25A). For the methicillin-sensitive human *S. aureus* isolate 1302, originally retrieved from a wound (Table 1), injections of 10^5 or 10^6 CFUs resulted in 80% embryo lethality (Figure 25B). *Staphylococcus chromogens* 1401 was recovered from an infected T4 vertebra of a chicken with "Kinky back" (Table 11). Injections of 10^6 CFUs resulted in only 7% embryo death, less than the 1xPBS control for that experiment (Figure 25C). *E. coli* 1409 was recovered from a tibial head necrosis lesion (Table 11). Injection of 10^5 to 10^6 CFUs resulted in no lethality through day 4 (Figure 25D). *E. coli* 1413 was isolated from the blood of a lame bird with bilateral BCO of the tibiae and femorae, where *E. coli* was also recovered from multiple lesions (Table 11). As before, injections of 10^5 or 10^6 CFUs into LCL resulted approximately 80% embryo lethality (Figure 25E). *E. cecorum* 1415 was isolated from a tibial head abscess in a case of bilateral tibial dyschondroplasia (Table 11). ELA results for 10^5 CFU actually showed more lethality than 10^6 CFUs but neither was statistically different from the PBS control (Figure 25F). We used two isolates (1510 & 1514) of *S. aureus* obtained from BCO lesions from two different birds in a commercial broiler house lameness outbreak where draft genome assemblies were highly related (Table 11; Ekesi 2020). The isolates showed different ELA results with 1510 lethality of 60% for 10^6 CFU, while 1514 produced 47% but only the 1510 results were statistically significant (Figure 25G & 25I). *E. coli* 1512 and 1527 were recovered from the left and right femoral lesions of the same bird (Table 11; Ekesi, 2020). Draft genome assemblies for both 1512 and 1527 were determined to be virtually identical. ELA results for 1512 yielded 87% lethality for 10^6 CFUs and 52% lethality for 10^5, but only the 10^6 results were statistically different from the

PBS control (Figure 25H). Therefore, only the human isolate *S. aureus* 1302, and chicken isolates *E. coli* 1413, and *S. aureus* 1510 and *E. coli* 1512, were found to cause significant lethality using LCL embryos.

We then chose to extend the analyses by comparing the results for ELA with layer embryos to ELA using Broiler Chicken Line (BCL) embryos. As shown in Figure 26, significant embryo lethality was obtained with *S. aureus* isolates1302 and 1514, plus *E. coli* 1413 and 1512. *S. agnetis* 908, *S. chromogenes* 1401, *E. coli* 1409, and *E. cecorum* 1415, showed no virulence for either CFU quantity. We did note that for all four isolates that showed lethality for BCL, both the 10^5 and 10^6 CFU injections showed significant embryo mortality (Figure 26 panels B, E, G and H). For LCL the 10^5 injections were only different from the controls for *S. aureus* isolate 1302 and *E. coli* 1413. However, for the other two isolates we might reach significance for the 10^5 CFU injections with more embryos. We also note that lethality was more rapid in the BCL than with LCL embryos (Figure 25 and 26).

Is ELA Virulence Readily Transferable by Electroporation?

To study the potential mechanism(s) of bacterial virulence acquisition, we used electroporation to transfer DNA from *E. coli* 1413 into *E. coli* 1409 to produce 1540T. We then passaged these cells through three rounds of selection by ELA in LCL embryos. After each round bacteria were recovered from one of the living embryos and verified on chromogenic media as *E. coli*. The rescued bacteria from each round were designated 1541P, 1544P, and 1547P (Table 11). We then repeated the ELA assay to compare the lethality of the transformant and recovered populations to 1409. Isolate 1409 inoculated into 10^6 CFUs 1409 into LCL embryos produced higher embryo lethality that previously for LCL embryos and was statistically different from 1xPBS control (P = 0.002). Injection of 10^6 CFU of 1540T resulted in a 27% lethality of LCL that was not different

133

from the negative control (Figure 27A). The bacterial culture recovered from live embryos was designated *E. coli* 1541P and confirmed as *E. coli* on chromogenic CO plates (Table 11). Injection of 10^6 CFU of 1541P into LCL embryos resulted in 20% lethality which was not different from the PBS control (Figure 27B). The culture recovered from live embryos was designated 1544P and confirmed as *E. coli* as before. When we injected 1544P injected into LCL at 10^6 CFU there was 40% embryo lethality which was statistically different from the PBS control (Figure 27C). The recovery from live embryos was repeated and designated *E. coli* 1547P. Injection of 10^6 CFUs of 1547P into LCL embryos resulted in 73% lethality (Figure 27D).

Discussion

We performed ELA with different bacterial isolates isolated from lame broilers to estimate relative pathogenicity. We observed that *S. agnetis* 908 is not pathogenic in the ELA even though we have shown that this isolate readily infects young broilers when administered at 10 to 105 CFU/ml in drinking water at 20 and 21 days of age (1, 3, 5, 6). Those broilers then begin to develop lameness by 41 days of age and by 56 days of age 50% of the birds will be clinically lame with BCO lesions in proximal femoral and tibial heads. Many of the birds develop bacteremia with hundreds to thousands of CFU/ml. Additionally, the infected birds spread the infection to birds within the same room and 30-40% of those birds will be lame by 56 days of age. ELA has been used to compare virulence of *E. cecorum* from BCO birds (primarily kinky back) and *E. cecorum* from feces (32). We used an *E. cecorum* we collected from an infected vertebrae in a kinky back bird (35) but it showed no significant virulence in the ELA. We compared three *E. coli* isolates from BCO lame birds (35) and found they had very different apparent ELA virulence. Even though these three isolates were from three different commercial

134

broiler farms in Arkansas that were experiencing BCO outbreaks, we have shown that all 3 are very different based on whole genome comparisons (35). This is surprising given that 1409 and 1413 were isolated on the same day from two different farms within 5 km of each other that were operated by the same integrator and supplied from the same hatchery. *S. aureus* isolates showed virulence in the ELA, including an isolate from a human infection, and isolates from a BCO outbreak on a different farm operated by a different integrator. We sampled 11 lame birds from that farm and determined that 7 of the birds were infected with *S.* aureus. Genome analysis showed that the *S. aureus* isolates were highly related and very closely related to numerous *S. aureus* isolates obtained from diseased birds or broiler meat dating back to the 1970s in Europe (35, 36). The clade has been isolated multiple times in Arkansas and Oklahoma for at least a decade. The clade appears to be exclusively associated with poultry so virulence in the ELA is not surprising. The genomic comparisons led us to propose that *E. coli* association with BCO is not exclusively poultry specific and that this species appears to be more of a generalist, whereas *S. aureus* and *S. agnetis* appear to be specialists and do not readily jump back and forth infecting different host species.

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Table 13. Sources (strain designation for species, and host) of bacterial isolates utilized for ELA. In Isolate Source Lame indicates a bird number from BCO sampling. Abbreviations include LT: Left Tibia, RT: Right tibia, LF: Left femur, and RF: Right femur.

Species	Designation	Host	Isolate Source	Citation
S. agnetis	908	Broiler	Femoral BCO; UA Research Farm	Al-Rubaye, *et al.*, 2015
S. chromogens	1401	Broiler	Thoracic Vertebrae; Lame3	Ekesi, 2020
E. cecorum	1415	Broiler	LT/RT; Lame5	Ekesi, 2020
E. coli	1409	Broiler	RT; Lame3	Ekesi, 2020
	1413	Broiler	Blood; Lame12	Ekesi, 2020
	1512	Broiler	LF; Lame18	Ekesi, 2020
	1527	Broiler	RF; Lame18	Ekesi, 2020
	1540T		1409 transformed with 1413 Plasmids	This work
	1541P		Recovered from live embryo injected with 1540T	This work
	1544P		Recovered from live embryo injected with 1541P	This work
	1547P		Recovered from live embryo injected with 1544P	This work
S. aureus	1510	Broiler	LT Lame14	Ekesi, 2020
	1514	Broiler	RF Lame15	Ekesi, 2020
	1302	Human	Wound	ATCC-29213
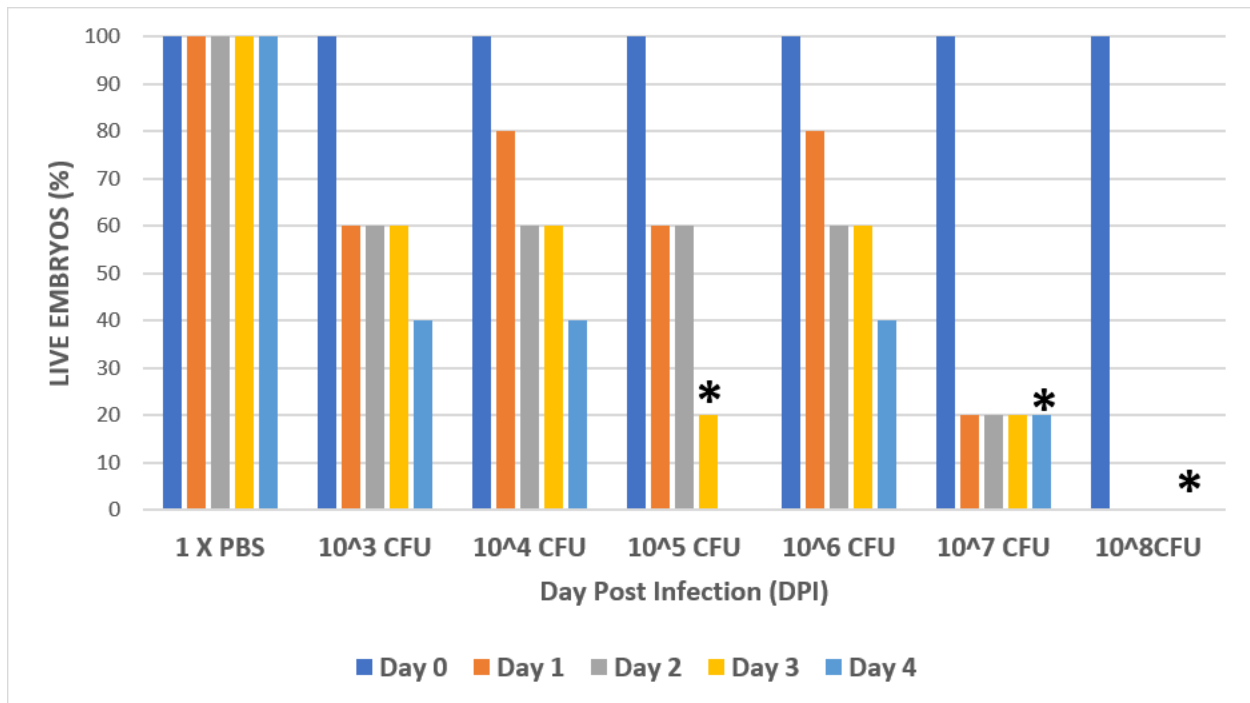
Figure 24. Embryo lethality assay in layer chicken line embryos to estimate the lethal dosage of bacterial isolates. The number of live embryos (Y-axis) for different quantities of *E. coli* 1413 is plotted over four days post-injection (X-axis). Isolate abbreviations and their sources are described in Table 11. For each treatment n = 5. Asterisks (*) indicates that particular treatment was significantly different from 1xPBS ($P < 0.05$).

Figure 25. Layer chicken line embryo lethality for injection of 10^5 or 10^6 CFU of bacterial isolates vs. phosphate saline vehicle. Isolates were: A. 908; n=14, B. 1302; n=15, C. 1401; n=14, D. 1409; n=15, E. 1413; n=15, F. 1415; n=9, G. 1510; n =15, H.1512; n=15, and I. 1514, n=15. Details are as in Figure 24. Asterisks (*) indicates that particular treatment was significantly different from 1xPBS (P <0.05).
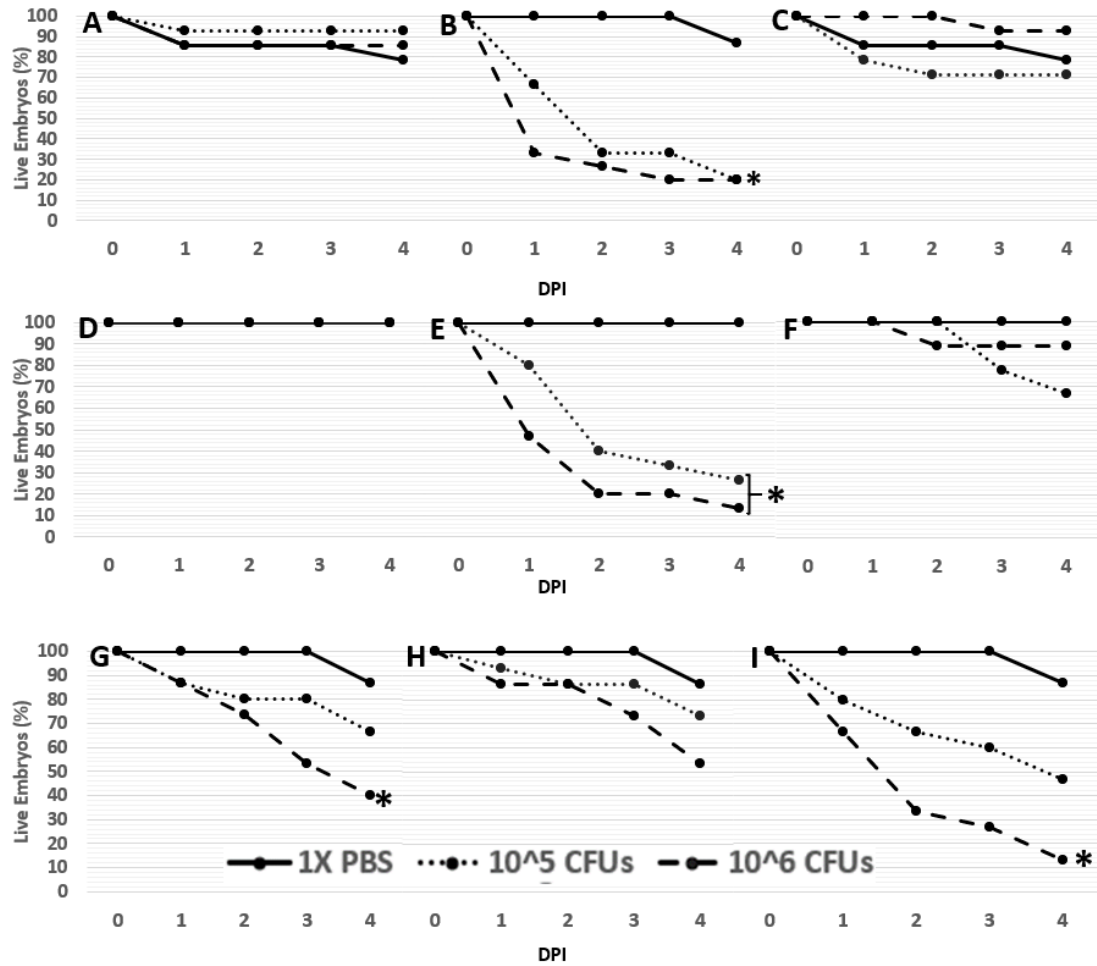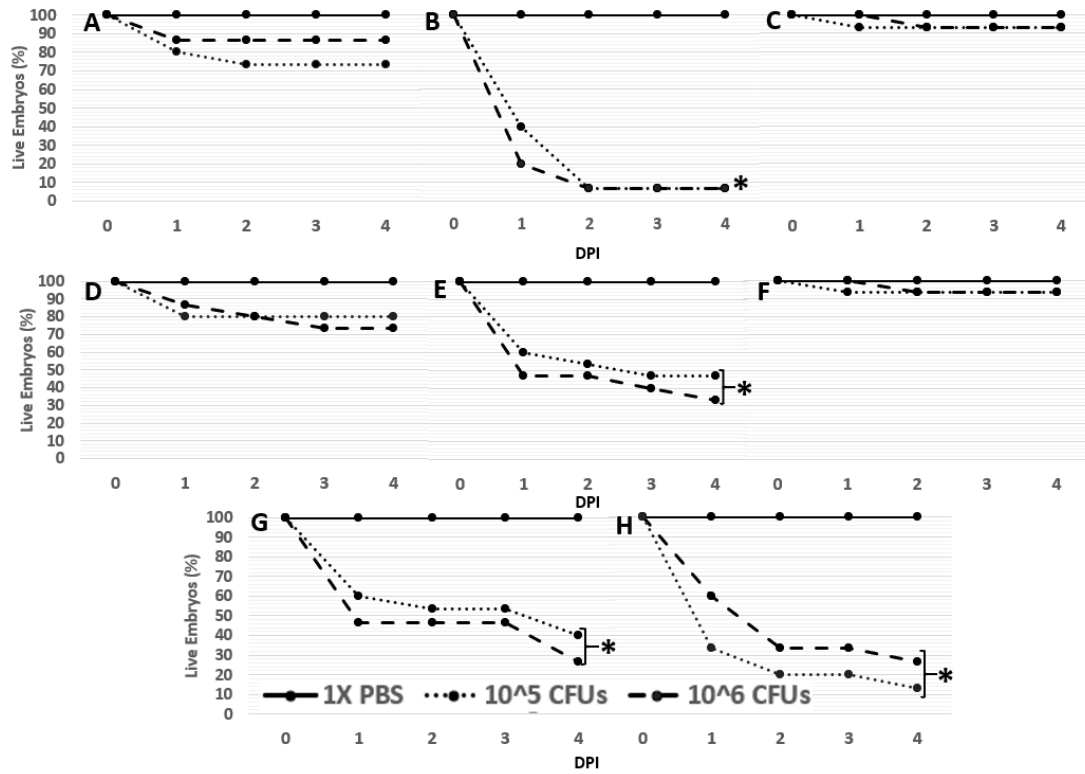
Figure 26. Broiler chicken line embryo lethality for injections of 10^5 or 10^6 CFU of bacterial isolates vs. phosphate saline vehicle. Isolates were: A. 908, B. 1302, C. 1401, D. 1409, E. 1413, F. 1415, G. 1514, and H.1527. Details are as in Figures 24 and 25. For all trials n=15 per treatment.
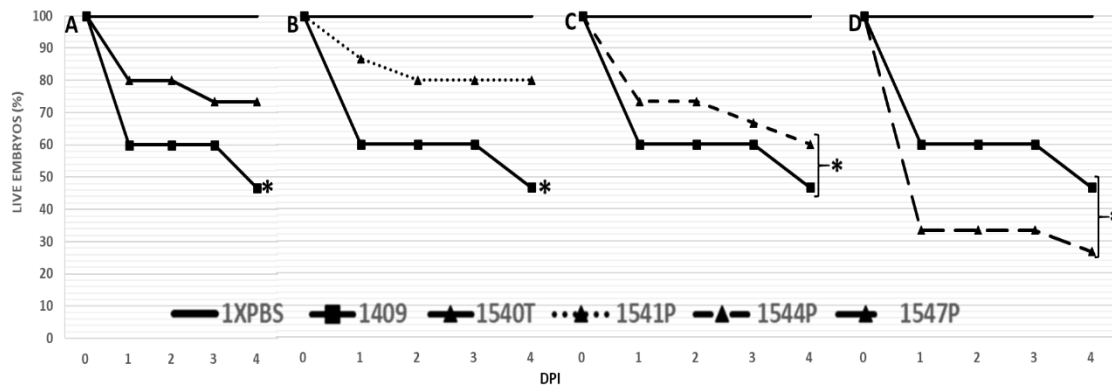
Figure 27. Embryo lethality is transferable from *E. coli* 1413 to 1409. Embryo lethality assays for 15 embryos per treatment for the transformant 1540T (Panel A), and recovered *E. coli* cultures after 1 passage, 1541P (Panel B), 2 passages, 1544P (Panel C), and 3 passages, 1547P (Panel D). All four panels compare to the 1xPBS control and 1409. Additional details are as in Figures 24 and 25.

CHAPTER 4

Chondronecrosis with Osteomyelitis in Broilers: Further Defining Lameness-Inducing

Models with Wire or Litter Flooring, to Evaluate Protection with Organic Trace Minerals

Disclaimer

In this trial, I was part of the animal trial at the farm where we administered Availa-ZMC the commercial product in broiler feed. I was part of the histopathological evaluation of the intestinal villi. I was part of the sample collection team for the assay of intestinal gene expression. In the phagocytosis assay, I collected the blood and helped purify the PBMCs.

Chondronecrosis with Osteomyelitis in Broilers: Further Defining Lameness-Inducing Models with Wire or Litter Flooring, to Evaluate Protection with Organic Trace Minerals

Abstract

The feed additive Availa-ZMC was investigated for the ability to reduce lameness in broilers using two alternative models for inducing lameness. The mixture of organic trace minerals was effective in reducing lameness by 20% in the wire-flooring model and 25% in the bacterial challenge on litter flooring model. Lameness in both models is overwhelmingly attributable to bacterial chondronecrosis with osteomyelitis. The reduction in lameness was associated, at least in part, with enhanced intestinal barrier integrity mediated by elevated expression of tight junction proteins and stimulation of bactericidal killing of adherent peripheral blood monocytes obtained from the birds on Availa-ZMC. Lameness is a major animal welfare concern in broiler production. The wire-flooring and bacterial challenge on litter-flooring models are effective models for evaluation of management strategies for mitigating infectious causes of lameness.

Keywords: Broiler; Lameness; Chondronecrosis; Staphylococcus; Organic trace mineral

Introduction

Lameness is one of the most significant animal welfare issues in the broiler industry, resulting in annual losses of millions of dollars (Siegel, *et al*., 2019; Wideman, 2016). A wire-flooring model has been shown to induce a high incidence of lameness in broilers (Wideman, 2016; Wideman, *et al*., 2013; Wideman, *et al*., 2014; Wideman, *et al*., 2012; Wideman and Prisby, 2013). Lameness induced in this system is overwhelmingly bacterial chondronecrosis with osteomyelitis (BCO) of the proximal tibiae and femora (Wideman, 2016; Wideman, *et al*., 2013; Wideman, *et al*., 2012; Wideman and Prisby, 2013). The predominant isolates from BCO lesions using the wire floor model on our research farm are *Staphylococcus agnetis* and the BCO lameness is sometimes associated with a significant bacteremia (Al-Rubaye, *et al*., 2015). The type strain, *S. agnetis* 908, when administered in drinking water can induce high levels of lameness for birds grown on wire or on litter (Al-Rubaye, *et al*., 2015; Alrubaye, *et al*., 2020). The BCO lameness model has demonstrated: i) translocation of bacteria into the blood for birds on litter, with higher translocation in birds on wire flooring (Al-Rubaye, *et al*., 2017); ii) transmission of BCO-inducing pathogens within a flock (Al-Rubaye, *et al*., 2017) or within a facility (Alrubaye, *et al*., 2020); and iii) protection against BCO inducing pathogens by probiotics and prebiotics (Alrubaye, *et al*., 2020; Wideman, 2016; Wideman, *et al*., 2015; Wideman, *et al*., 2012). We now extend these investigations to the investigation of the commercial, complex organic trace mineral, Availa-ZMC. Organic zinc is reported to enhance epithelial integrity, gut health, and immune function (Hudson, *et al*., 2004; Star, *et al*., 2012; Zakaria, *et al*., 2017). The data reported herein demonstrate that this product can reduce lameness in both the wire-flooring model, and the bacterial challenge on litter-flooring model. Additionally, we observed that Availa-ZMC shows a dose-dependent enhancement of bacterial killing activity by adherent

146

peripheral blood monocytes cultured from treated birds. These data extend the range of products that can be used to reduce BCO lameness, highlight the importance of organic trace minerals in improving animal well-being, and provide further validation of the two models we have developed to investigate treatments and management strategies for reducing BCO lameness in broiler operations.

Materials and Methods

Lameness Trials

All animal experiments were approved by the University of Arkansas Institutional Animal Care and Use Committee under protocols 18010, and 18075. One day (d) old chicks representing surplus males from a female broiler-breeder product were kindly provided by Cobb-Vantress (Siloam Springs, AR). Chicks were placed in 5 x 10 ft. pens on either suspended wire flooring (Wideman, 2016; Wideman, *et al.*, 2012) or on standard wood-shaving litter at 60 per pen. Nipple water lines were supplied with city tap water on one side of the pen and two feeders were placed on the opposite side. Feed was standard starter through d35 and finisher through d56. Computer controllers regulated the temperature, photoperiod and ventilation. Tunnel ventilation and evaporative cooling pads were automatically activated when needed. The photoperiod was set for 23 h light:1 h dark for the duration of the experiment. Thermoneutral temperature targets were as follows: 90 °F for d1 to d3, 88 °F for d4 to d6, 85 °F for d7 to d10, 80 °F for d11 to d14, and 75 °F thereafter. On d19 all pens were culled to 50 birds. For pens challenged with *S. agnetis* in the drinking water the tap water supplying the nipple waterer was replaced with a gravity flow from an elevated 20L carboy of tap water. The bacteria (stationary overnight culture) were mixed into tap water in the carboy to 10^4 CFU/ml (colony forming units per ml). After d21 the nipple supply was returned to the tap water. All water lines were flushed with

dilute bleach and fresh tap water prior to each experiment. Beginning on d20 all birds were encouraged twice per d to move using standard kitchen brooms. Any bird that was reticent to move was marked with spray paint. Birds that continued to be unwilling or unable to walk were diagnosed as "clinically lame" and euthanized. All birds that died or were diagnosed as clinical lame were recorded by date, and pen number. Necropsy for BCO lameness was as described (Wideman, 2016) to categorize as either: N = Normal proximal femur head or proximal tibia head; KB = Kinky Back (Spondylolisthesis); FHS = Proximal Femoral Head Separation (epiphyseolysis); FHT = Proximal Femoral Head Transitional degeneration; FHN = Proximal Femoral Head Necrosis; THN = Proximal Tibial Head Necrosis; Other = symptoms other than BCO, and Total Lame included all birds with any FHS, FHT, FHN, THN, or KB lesions.

For administration of Availa-ZMC the commercial product was added to the feed (Table 12) prior to pelleting. Samples of the pelleted feed were shipped to the supplier for verification of proper mixing prior to any experiment. Details on the feed formulations and treatment groups are described in Table 12.

Histological Evaluation of Intestinal Villi

Intestinal samples (3 cm section) for histopathology were the distal jejunum (1 cm proximal to Meckel's diverticulum) and proximal ileum (1 cm distal to Meckel's diverticulum). Samples from freshly euthanized birds were flushed with 1 x PBS and fixed in phosphate buffered formalin. The fixed samples were processed through the histology laboratory in the Department of Poultry Science at the University of Arkansas. Hematoxylin-Eosin stained sections were imaged on an Olympus inverted scope at 400x using a CCD camper to display on an LCD monitor. Villus length was measured on a 21-inch diagonal LCD monitor with a flexible ruler. Calibration was based on a stage micrometer. For villus length and pathology, at least 4 sections

148

were examined for each tissue for each bird (8 to 10 birds per treatment). For each section, villus length was measured and gross pathology (villus tip integrity) was scored, on four sides (top, left, right and bottom). For some sections, villus length and tip integrity could not be measured on all four sides owing to tissue damage in sectioning.

Assay of Intestinal Gene Expression

One µg of total RNA was extracted from tissue samples by homogenization using Trizol Reagent (ThermoFisher Scientific, Rockford, IL) in accordance with the manufacturer's recommendations. RNA concentration, quality, and integrity were assessed by the ratio of absorbance (260/280), and electrophoresis in 1% agarose gels using a Take 3 micro volume plate and the Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). RNAs were treated with DNAseI, and reverse transcribed via qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The cDNA was then amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (ThermoFisher Scientific, Rockford, IL) in triplicate 20 µL per reactions. Oligonucleotide primers specific for chicken occludin (OCLN): forward 5'-CGCAGATGTCCAGCGGTTA-3' and reverse 5'- GTAGGCCTGGCTGCACATG; claudin 1 (CLDN1); forward 5'- CCCACGTTTTCCCCTGAAA-3' and reverse 5'-GCCAGCCTCACCAGTGTTG-3' ; gap junction protein alpha 1 (GJA1): forward 5'-TGGCAGCACCATCTCCAA -3' and reverse 5'- GGTGCTCATCGGCGAAGT-3'; and catenin beta 1 (CTNNB1): forward 5'- TGCCCCACTGCGTGAAC-3' and reverse 5'-TGCTCTAACCAGCAGCTGAACT-3'. Primers for the reference, housekeeping gene r18S have been published previously (Dhamad, *et al*., 2019; Greene, *et al*., 2019; Lassiter, *et al*., 2015; Piekarski-Welsher, *et al*., 2018). The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 58°C for 1 min with plate read. Post PCR, melting

curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude samples with non-specific products. PCR products were also confirmed for one specific size band by agarose gel electrophoresis. Negative controls lacked cDNA input as template for the PCR and were verified for absence of gel bands. Relative expression of target genes were determined by the $2^{-\Delta\Delta Ct}$ method using r18S as the reference and the control group as the calibrator (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Phagocytosis Assay

Blood (1 ml) was collected from a wing vein using a Vacutainer containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ). Monocytes were enriched and cultured using published protocols (Dawes, *et al*., 2014; Drechsler, *et al*., 2013). Medium was RPMI (VWR) with 1x GlutaMax (Life Technologies) and 10% low endotoxin Fetal Bovine Serum. After 5 d in culture (37 °C; 5% CO_2) we challenged the adherent cells in triplicate with an approximate multiplicity of infection (MOI) of 1:1 with *S. agnetis* 908 for 2 d following published methods (Campbell, *et al*., 1994; Drevets, *et al*., 2015). Specifically, the bacteria were added to the medium for 2 h, then the medium was replaced with media supplemented with gentamycin (50 µg/ml) for 6 h to kill non-internalized bacteria. The medium was replaced with antibiotic-free medium. After 2 d in culture the adherent cells are lysed by addition of pure water and a dilution series of the lysate was plated on Luria Broth agar plates for viable bacterial cell counts.

Statistical Analyses

Data were compared using either the T-test function in Microsoft Excel or a Generalized Linear Model (GLM) module in R.3.4.2 to produce P-values between treatments, as indicated. Gene expression data were analyzed by One-way ANOVA. If ANOVA revealed significant effects, the means were compared by Tukey's multiple range test using the Graph Pad Prism version 6.00 for

Windows (Graph Pad Software, La Jolla California, USA). Significant difference was accepted at P ≤ 0.05. Data are expressed as the mean ± SEM.

Results

Experiment 1 evaluated whether Availa-ZMC could reduce lameness for birds raised on wire-flooring to induce lameness. Chicks (1d old) were raised to d56 on wire-flooring with no direct administration of a bacterial challenge. There were four pens in each treatment group, the Control treatment received standard feed formulations (Table 12), whereas the Availa-ZMC normal treatment received the product at 1000 mg/Kg of feed, and the Availa-ZMC high treatment group received the product at 1500 mg/Kg of feed. Feed formulations were continuous through d56, the end of the experiment. Lameness began to appear in all three treatments on d37, but the trajectory of lameness accumulation was higher for the birds on standard feed (Figure 28). The final cumulative lameness for the Control was 66%, but the Availa-ZMC normal treatment had 47% lameness, and Availa-ZMC high had 57% lameness. Comparison of the lameness data by Generalized Linear Model (GLM) with the individual bird as the experimental unit showed that the Availa-ZMC normal treatment was statistically different from Control (P = 0.0003) and Availa-ZMC high (P = 0.03). Control and Availa-ZMC high were not statistically different (P = 0.15). Pen-to-pen variability for the three treatments in experiment 1 reveals a degree of variability in the total lame per pen within a treatment (Table 13). Loss of birds to mortalities unrelated to lameness, and final body weights were not different between treatments. Supplementation of feed with Availa-ZMC at either level had no discernable effect on the severity of BCO lesions for proximal tibiae and femorae from birds diagnosed as lame through the course of the experiment (Figure 29).

Experiment 2 evaluated whether Availa-ZMC could reduce lameness for birds raised on litter-flooring when a bacterial challenge is imposed. The feed supplementation was the same, but lameness was induced by the transmission of the hypervirulent strain *S. agnetis* 908 from birds challenged with the bacterium in drinking water for d20 and d21. There were 3 pens of birds on litter-flooring on standard feed that were the Source population. These three pens were "upwind," relative to the exhaust fans, of the treatment pens (Table 12). There were four pens for each of the three treatments: Control, Availa-ZMC normal, and Availa-ZMC high, arrayed in a randomized block design and separated by at least 3 meters from the Source pens. Lameness began to appear on Day-36 but lameness accumulation was accentuated in the Source (Figure 30). Accumulation of lameness in the three treatment groups lagged behind that for the Source population by about 3 to 4 d through d48 where the Control cumulative lameness continued to parallel that for the Source but the lameness accumulation is reduced for both Availa-ZMC treatments. Final percent lameness was Source 83%, Control 65%, Availa-ZMC normal 49%, and Availa-ZMC high 52%. GLM based comparisons of the lameness data with the individual bird as the experimental unit showed that the percent lameness was statistically higher in the Control treatment than in the Availa-ZMC normal (P = 0.002) and Availa-ZMC high (P = 0.006), treatments. Pen-to-pen total lame was more uniform in this experiment compared to experiment 1, and losses due to mortalities unrelated to lameness were lower (Table 13). Final body weights were comparable between experiments 1 and 2, but the body weights for Availa-ZMC normal were lower (T-test, P = 0.02) compared to the other two treatments in experiment 2. There was no clear difference in the distribution of BCO lesions between any of the four treatment groups in experiment 2 (Figure 31), however we have no explanation for the rather high percentage of Normal left femoral head diagnoses in the Source population.

Sections of distal jejunum and proximal ileum were collected from five apparently healthy birds on d57 from the Control and Availa-ZMC treatments. Villus length was assessed for multiple sections from each bird and the average villus lengths were computed (Figure 32). Student T-test of the villus length data indicate that villus length in the ileum was reduced in the Availa-ZMC treatments (P < 0.001) but villus length in the jejunum increased (P < 0.001). There was no difference in villus length between the two different levels of Availa-ZMC. In terms of important determinants of villus integrity, we examined expression of critical tight junction genes using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Ileum and jejunum from the Availa-ZMC high samples showed significantly upregulated expression for CLDN1, OCLN, GJA-1, and CTNB1, compared to the Control (Figure 33). Complimentary data for histology and expression from experiment 1 is not provided as the stress imposed by the wire-flooring appears to significantly impact intestinal development and villus formation (A. Hasan, unpublished).

Wing vein blood was collected from the same birds examined for intestinal histopathology. Monocytes were enriched and adherent cells cultured for 5 d. The cells were then used in phagocytosis assays against *S. agnetis* 908 at an approximate MOI of 1:1. Bacterial survival was assessed after 2 d (Table 14). The bactericidal activity was variable between birds within each treatment. The most variation was in the Availa-ZMC normal birds where the adherent cells for bird 3 were highly active in killing *S. agnetis* 908. For the lowest dilution plated, 10^{-2}, there were only 5 colonies from one of the three triplicate wells. Therefore, verifying that bacteria were added but the bacterial survival was very low within the monocytes from this broiler. The high variability for the five birds from Availa-ZMC normal meant that this treatment group was not statistically different from either the Control or Availa-ZMC high cells. Bactericidal activity of

cells from the Availa-ZMC high birds was higher than the activity of cells from the Control treatment (T-test, P = 0.00085).

Discussion

In our previous research publications we had reported that *S. agnetis* 908 can induce lameness at 80-90% when administered to birds raised on wire flooring (Al-Rubaye, *et al*., 2015; Al-Rubaye, *et al*., 2017). We also demonstrated that *S. agnetis* 908 can induce lameness of 50 to 80% when administered in drinking water to birds raised on litter flooring (Alrubaye, *et al*., 2020). Further, the lameness can be transmitted from the birds challenged with bacteria to unchallenged birds within the same broiler house. We also demonstrated that specific probiotics in the feed can protect broilers when raised on wire-flooring (Al-Rubaye, *et al*., 2017; Wideman, *et al*., 2012), but different probiotics in the feed can protect the unchallenged birds in the litter-flooring with bacterial challenge model (Alrubaye, *et al*., 2020). The combination of organic zinc, manganese, and copper has been reported to improve poultry health, reduce bacterial pathogen colonization, and reduce femoral head necrosis (McKnight, *et al*., 2020; Sirri, *et al*., 2016). We therefore hypothesized that supplementation with Availa-ZMC complexed trace minerals could be efficacious in reducing BCO lameness in our two models for inducing BCO lameness. The results from experiments 1 and 2 confirm that Availa-ZMC is effective in reducing lameness in both models for inducing lameness. When birds were raised on wire-flooring with no direct bacterial challenge, the mineral supplement reduced lameness by 14 to 29% (Figure 25) and appears to have reduced mortality due to causes not attributable to lameness (Table 13). In the litter-flooring with bacterial challenge model the Availa-ZMC similarly reduced lameness by 20 to 25% relative to the Control treatment (Figure 27). Most importantly, this latter model employs the contagious spread of the infection observed in some broiler operations. Although

154

our model uses a hypervirulent bacterial strain, the reduction could be greater against less

virulent species/strains. Substituting complexed organic trace minerals for inorganic minerals

appears to have reduced villus length in the ileum and increased villus length in the jejunum

(Table 13). Further investigations are warranted to determine whether these changes arising from

the difference in source of trace minerals alters any aspect of assimilation or feed conversion

rates in infectious models. In these studies, the final body weights did not seem to have been

impacted (Table 13). It is important to consider the influence of different stocking density

induced by different mortality rates. Interestingly, the organic trace mineral upregulated the

expression of the genes for tight junction (CLDN1, OCLN), gap junction (GJA-1), and

desmosome (CTNB1), consistent with improved gut barrier integrity. Although the exact

functions of the individual tight junction proteins remain elusive, in avian species, occludin has

been reported to be an integral component in tight junction barrier function (Balda, *et al*., 2000).

Studies conducted in occludin-deficient mice showed gut inflammation and defective epithelial

barrier function (Schulzke, *et al*., 2005). Similarly, it has been reported that down regulation of

CLDN1 can drastically reduce barrier integrity (Zeissig, *et al*., 2007). Upregulation of these

genes is consistent with Availa-ZMC enhancing barrier functions and reducing translocation of

bacteria into the blood, a critical first step in the progression of BCO lameness (Al-Rubaye, *et

al*., 2017; Wideman, 2016; Wideman and Prisby, 2013). Further, the organic trace minerals

appear to enhance the bacterial killing activity of adherent peripheral blood monocytes (Table

14). Availa-ZMC has been reported to improve intestinal health, epithelial integrity, and

immune function (Hudson, *et al*., 2004; Star, *et al*., 2012; Zakaria, *et al*., 2017). The reduction in

bacterial lameness in both models could result from either, or both, of enhanced barrier function

and enhanced bactericidal activity of phagocytes. Growth on wire-flooring increases

translocation of bacteria into the blood relative to growth on litter-flooring (Al-Rubaye, *et al*., 2017). The bacterial challenge on litter-flooring model involves non-contact spread of the infection from the Source population and we have speculated on whether the infection is through the pulmonary or gastro-intestinal path (Alrubaye, *et al*., 2020). The adherent peripheral blood monocyte phagocytosis results suggest that the Availa-ZMC reduces lameness in part by enhanced killing of bacteria that translocate into the blood on either type of flooring (Al-Rubaye, *et al*., 2017). The enhanced gene expression data for gut integrity markers argues that both immunity and barrier functions have been enhanced for the Availa-ZMC treated birds on litter-flooring. Most intriguing is the high bactericidal activity of the monocytes from bird 3 from the Availa-ZMC normal treatment. Only one of 15 birds displayed such superior activity. However, we cannot discern whether the activity for cells from this bird were inherent to that bird or resulted from stimulation by the Availa-ZMC supplementation. There could be a small percentage of birds with superior innate immunity, or a small percentage of birds with immune systems that are highly activated by organic trace minerals. Regardless, identification of these birds would provide a major new tool for improving animal welfare.

The work presented here and our recent work demonstrating that certain probiotics can also reduce BCO lameness (Alrubaye *et al*., 2020) strongly support investigations pairing probiotics with Availa-ZMC to determine whether the protective effects are overlapping, additive or synergistic. The bacterial challenge on litter flooring model provides an excellent system for evaluation of these interactions. Development of effective management strategies that can be employed in the broiler industry will improve productivity and reduce animal welfare concerns.

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Appendix

Table 14. Feed supplementation for the three treatments

Treatment	Inorganic Sources, SO_4[a]			Availa⁻ZMC[ab]			
	Zn	Mn	Cu	mg/Kg of feed	Zn	Mn	Cu
Control	100	100	20.5	0	0	0	0
Availa-ZMC normal	40	40	10	1000	40	40	7
Availa-ZMC high	40	40	10	1500	60	60	10.5

[a]-Values are in parts per million, with the same levels in starter and finisher feeds.
[b]-Availa-ZMC: Zn, Mn and Cu amino acid complex; Zinpro Corporation, 10400 Viking Drive, Eden Prairie, MN 55344

Table 15. Lame, and Mortality by pen, and ending BW for three treatments in Experiment 1 and 2

Experiment	Pen	Count Lame					Count Mortality[a]					BW (kg)[c]	
		1	2	3	4	Avg[c]	1	2	3	4	N		Avg[c]
1	Control	36	24	27	40	31.8 ± 3.2	2	2	4	0	10		4.27 ± 0.17
1	Availa-ZMC normal	28	25	23	16	23.0 ± 2.2	0	1	0	2	12		4.27 ± 0.07
1	Availa-ZMC high	26	25	43	19	28.3 ± 4.5	0	0	0	0	10		4.23 ± 0.09
2	Control	32	35	32	30	32.3 ± 0.9	0	1	0	0	5		4.37 ± 0.05
2	Availa-ZMC normal	22	23	31	26	25.5 ± 1.8	0	0	0	0	5		4.18 ± 0.05
2	Availa-ZMC high	26	26	21	28	25.3 ± 1.3	0	0	0	0	5		4.40 ± 0.07

[a]-Mortality from issues other than lameness
[b]-BW for apparently healthy birds (N) at the end of the experiment (d56)
[c]-Average (Avg) ± SEM

Table 16. Bacterial survival in adherent peripheral blood monocytes from five birds from the three treatments in experiment 2

| | | Treatment | |
| | | Availa-ZMC | Availa-ZMC |
Bird	Control[a]	normal[a]	high[a]
1	1.3×10^7	1.6×10^7	5.8×10^6
2	1.7×10^7	1.2×10^7	1.1×10^7
3	1.7×10^7	1.7×10^2	9.9×10^6
4	1.6×10^7	1.1×10^7	7.6×10^6
5	1.2×10^7	2.0×10^7	8.6×10^6
Average	1.5×10^7	1.2×10^7	8.6×10^6
SEM	9.2×10^5	3.0×10^6	8.2×10^5

[a]-CFU average from triplicate wells, details on the assay in Materials and Methods
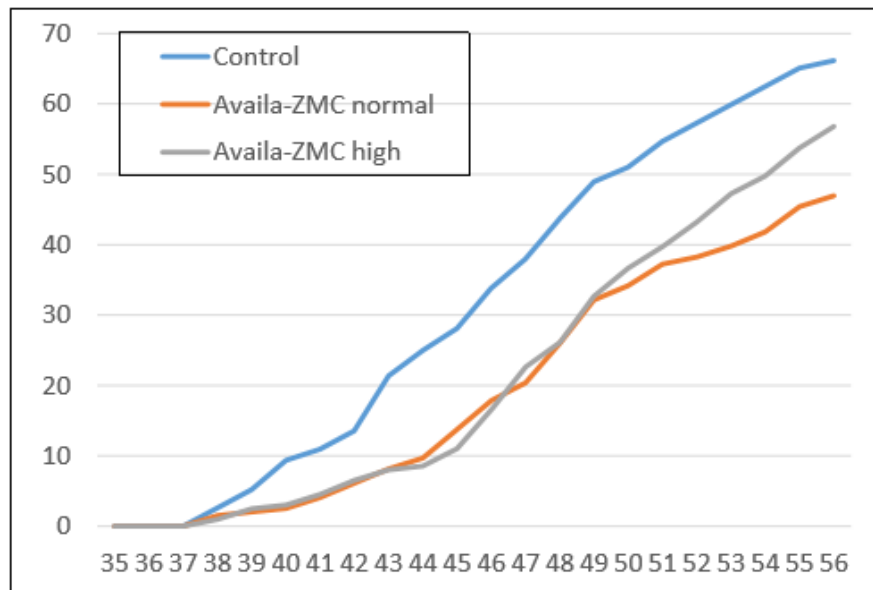

Figure 28. Cumulative lameness for broilers raised on wire-flooring on control feed or feed supplemented with Zinpro Availa-ZMC. Cumulative percent lameness (vertical axis) is plotted from d35 to d56 (horizontal axis). Details of the three treatments are in Table 9.
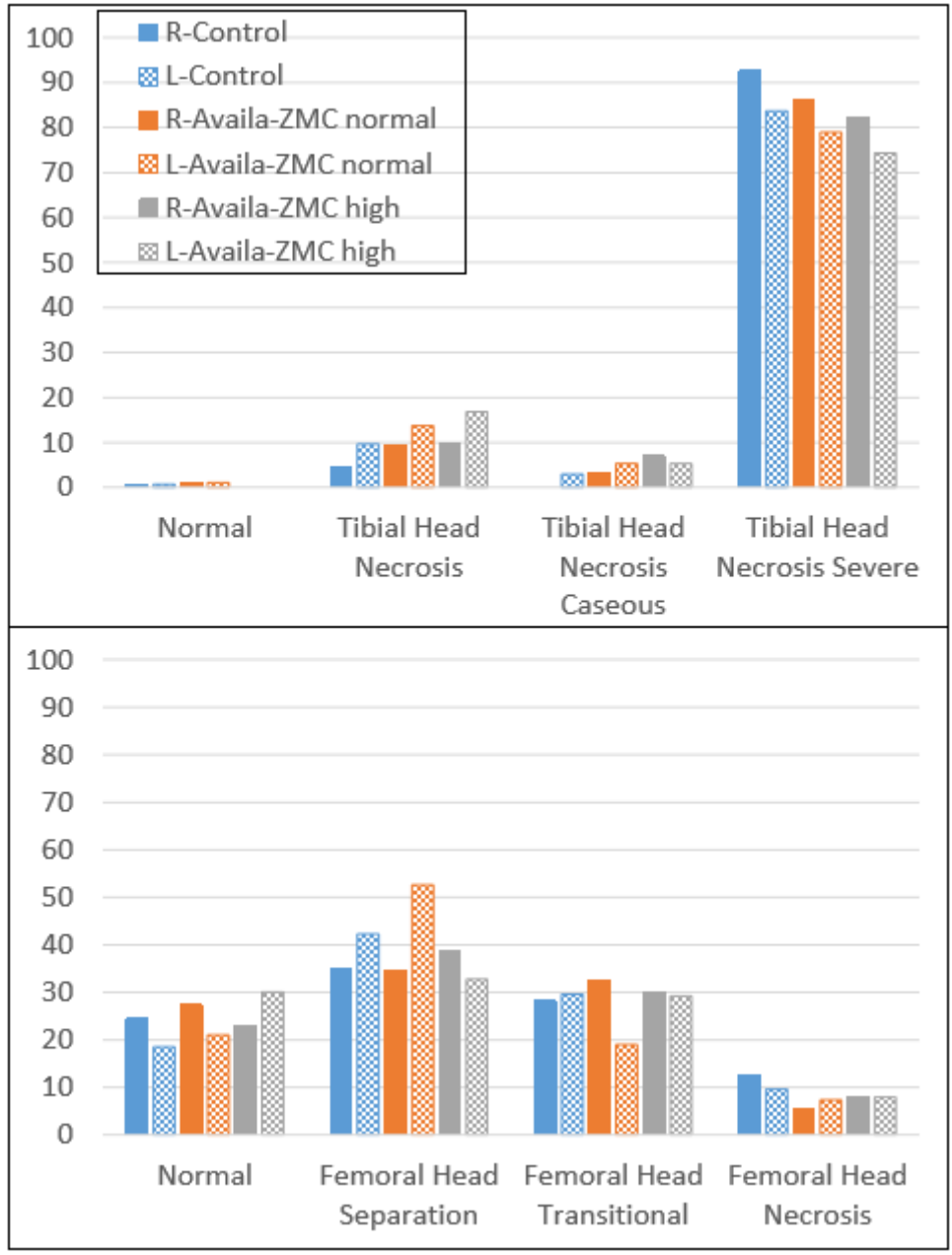
Figure 29. Tibial (upper) and femoral (lower) lesion diagnoses for all lame birds raised on wire-flooring in experiment 1. Proximal heads were diagnosed at necropsy for R- right; L- left leg bones, for each of the three treatments.
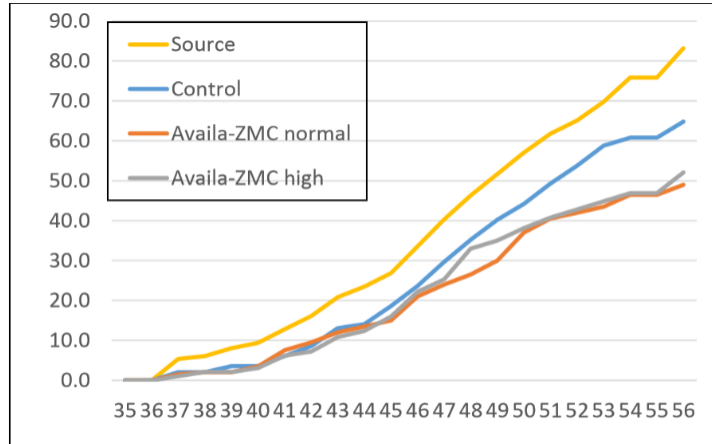
Figure 30. Cumulative lameness for broilers with a bacterial challenged, raised on litter-flooring, on control feed or feed supplemented with Zinpro Availa-ZMC in experiment 2. Cumulative percent lameness (vertical axis) is plotted from d35 to d56 (horizontal axis). Source was the same as Control but was challenged with *S. agnetis* 908 at 10^4 CFU/ml in drinking water for d20 and d21.
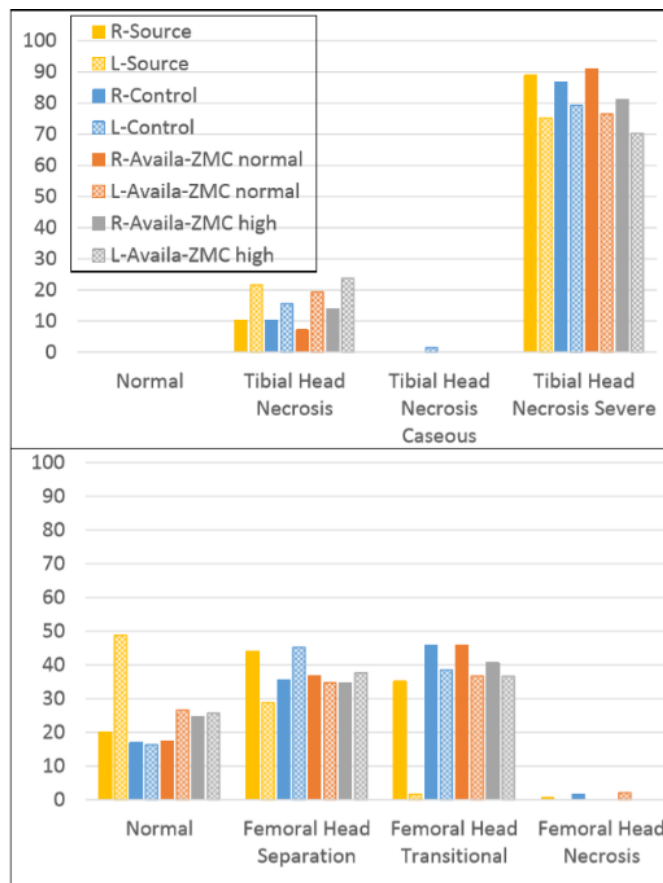

Figure 31. Tibial (upper panel) and femoral (lower panel) lesion diagnoses for all lame birds raised on litter-flooring in experiment 2. Proximal heads were diagnosed at necropsy for R- right; L- left leg bones, for each of the four treatments.
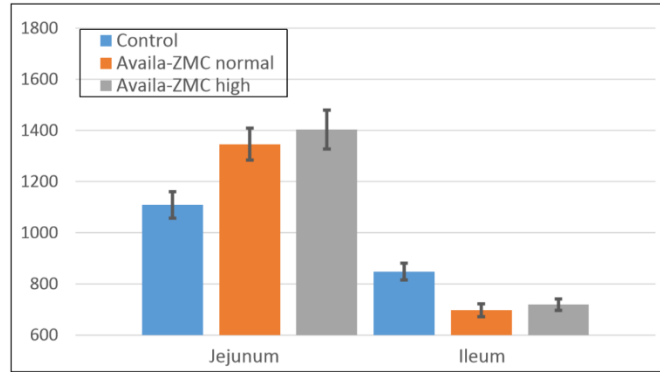
Figure 32. Villus length at d57 for apparently healthy birds from three treatment groups in experiment 2. Average villus length (µm; vertical axis) for distal jejunum and proximal ileum for five birds from each treatment. Error bars indicate SEM.

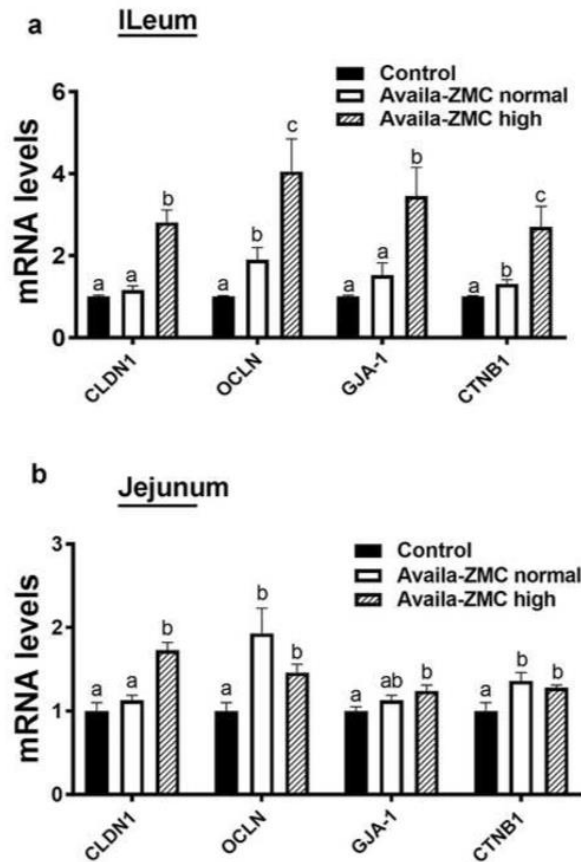Figure 33: Expression of intestinal barrier integrity-related genes from three treatment groups in experiment 2. The relative expression of ileal and jejunal OCLN, CLDN1, GJA-1, and CTNB1 was determined by qPCR and analyzed by $2^{-\Delta\Delta Ct}$ method using control group as the calibrator. Data are presented as mean ± SEM (n = 6/group). Different letters indicate significant difference at $P < 0.05$.

CHAPTER 5

Typhoid Mary Experiment: BCO incidence variation due to bird-to-bird intra-pen transmission when administering *Staphylococcus agnetis* in the water

Disclaimer

In this trial, I was part of a team that performed the animal trials in this project.

Typhoid Mary Experiment: BCO incidence variation due to bird-to-bird intra-pen transmission when administering *Staphylococcus agnetis* in the water

Summary

In our series of BCO trials, we have observed that some pens seem to show lameness earlier than others. We contended that this is because of one of two alternatives: i) a variable incidence of some additional infectious agent (virus, bacteria, coccidian), or ii) a carrier infecting other pen-mates. Since we have determined that *S. agnetis* is a causative agent of BCO and lameness, we tested whether this pathogen can be easily transmitted in a floor pen environment. *S. agnetis* administered in drinking water at Day 20 to birds reared on wire flooring results in a 40-80% incidence of lameness. Without bacterial administration we see 30-50% lameness depending on the experiment, and the major (78%) bacterial species recovered from BCO lesions on our farm is *S. agnetis*. In this experiment we wanted to determine if broilers challenged with *S. agnetis* can transmit the bacterium to birds in the same pen, thereby spreading BCO and lameness. We discovered that young broilers exposed to *S. agnetis* at an early age of Day-20 may harbor the bacterium and if mixed with unexposed birds 10 days later can transmit the bacterium to their pen mates via unknown mechanism(s).

Materials and methods

    Animal Housing, Care, and Treatment

Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol #11002). The experiments were conducted using pens in A365W at the University of Arkansas Poultry Research Farm. Carboys and nipple waterers were flushed with dilute bleach (5%) followed by a tap water flush to remove bacterial biofilms at least two days before the chicks were placed. All birds were on 23 hours of light per day,

Thermoneutral/Optimal brooding and growing temperatures throughout (32.2 ℃ for d1 to d3, 31.1 ℃ for d4 to d6, 29.4 ℃ for d7 to d10, 26.7 ℃ for d11 to d14, and 23.9 ℃), and Optimal ventilation throughout. The starter diet was a commercial corn and soybean meal-based chick starter (crumbles), and on d35 (10/14/2014) all birds were switched to a pelleted commercial corn and soybean meal-based finisher diet. Feed was formulated without meat or animal byproducts to meet or exceed minimum National Research Council (1994) standards for all ingredients. Starter and finisher feeds were provided *ad libitum*. Body weights were not recorded during the experiments, to avoid imposing additional stress on birds that already were under significant stress due to the wire flooring (Wideman & Pevzner., 2012; Wideman & Prisby., 2012).

Cobb500 surplus fast feathering breeder male chicks from the Fayetteville Hatchery were placed at 70 chicks per pen on suspended wire flooring as described (Wideman *et al*., 2012). Bird densities initially were approximately 1.65 ft^2/chick in all pens. A365W is equipped with computer controllers to regulate the temperature, photoperiod, and ventilation. On day 14 all pens were culled to 60 birds per pen. Beginning on day 15 all birds were "walked" daily by being prompted with a broom. For days 20 and 21 the birds in pens 11, 12, 16-21, 23, and 24 were treated with *S. agnetis* isolate 908 at 10^5 CFU/ml in their drinking water from 20 L carboys then the lines were flushed well with tap water and returned to city tap water. On day 30, we reciprocally exchanged half of the birds between pens 4&16, 5&17, 6&18, 7&19, 8&20, and 9&21. Beginning on day 22 we began recording lameness per pen and the actual birds diagnosed as lame. All broilers that died or that developed clinical lameness were recorded by date, gender, and tag number, and then necropsied to assess BCO lesion distributions.

Clinical Diagnosis of Lameness

The birds were "walked" and observed for lameness every two days beginning on Day 15. Affected broilers had difficulty standing, exhibited an obvious limping gait while dipping one or both wingtips and, if not removed, became completely immobilized within 48h. Birds were humanely euthanized as soon as the onset of lameness was noticed, and were necropsied within 20 min post-mortem. Lame birds were humanely euthanized with CO_2 gas or by cervical dislocation. Birds succumbing to BCO can die quickly because they have difficulty accessing food and water, and they can be trampled by their flock mates. Therefore, birds found dead also were necropsied to ascertain the cause of death and assess leg lesions. All broilers that died or that developed clinical lameness were recorded by date, gender, and wing band, and they were necropsied to assess BCO lesion distributions.

Birds that were unable to walk were diagnosed as "clinically lame" and humanely euthanized. All birds that died or developed clinical lameness were recorded by date, tag number, and pen number. They were necropsied and assigned to one of the following categories:

Normal = Femur head and proximal tibia appear entirely normal
Cull = Runts and individuals that failed to thrive or appeared to be clinically ill
U = Unknown cause of death
NE = Necrotic Enteritis
SDS = Sudden Death Syndrome (Flip over, Heart Attacks)
PHS = Pulmonary Hypertension Syndrome, Ascites
KB = Kinky Back (Spondylolisthesis)
TW = Twisted Leg or Slipped Tendon (perosis)
TD = Tibial Dyschondroplasia
Lame-UNK = Lameness for undetermined reasons
FHS = Proximal Femoral Head Separation (epiphyseolysis)
FHT = Proximal Femoral Head Transitional degeneration
FHN = Proximal Femoral Head Necrosis (bacterial chondronecrosis with osteomyelitis, BCO)
THN = Mild Proximal Tibial Head Necrosis, a sub-category of BCO in the tibiotarsus
THNS = Proximal Tibial Head Necrosis Severe, THN in which the growth plate was imminently threatened or damaged

THNC = Proximal Tibial Head Necrosis Caseous, THN in which caseous exudates or
bacterial sequestrate were macroscopically evident
Total Lame = FHS + FHT + FHN + THN + THNS + THN
Percent Lame = Total Lame/ (Total Birds - [sick or dead from other reasons])

Previously published photographs illustrate typical BCO lesions of the proximal femora and tibiae (Wideman & Prisby., 2012; Wideman *et al*., 2012). Proximal femoral head lesions (FHS, FHT, FHN) and tibial head lesions (THN, THNs, THNc) were categorized separately to emphasize the progressive development of BCO (Wideman etal.,2012).On day 56 representative surviving birds were euthanized and necropsied to assess sub-clinical lesion incidences: Normal proximal femoral head; Femoral Head Separation; Femoral Head Transitional degeneration; Femoral Head Necrosis; Tibial Head Necrosis, and Tibial Dyschondroplasia.

Results

The layout of the pens for the experiment is presented in Figure 34 and the protocol followed is in Table 15. There were four treatment groups assigned for this experiment that was classified as **Y**: Challenged with *S. agnetis*; mixed with no challenge; N: No challenge; mixed with *S. agnetis* challenged; C+: *S. agnetis* challenged and not mixed; and C-: No challenge and not mixed. Death due to causes other than BCO lameness was minimal for all four treatments (Y: 3; N: 2; C+: 1; N: 3). The cumulative percentage of lameness per treatment throughout the experiment is presented in Figure 35. The % lameness for treatments Y, N, and C+ diverges from the unchallenged unmixed control (C-) after Day 42, and then continue to near 70% with C- finishing at 50%. The Y and C+ *S. agnetis* challenged groups had nearly identical total % lameness of 73.4 and 75.5% lameness, respectively. Unchallenged and mixed treatment (N) were slightly less at 69%, but N was closed to Y+ and C+ challenged groups. As the accumulation of lameness in the unchallenged N treatment group appears to follow a similar accumulation to the Y and C+ treatment groups which were challenged with *S. agnetis,* this

strongly suggests that the Y treatment birds transmitted the bacterium to their N treatment unchallenged pen-mates. Table 16 presents the average lameness per pen for the treatment groups. In Table 16, we pooled each of the Y and N birds for the pens that were mixed to maintain approximately 60 birds per pen (we summed the lame birds in the Y treatment for pens 4 and 16, and we summed the lame birds in the N treatment for pens 4 and 16). The Student T-tests on the lameness counts for treatment groups by pen showed no significant difference between treatments Y, N, and C+ (P≥0.187). The three treatments were all significantly different from C- (P<0.013). Therefore, this experiment demonstrates that if we expose broiler chicks to a two-day inoculation with *S. agnetis* at day 20, and 10 days later mix those exposed birds with unexposed birds then the incidence of lameness in the mixed birds is equivalent throughout the pen. Thus, the exposed birds transmit the bacterium to their unexposed pen-mates. The mechanism of transmittal could be direct physical contact, contamination of the waterers from exposed birds, or vectored transmittal. The exposure should not be from residual *S. agnetis* on the nipple waterers because those were flushed after the challenge administration and we did not see any difference in lameness incidence between pens 4-9 and 14-21 (pens 14-21 were the pens with *S. agnetis* administration).

Assessment of all types of femoral and tibial lesions diagnosed for each treatment group showed little or no difference in the range of lesions or particular proximal leg bone head afflicted (Figure 35) except for perhaps a lower incidence of tibial lesions in the C- group.

Discussion

The Typhoid Mary experiment shows that young broilers exposed to *S. agnetis* at an early age (20 days) harbor the bacterium and if mixed with unexposed birds 10 days later can transmit the bacterium to their pen mates via some mechanism, most likely direct physical transfer,

171

contaminating the waterers, or other vectors. The transfer must be rapid because as the incidence

of BCO lameness begins to accumulate around 40-42 days that is only 10-12 days after the

mixing of the two treatment groups. We have long suspected the Typhoid Mary aspect of BCO

lameness because in the absence of our bacterial challenge we often experienced a few pens

where BCO lameness appeared early and reached much higher levels than other wire flooring

pens. We suspected there might be one or more pre-disposing subclinical infectious agents

(mycoplasma, virus, etc.) but this experiment suggests it is more likely a carrier of the eliciting

bacterial species, in this case, *S. agnetis.* One caveat is that we did not do microbial surveys of

the BCO lesions in this experiment. Therefore, we cannot explicitly state that the lesions in the

N treatment birds contained predominantly *S. agnetis.* However, the data are most consistent

with the Y treatment birds transmitting the *S. agnetis* to the N group of birds. In prior

experiments, we have shown that *S. agnetis* is the predominant species isolated from BCO

lesions on our research farm in multiple facilities, and that if a non-BCO pathogen is

administered (human *S. aureus*) we still recover *S. agnetis* from the BCO lesions (Al-Rubaye *et

al*., 2015). One major suspect for horizontal transmission is mites. Chickens are known to have

several different types of endemic mites. Metagenomics has detected the presence of *S. agnetis*

DNA in the guts of sheep scab mites (Hogg & Lehane., 1999). That does not mean there are

viable organisms, but the presence of the DNA suggests at least a transient presence. Intense

poultry production systems have been known to harbor more than 30 mite genera (Horn *et al*.,

2015) and molecular surveys of poultry red mites have detected multiple chicken viruses and

mycoplasma (Huong *et al*., 2014). Therefore, chicken mites should be suspect for vectoring the

bacterium. As the C- treatment was in the same room but separated by an unused pen the vector

does not appear to transmit readily over 8-10 feet which argues against vectoring by flies or

gnats. Although the C- pens which were closest to the challenged pens did have a slightly higher percentage of lameness. Our experimental data is consistent with the spread of BCO lameness within a broiler house or within a region of a broiler house that conforms with anecdotal narratives from growers. Future experiments should be designed to examine whether birds in neighboring pens can transmit BCO lameness when direct physical contact is reduced and there are no shared nipple waterers. There should also be concern about vertical transmission of agents of lameness through eggs or egg facilities. In conclusion, the bacterial species we have identified as intimately involved in BCO lameness in young broilers is communicable between broilers raised in a shared pen. And further work on the mechanism of horizontal transmission of the bacterium is warranted.

Pen 1 Control; No Challenge		Pen 13 Control; No Challenge
Pen 2 Control; No Challenge		Pen 14 Control; No Challenge
Pen 3: empty		Pen 15: empty
Pen 4 No Challenge mix 50:50 with 16		Pen 16 Challenge mix 50:50 with 4
Pen 5 No Challenge mix 50:50 with 17		Pen 17 Challenge mix 50:50 with 5
Pen 6 No Challenge mix 50:50 with 18		Pen 18 Challenge mix 50:50 with 6
Pen 7 No Challenge mix 50:50 with 19		Pen 19 Challenge mix 50:50 with 7
Pen 8 No Challenge mix 50:50 with 20		Pen 20 Challenge mix 50:50 with 8
Pen 9 No Challenge mix 50:50 with 21		Pen 21 Challenge mix 50:50 with 9
Pen 10: empty		Pen 22: empty
Pen 11 Challenge positive control		Pen 23 Challenge positive control
Pen 12 Challenge positive control		Pen 24 Challenge positive control

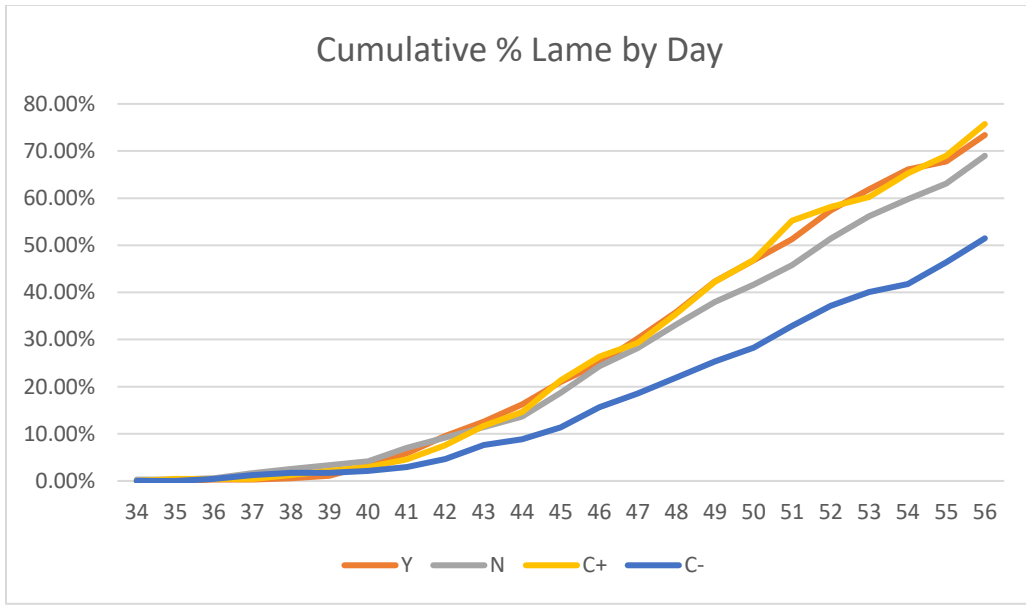Figure 34. Pen Setup in A364 for the Typhoid Mary experiment.

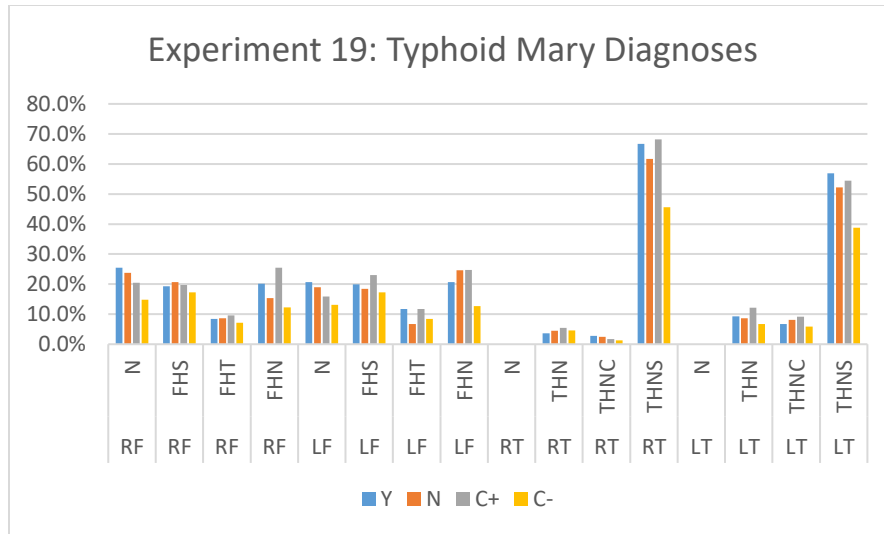Figure 35. Cumulative % lameness per treatment from day 34 through day 56.

Figure 36. Tibial and Femoral BCO lesion diagnoses for the four treatment groups. Percent incidence in lame birds is presented for RF right femur, LF- left femur, RT- right tibia; and LT- left tibia. Lesion type are as in Materials and Methods

Table 17. Protocol for Typhoid Mary Experiment

Day of Age	Comments
1	Place chicks on wire flooring. Cobb starter. All chicks tagged and recorded
14	cull to 60 birds per pen
20	Administer *S. agnetis* at 10^5 cfu/ml in drinking water for 2 days to pens 11,12,16-21,23,24
22	Begin recording all deaths, lame and infirmed
30	Swap birds 50:50 between pens 4&16,5&17,6&18,7&19,8&20,9&21
35	Switch to Cobb finisher
56	Complete experiment. Weigh all remaining birds and necropsy 5 apparently healthy birds from each pen

Table 18. Total and percent lameness for the four treatment groups along with average lame birds per pen. Note that for the Y and N treatment groups the per pen averages were for the two pens that were mixed so that all averages are for the same number of treated birds. Treatments that are different (P < 0.05) have Average ± std with superscript letters that are different.

Treatment Group	Y	N	C+	C-
Final Count	357	358	239	237
% Lame	73.4%	69.0%	75.7%	51.5%
Average lame ± std per pen	43.7±6.8[a]	41.2±7.8[a]	45.3±5.9[a]	30.5±4.8[b]

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Huong CTT, Murano T, Uno Y, Usui T, Yamaguchi T. Molecular Detection of Avian Pathogens in Poultry Red Mite (Dermanyssus gallinae) Collected in Chicken Farms. The Journal of Veterinary Medical Science. 2014;76(12):1583-7. doi: 10.1292/jvms.14-0253. PubMed PMID: PMC4300372.

CHAPTER 6

Conclusions

Conclusions

The poultry industry has seen significant intensification since the 1940s. This has led to shift in poultry production from a small scale "Backyard" farming to a more intensified and integrated production structure. Poultry industry has been successful due to its ability to produce marketable products. Post 1940s the introduced poultry breeding programmes employed numerous technologies to select important production traits. Today, broilers grow faster, have improved meat quality, have reduced feed conversion ratio, reduced mortality, and their production is more eco-friendly compared to production of pork or beef. These gains seem to come at a price, as the poultry industry is faced with the issue of lameness. Numerous factors combine to cause the incidence of lameness in broilers. However, Bacterial chondronecrosis with osteomyelitis is the leading cause of the disease. The full etiology of the ailment is not understood, but we have hypothesized that bacteria from vertical and horizontal transfers translocate through compromised gut, integuments and respiratory pathways into birds that develop lameness. These bacteria survive and colonize the long bones and T4 vertebrae causes various forms of necrosis that have been described. We studied lameness the Wideman's wire-, litter-flooring, or a combination of both. We have isolated *S. agnetis* as a major BCO agent in lame birds on our research facility, that can cause 50% incidence of lameness. Other BCO species like *E. coli and S. aureus* are frequent causative agents in other farms.

In this dissertation, we covered the analysis of genomes of *E. coli and S. aureus* that were isolated from three farms in Arkansas area. We found that the *E. coli* were more generalist in their pattern of infections regardless of geolocation. *S. aureus* seemed were closely related to isolates from Europe and were more specialized to infect chickens. Further analysis of their

genomes indicates the *S. aureus* we isolated have only been in the area for a few decades and may have acquired virulence factors important for its adaptation and disease mechanism.

We used embryo lethality assay to access the pathogenicity of BCO isolates and for the different reasons highlight in chapter 3, we do not trust that ELA is an efficient mechanism for determining pathogenicity of BCO isolates. And although this work is on-going, we find that a non-pathogenic *E. coli* electroporated with plasmids from a more pathogenic strain and passaged over a few generations, acquired embryo lethality properties.

We used the *Staphylococcus*-Lameness model to test the efficacy of Zimpro Micronutrients for reducing lameness in broilers and found that at certain levels these products can significantly reduce the incidence of BCO lameness. We also analysed the levels of expression of mucin and gut integral proteins. While there are certain gut integral proteins expressed in a differentially important manner to the incidence of BCO, we cannot say the same of Mucin genes as our analysis was inconclusive. The gut length and integrity for birds that received treatments of Zinpro Avalia micronutrients were also significantly improved. We therefore recommend Zinpro Avalia for it beneficial effects in commercial broiler productions.

Finally, we used the wire-flooring model to determine whether BCO can be transmittable between birds of the same pen. We transferred birds exposed to *S. agnetis* in drinking water for two days on Days 20, after found that birds were capable of transferring bacteria to their pen-mates. The mechanism of this transfer is still not known. Research is on-going in many aspects of BCO to better characterize the etiology of this disease, as therein lies the remedial.

Chapter 7

Appendix

UNIVERSITY OF ARKANSAS.

Office of Research Compliance

To: Douglas Rhoads
Fr: Craig Coon
Date: September 8th, 2017
Subject: IACUC Approval
Expiration Date: August 31st, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18010**: *Evaluation of Zinpro micronutrients for protection against BCO lameness and improving bone health for broilers raised on wire flooring.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond August 31st, 2018 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: . Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

UNIVERSITY OF ARKANSAS.

Office of Research Compliance

To:	Douglas Rhoads
Fr:	Craig Coon
Date:	February 12th, 2018
Subject:	IACUC Approval
Expiration Date:	February 2nd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18075**: *Evaluation of Zinpro micronutrients for protection against BCO lameness and improving bone health for broilers raised on litter flooring with bacterial challenge.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 2nd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Douglas Rhoads, Adnan Al-Rubaye, Nnamdi Ekesi, and Amar Hasan. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp