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Pathogenicity of Staphylococcus agnetis Associated with Lame Broilers with Osteomyelitis

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Pathogenicity of Staphylococcus agnetis Associated with Lame Broilers with Osteomyelitis

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of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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ABSTRACT

We developed High Resolution Melt Analysis (HERMA) as a rapid and reliable molecular diagnostic assay for the detection and identification of the main bacterial species recovered from the blood and lesions of bacterial chondronecrosis with osteomyelitis (BCO) from the lame broilers at the University of Arkansas poultry research farm. Also, the present study confirms that raising young birds on suspended wire flooring has been proved to successfully induce lameness attributable to BCO with birds being more susceptible to bacteraemia than those that were raised on litter flooring. The newly described pathogen, Staphylococcus agnetis, has been reported to be overrepresented in blood and BCO lesions from lame broilers. In this study, we tried to understand the role of macrophage killing in the virulence of this emerging pathogen as survival the killing mechanisms exerted by macrophage may be a critical component for bacterial systemic dissemination (bacteraemia). Our chicken isolate S. agnetis 908 were considered to be hyper virulent strain by resisting the killing strategies by chicken macrophage cells in tissue culture. Moreover, we assess the process of adding organic trace minerals (OTM) supplements to the pelleted feed to improve flock health and performance through enhancing the immune capacity of the broiler chicken

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CHAPTER 1. Review of literature

1. 1. Poultry industry

1. 1. 1 Ancestry and domestication of chickens:

Poultry are domesticated birds which are raised by humans for their eggs, meat, and feathers (Vaarst et al.,2015). This includes birds such as chicken, turkey, fowl, peafowl, goose, duck, guinea, pigeon, quail, and pheasant (Al-Nasser et al. 2007). Chickens are the most popular poultry species around the world. Chicken constitutes approximately 90% of the poultry population (Alders et al., 2018). Chickens are the descendants of the wild red Junglefowl (Al-Nasser et al. 2007; Caplen et al., 2012). Based on the archeological evidence, domesticated chicken (Gallus gallus domesticus) originally existed in China at least 8,000 years ago with subsequent dispersal to other regions over the world by land and sea (Alders et al., 2018). Historically, the exploitation of chickens was confined to symbolic and social purposes (Perry-Gal et al., 2015). It is unclear when and where chickens were first used for economic purpose as an important livestock species. Using chicken for entertainment (cockfighting) continued until mid-1800s, when it was made illegal (Al-Nasser et al. 2007). Afterward, the chicken has been bred for exhibition by poultry breeders in USA and Europe. For this purpose, chickens were selected for particular features such as uniformity and colored plumage designs. These attempts were significant in the improvement of standard breeds and variations of chickens which were utilized in the production of today's chickens.

1. 1. 2 Structure of the poultry industry:

At the beginning of the 20th century, animal genetics and breeding technologies have focused on commercial application for livestock production (Johnson and Ruttan, 1997). Breeding technologies have an immense impact on the farm-level productivity, especially in the poultry

sector which has experienced almost 100% adoption of these technologies in order to significantly increase productivity. Broiler production for meat consumption is a highly industrialized process (USDA, 2011). The broiler supply chain has several distinct components starting with primary breeders, the genetic stock for the industry. These breeders composed of elite (pedigree/foundation), great grandparents (GGP), and grandparent (GP) generations which are owned by primary broiler breeding companies such as Cobb-Vantress and Aviagen. Eggs from pedigree flocks are hatched in a special pedigree hatchery and their progeny then go on to produce GGP flocks (USDA, 2013). Eggs from GGP line produce chicks for the GP generation. Grandparent flocks have two separate male and female lines which are 2-way cross hybrids. Day-old chicks from GP stock then go to special GP hatcheries world-wide to produce the final generation of breeding stock (PS; multiplier/parents/broiler breeder) which passes to the production sector where eggs from multiplier flocks are hatched to yield production birds (broilers, layers, and market turkeys) for human consumption (Figure1.1). Primary breeding stocks are kept on high level biosecure farms due to their value and critical importance in the production pyramid (Figure 1.2) (Paxton et al., 2010). Breeding companies have the opportunity to influence both the welfare, robustness, as well as productivity of broiler chickens through genetic selection (EFSA, 2010).

In commercial broiler breeding programs, generally, a four-way cross is implemented (van Eenennaam et al., 2014). To emphasize hybrid vigor or heterosis exploitation systematic matings which involve crossing different breeds, strains, or inbred lines are applied. For example, female lines are selected for their fertility egg quality while male lines are selected for growth qualities (Oldenbroek and van der Waaij, 2015). Therefore, crossbreeding produces a high number of healthy chicks that meet the requirements of chicken producer/processing companies. A faster

rate of genetic improvement in broilers can be accomplished due to the combination of several factors such as shorter generation intervals, large numbers of offspring (each female can produce more than 200 chicks each generation), and defined closed breeding populations (van Eenennaam et al., 2014).

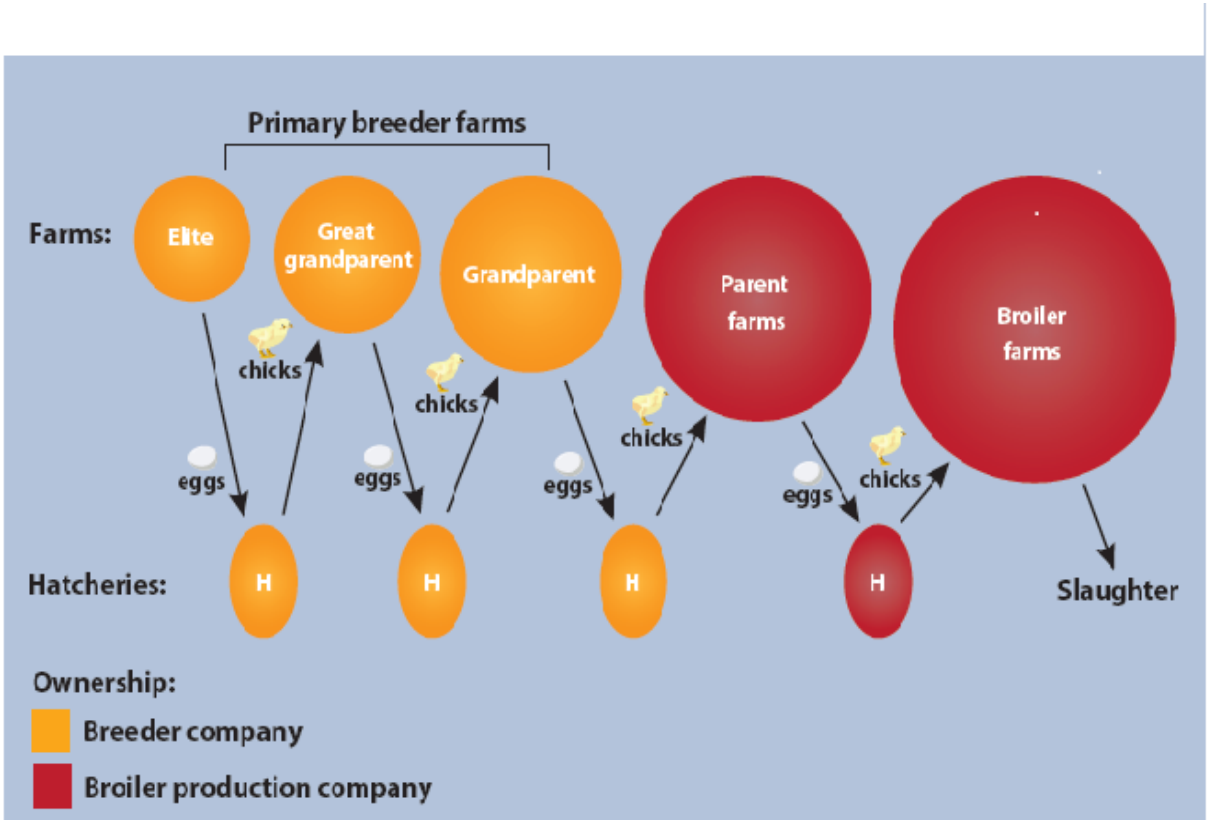

Figure 1.1: Primary breeders, parent breeders, and commercial broilers. Eggs from each generation are hatched to produce the flocks of the next generation. Ultimately, broilers are the product of multipliers in the parent line. Image reproduced from (USDA, 2013).

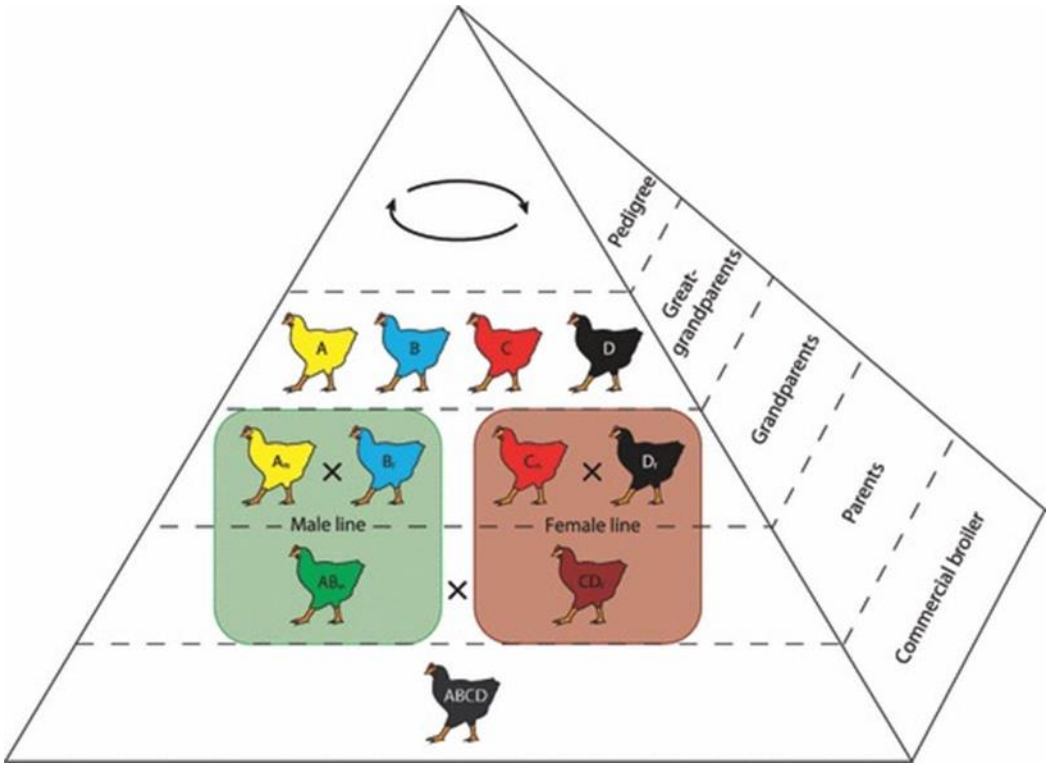

Figure 1.2: Poultry breeding structure. This pyramid presents a typical commercial broiler chicken breeding program where each segment represents a generation responsible for the transmission of selection response from purebred lines (on the top of the production line where selection of economically important traits takes place and maintains) to the billions of commercial broiler descendants (the fifth generation), which is produced from the significant multiplication through grandparent and parent lines. The time interval between selection in the pure lines and gains in the commercial broilers is typically around 4 years. Image reproduced from (Paxton et al., 2010).

1. 1 .3 Commercialization of the poultry industry:

Modern chicken breeds are the product of evolution over billions of years due to natural selection, on which artificial genetic selection of desirable traits for commercial purposes has been implemented (Schmidt et al., 2009; Tallentire et al., 2016). Commercial selection to obtain the modern broilers has changed (Laughlin, 2007; Paxton et al., 2010). In the USA, instead of focusing only on simple mass selection for live weight at market age, multiple selection criteria for growth related characteristics which positively influence economic gains and fit the demands of the consumer were included in the selection program. Early breed selection dedicated mainly

to egg production, and chicken meat was viewed as a by-product of egg production until the late 1920s (van Eenennaam et al., 2014). However, the advent of industrial scale agriculture at the beginning of 20th century enhanced intensive selection of high yield meat breeds (Schmidt et al., 2009). Since their domestications, the greatest advancement made in chicken genetics for the large-scale commercialization of the poultry industry was observed in the second half of the 20th century due to progress made in quantitative genetics and the success of the industries commercial objective (Tallentire et al., 2016). During domestication, particularly the later stage of breed improvements, chicken body mass and growth traits have increased from between twofold to reach more than fivefold as compared to their wild ancestor; the Red Junglefowl (Johnsson et al., 2018). Therefore, improved production profitability has been observed throughout increasing egg production in layers and minimizing the number of raising days so that the birds reach their market weight earlier than ever before (Buzala and Janicki, 2016). In 1920, 16 weeks were needed for a broiler chicken to reach only 1 kg (2.2 lb), while today's broiler strains may reach 2.6 kg (5.9 lb) in only 6 weeks (HSUS, 2013). Genetic selection has been decreasing the age by which broilers reach slaughter weight by as much as one day every year. As a result of increased growth rate, a broiler chicken has undergone more than 400% increase in growth rate between the years of 1950 and 2005 (Figure 1.3), and the feed conversion ratio (FCR) has reduced by 50% concurrently (Zuidhof et al., 2014; Buzala and Janicki, 2016). Besides that, artificial selection also improves the muscle yield by 30% higher in male and 37% higher in female for the pectoralis minor while pectoralis major yield increase by 79% in male and 85% in female. In addition, as broilers reach their market weight more quickly, the amount of feed consumed by those rapidly growing chickens has reduced dramatically, which results in improving the environmental sustainability of broiler productions (Tallentire et al., 2018). Rapid

growth rate played a significant role in the productivity of the US broiler industry, which produced around 36 billion bounds of broiler meat in 2007 (Schmidt et al., 2009). In 2013, more than 8.5 billion broiler chickens were slaughtered for human consumption in the United States annually (HSUS, 2013). The global demand for poultry products continues to grow remarkably (Al-Nasser et al., 2007). This is because of a high nutrition value and relatively low cost. Chicken meat and eggs are the main sources of protein in the human diet. Also, chicken meat consumption has been increasing annually (Tavárez and Santos, 2016). Unlike beef and pork, global prices of chicken meat are relatively more affordable. According to USDA Economic and Research Service (2019), the recorded retail price of chicken meat is ($1.885/lb) which was 32% and 48% cheaper than beef ($5.89/lb) and pork ($3.88/lb), respectively. Additionally, there is the contribution of other associated factors such as lack of cultural/ religious constraints regarding chicken meat consumption, rise in health awareness of consumers (healthier alternative to red meat), the efficacy of production, and the increasing size of human population (Tallentire et al., 2018). Broilers predominate the world poultry market (Al-Nasser et al., 2007). It constitutes 70% of the market while turkeys and other poultry represent around 8% and 22%, respectively. This shows that broiler meat is more favorable over other poultry meats.

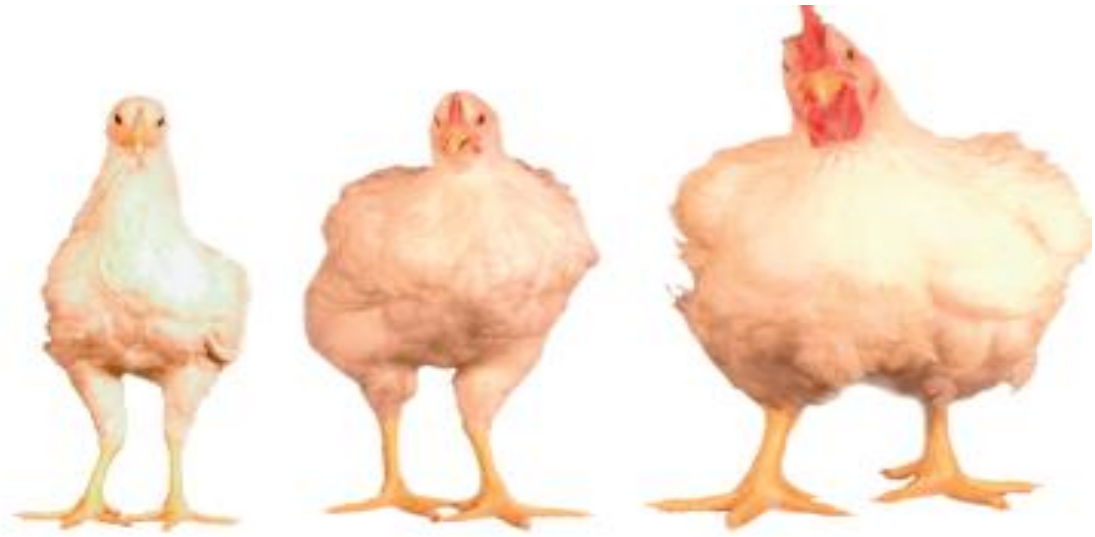

Figure 1.3: The change in the size of commercial broiler chickens due to the application of artificial selection technologies. The photos show broilers produced in the 1950s (left), in 1970s (middle), and in 2005 (right). The birds of these years were grown in identical environment and were at the same age (65 days). They weigh 905, 1808, and 4202 g, respectively. Image reproduced from (Zuidhof et al., 2014).

1. 1. 4 The impact of genetic selection on the welfare of broiler chickens:

After the second world war, the success of the poultry meat industry in delivering high-quality affordable food in significant quantities is well documented (Hocking, 2014). Intensive commercial selection has led to improved productivity and efficiency in the modern poultry industry, which over the last few decades has experienced a significant increase in the growth rate or the amount of weight gain over a time period and an evident improvement in the body conformation (Paxton et al., 2010).

Animal welfare is influenced by multiple factors including genetic selection, production environment, feed quality, disease challenges, housing systems, and stocking density (Rauw and Gomez-Raya, 2015). Animal welfare standards can be expressed as trouble-free production, lack of any abnormalities that obstruct production potential, low mortality, better fitness, and well-being, in the context of specific market or commercial production environment (Hiemstra and Nabel, 2013). Genetic propensity to compromised welfare may be masked by favorable

conditions in the higher levels of the breeding programs. However, welfare problems in broiler breeders are expressed in commercial production systems which are less controlled as compared to parent stock (Duncan, 2001). Intentional selection pressure placed on broilers to improve productivity represented by rapid growth rate, high meat yield, and enhanced feed efficiency (Schmidt et al., 2009; Dawkins and Layton, 2012; Tavárez and Santos, 2016), has been accompanied by unfavorable side effects for several reproductive, physiological, and immunological traits; accordingly, affecting the welfare of farm animals (Hocking, 2014; Rauw, 2016). Many studies have reported the undesirable correlated responses to the intensive system of poultry production linked to genetic selection such as skeletal deformities (Lilburn, 1994; Rath et al., 2000; Zuidhof et al., 2014; Tavárez and Santos, 2016), ascites or pulmonary hypertension syndrome (PHS) (Julian,1998; Wideman & French, 1999; Wideman et al., 2013), sudden death syndrome (SDS) due to cardiovascular failure (Bessei, 2006; Schmidt et al., 2009; Federici et al., 2016), compromised immune system (Cheema et al., 2003; Zuidhof et al., 2014), as well as poor reproductive performance (Hocking, 1993; Schmidt et al., 2009). These mounting studies report that human-directed evolution throughout the implementation of artificial selection for higher production potential can disturb genetic homeostasis, leading to reduced welfare and increased mortality (Cheema et al., 2003; Tickle et al., 2014), with poor walking activity or locomotion (Knowles et al., 2008). Furthermore, these studies have highlighted how selection for high growth rate and early carcass development have influenced particular tissues and organs along with enhancing the perception of how alterations in one organ system can have pleiotropic outcomes on others (al., 2009). More information is required to better understand how rapid growth and increasing body mass has shaped the broilers' anatomical and physiological changes across ontogeny (Tickle et al., 2014).

1. 2 Lameness in broiler chickens

1. 2. 1 Leg disorders

Broiler chickens are the fastest-growing and lowest-priced source of protein among farm animals (Tullo et al., 2017). They have been developed for high growth rate and improved meat yield through intensive selection programs. This produces differential growth of body organs such as early muscle growth without concurrent increase in skeletal development leading to leg weakness and disorders (Manohar et al., 2015; Aydin, 2018). Ability to walk normally can be characterized as an integrated function of the nervous, muscular, and skeletal systems (Manohar et al., 2015). Therefore, any failure in one of these components will lead to impaired leg activity or lameness. Leg weakness, lameness, and other bone disorders associated with various metabolic diseases represent a major animal welfare issue (Bessei, 2006; Dawkins and Layton, 2012; Toscano et al., 2013; Gocsik et al., 2017), and production concern (Bradshaw et al., 2002; Toscano et al., 2013; Gocsik et al., 2017) in fast growing meat-type chickens leading to increased morbidity and mortality in broiler chickens worldwide (Gocsik et al., 2017).

As a consequence of breeding program goals at achieving the greatest muscle mass in broilers, an inverse correlation has been found between the increase in wing muscle mass and decease in leg muscle perimeter leading to adverse effect on the performance and well-being of the young, heavy meat-type birds (Rath et al., 1999; Kierończyk et al., 2017). Moreover, the physical instability due to pectoral hypertrophy (Paxton et al., 2010), results in displacing the avian center of mass (CoM) cranially (Abourachid, 1993), and hence instability in the leg to achieve sustainable balance to the whole body weight (Nääs et al., 2009). Increased muscle mass builds up and changes in body conformation due to displaced center of the mass and reduced leg length (Caplen et al., 2012), impose enormous strain on the immature skeleton of broilers (Kierończyk

et al., 2017). Therefore, these anatomical changes are found to be strongly associated with skeletal abnormalities such as weakening, deformity, infections, contusion, and osteoporosis (Rath et al., 1999; Kierończyk et al., 2017). This suggests that the rapidly growing bodies of the commercial broilers are not evolving in harmony with these traits and possibly predisposing them to lameness (Paxton et al., 2010).

Lameness is more pronounced in broilers with reduced physical activity (Weeks et al., 2000; Sherlock et al., 2010). The lame birds spend more time laying and sleeping. The percentage of lying down time of broilers has increased from approximately 76% in the normal birds to 86% in the lame ones. Time spent walking at processing weight is reduced from 3.3% to 1.5% in the cases of severe lameness. Because of the increased gain in the body weight, the incidence of lameness is more frequent with increase in broiler age.

1. 2. 2 Prevalence and cost

The poultry industry has witnessed a profound proliferation over the last fifty years and today more than 20 billion broiler chickens are produced annually (Aydin, 2018). The economic advantages of increased body weight have led to the development of skeletal abnormalities. Lameness in broilers is one of the leading causes of high incidence of morbidity than mortality. Therefore, the degree of lameness and the final body weight at delivery are negatively correlated (Gocsik et al.,2017). As a consequence of this negative impact of lameness on productivity, production performance (production cost and returns) has decreased (Bradshaw et al., 2002; Gocsik et al.,2017). Considerable economic losses in affected broiler flocks are due to poor growth, higher condemnation and downgrading at slaughter (McNamee and Smyth, 2000). Many studies have suggested that disabled birds with severe lameness suffer pain when they walk (Nääs et al., 2009; Sherlock et al., 2010; Caplen et al., 2014; Gocsik et al.,2017), therefore, they

may have trouble in performing essential behaviors such as accessing feed and water (McNamee and Smyth, 2000; Bessei, 2006; Gocsik et al.,2017; Aydin, 2018).

Bone deformities have been considered as the prominent cause of losses in Canadian broilers (Riddell and Springer, 1985). The average incidence of broilers with bone disorders was 1.72% which comprised of 1.1% in the field cull and 0.62% were condemned (McNamee and Smyth, 2000). Another study which was conducted on male and female broilers showed that lameness prevalence was 0.52% in males and 0.38% in female. According to the United Kingdom chicken association, a survey of 176 commercial broilers estimated more than 27% of birds showed poor locomotion and about 3% were disabled (Knowles et al., 2008; Toscano et al., 2013; Kierończyk et al., 2017). In addition, 30% of broiler chickens and 90% of turkeys were lame due to tibial dyschondroplasia (Kierończyk et al., 2017). One study indicated that lameness attributed to bacterial chondronecrosis with osteomyelitis (BCO) was estimated to reach 0.75% in all birds placed in a study (McNamee & Smyth, 2000). Leg disorders are estimated to cost the poultry industry several hundred million dollars annually (Bradshaw et al., 2002). Approximately 12.5 billion birds suffer from leg disorders each year (Cook, 2000). Moreover, cost and losses due to these disturbances in growth rate and the development of musculoskeletal systems in poultry industry reached 120 million dollars in the USA alone. It was reported that skeletal disorders could indirectly result in decreased profitability from further broiler meat processing leading to total profit reduction by 10-40% of the cost (Kierończyk et al., 2017). The negative impact on the economy attributed to bone weakness and lameness can be reduced by determining specific risk factors and adopting effective management practices that affect the overall level of leg problems and consequently reduce lameness in broilers (Knowles et al., 2008).

1. 2. 3 Etiology of lameness:

Many factors were involved in the development of leg disorders including genetic determinants, species, sex, age, nutrition, infectious agents, growth rate and body weight (Kierończyk et al., 2017; Aydin, 2018). Other contributing factors include mechanically induced or trauma-associated problems, toxins, feed conversion efficiency, and handling and movement (Aydin, 2018). In addition to these factors Knowles et al., (2008) emphasized the importance of flock effective practices which exacerbate the problem of bone disorders if they are disregarded. However, the causes of lameness can be classified as developmental, generative or infection based on the pathological agent involved in causing the disease (Figure 1.4) (Bradshaw et al., 2002; Sherlock et al., 2010; Kierończyk et al., 2017). Developmental abnormalities include varus-valgus deformity (VVD), tibia dyschondroplasia (TD), rickets, chondrodystrophy (Julian, 1998; Cook, 2000; Aydin, 2018), spondylolisthesis (kinky back), epiphyseal separation, ruptured gastrocnemius tendon, (Julian, 1998), and contact dermatitis (Aydin, 2018). Infectious disorders were classified by North Central Regional Committee (NC-187) as inducers of bone problems (Cook, 2000). Bacteria, mycoplasma, and viruses do not directly impact skeletal system. These infectious agents infect the soft tissues (tendons and synoviae) and joint gap (fluid accumulation) causing severe signs of leg disorders. It is hard to identify the underlying etiology of non-infectious skeletal abnormality because they are not mutually exclusive in such a way that one of them may interact with one of the others (Bradshaw et al., 2002).

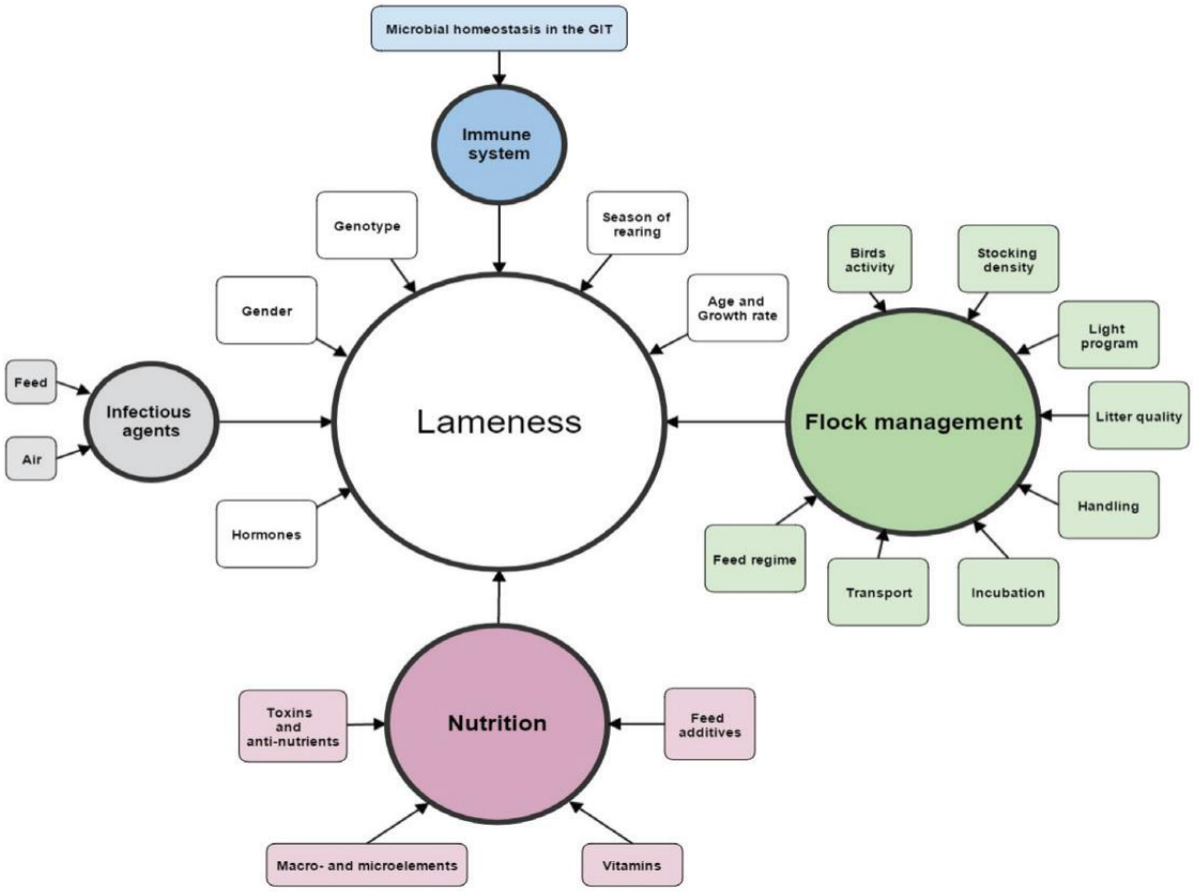

Figure 1. 4: Infectious and non-infectious factors involved in the development of lameness in broilers. Image reproduced from (Kierończyk et al., 2017).

1. 3 Bacterial chondronecrosis with osteomyelitis (BCO):

1. 3. 1 Terminology and incidence:

BCO is reported to be the most common cause of lameness in commercial broiler flocks (Cook, 2000; Wideman et al., 2012). It is manifested by necrotic degeneration and bacterial infection mainly within the rapidly growing bones and joints in broilers (McNamee and Smyth, 2000; Bradshaw et al., 2002; Wideman et al., 2012). This condition has been previously reported under different names including femoral head necrosis (FHN), osteomyelitis, long bone necrosis, proximal femoral degeneration. However, BCO is considered to be the most applicable because it describes the accurate pathology (necrotic degeneration) and also indicates the involvement of

microbial infection primarily within the proximal head of femur and tibia, as well as, other rapidly growing bones such as the T4 vertebra. Osteomyelitis is a common condition in chicken, turkey, and duck (Wise, 1975). It was first stated as cause of lameness in commercial broilers in Australia, and Staphylococcus aureus was recovered from the lesions (McNamee and Smyth, 2000; Dinev, 2012). BCO was subsequently reported in broilers from other countries such as Canada, Australia, Europe, and USA (McNamee and Smyth, 2000; Bradshaw et al., 2002; Dinev, 2012; Wideman et al., 2012). A study was conducted in Northern Ireland to investigate BCO in commercial broilers (McNamee, 1998; McNamee and Smyth, 2000; Wijesurendra et al., 2017). It was reported that the mean incidence of culling was 0.52% in male flocks and 0.38% in female. Furthermore, a study conducted by McNamee et al., found that 0.52% of all broilers culled were suffering from leg disorders, and BCO was reported in 17.3% of these cases (Bradshaw et al., 2002; McNamee et al., 1999). Moreover, a study carried out on 67 lame birds to investigate the prevalence of BCO in the proximal femora found that 64% showed gross degeneration of femoral end mainly because of osteomyelitis, 25% expressed epiphysiolysis while the remaining 11% showed microscopic bacterial or osteochondritis lesions (Thorb et al., 1993; Bradshaw et al., 2002). Investigation in Bulgaria also reported high incidence of lameness responsible for over 15% of mortality in some broilers, with BCO the most prominent cause affecting more than 90% of these cases (Dinev, 2009; Wijesurendra et al., 2017).

Because of the low incidence of lameness attributed to BCO, several experimental models have been developed to study the etiology and pathogenesis of BCO (McNamee et al., 1999; Gilley et al., 2014). Most of these models involve exposing birds to known pathogens tracheal administration, intravenous injection, or aerosol inhalation. Moreover, a recently developed wire-flooring model has been applied to induce significant levels of BCO which help to understand

the pathogenesis of this disease in systematic and reliable way (Wideman et al., 2012; Wideman and Prisby, 2013; Gilley et al., 2014). In this experimental system, young broilers are reared on flat or sloping wire flooring instead of wood-shavings litter to consistently promoting high growth rates and normal levels of activity at relatively low stocking densities while using unstable footing to create additional torque and shear stress on susceptible proximal leg joints.

1. 3. 2 Clinical signs of BCO

BCO is an age-related condition considered a severe degenerative disorder that mainly affects broilers towards the end of their growing time (Bradshaw et al., 2002). Lame birds may not show any apparent sign of leg deformities but are hesitant to move. Live, affected birds have a characteristic limping gait and when they walk, lame birds place their wing tips on the ground for support during locomotion (McNamee and Smyth, 2000). Moreover, many of these birds vocalize loudly during examination when pressure is applied to the affected proximal femur, indicating that birds suffer from pain in the advanced stages of the disease (Bradshaw et al., 2002). Affected birds have a reduced feed efficiency and lower growth rate because they are unwilling to walk to obtain food and water (Sherlock et al., 2010; Kierończyk et al, 2017. Aydin, 2018). Therefore, a sharp decrease in the body weight has been observed as a characteristic feature of both naturally occurring and experimentally stimulated BCO (McNamee and Smyth, 2000; Bradshaw et al., 2002). This negative impact on growth rate has been exploited to estimate the development of lesions in affected birds.

1. 3. 3 Pathogenesis of BCO

The pathogenesis of BCO is complex (McNamee and Smyth, 2000; Wideman et al., 2012). It is mostly attributed to the accelerated growth rates that impose excessive mechanical forces on the structurally immature growth plate of the thoracic vertebrae, and the proximal leg bones

(Bradshaw et al., 2002), leading to the formation of microfractures and clefts within the epiphyseal and physeal cartilage (osteochondrosis) (Wideman and Prisby, 2013; Wideman, 2016). Consequently, microscopic zones of focal ischemia and necrosis occur due to truncation of the blood vessels that penetrate this area. Biomechanical stresses and sluggish circulation in metaphyseal vessels contribute to the pathogenesis of osteochondrosis in different kinds of animals. Bacteria may localize within pre-existing pathologies such as disruptions of the physeal vasculature, microfractures, and clefts commonly observed in the affected areas, which serve as foci for bacterial invasion (Bradshaw et al., 2002). Microfractures of the growth plate allow colonization of osteochondrotic clefts (wound sites) by hematogenously disseminated opportunistic bacteria (Wise, 1975; Thorp et al., 1993; Wideman et al., 2012; Wideman, 2016). Infecting bacteria enter the blood circulation via translocation through the respiratory system or gastrointestinal tract and then can spread through bloodstream, exit the circulation via the fenestrated epithelium at the ends of epiphyseal and physeal vascular plexuses and pass into the cartilaginous matrix (Figure 1.5) (Emslie and Nade, 1983; Wideman et al., 2012; Wideman, 2016). Stress-mediated immunosuppression can facilitate bacterial translocation and proliferation (Wideman et al., 2012; Wideman and Prisby, 2013; Wideman, 2016). Translocated bacteria can create blocking bacterial emboli in the epiphyseal and metaphyseal vascular plexuses (Emslie and Nade, 1983; Emslie et al., 1983; McNamee and Smyth, 2000; Wideman et al., 2012; Wideman, 2016). The plexuses are important for the survival and maturation of chondrocytes. Bacteria often possess the ability to adhere to the exposed cartilage matrix and colonize osteochondrotic clefts sites. Pathogens that can reach these sites are able to multiply outside of surveillance by circulating leukocytes and antibiotics (Emslie and Nade, 1983; Emslie et al.,

1984; Wideman et al., 2012; Wideman, 2016). Terminal BCO is considered as necrotic degeneration due to the released lytic substances at sites of bacterial colonization.

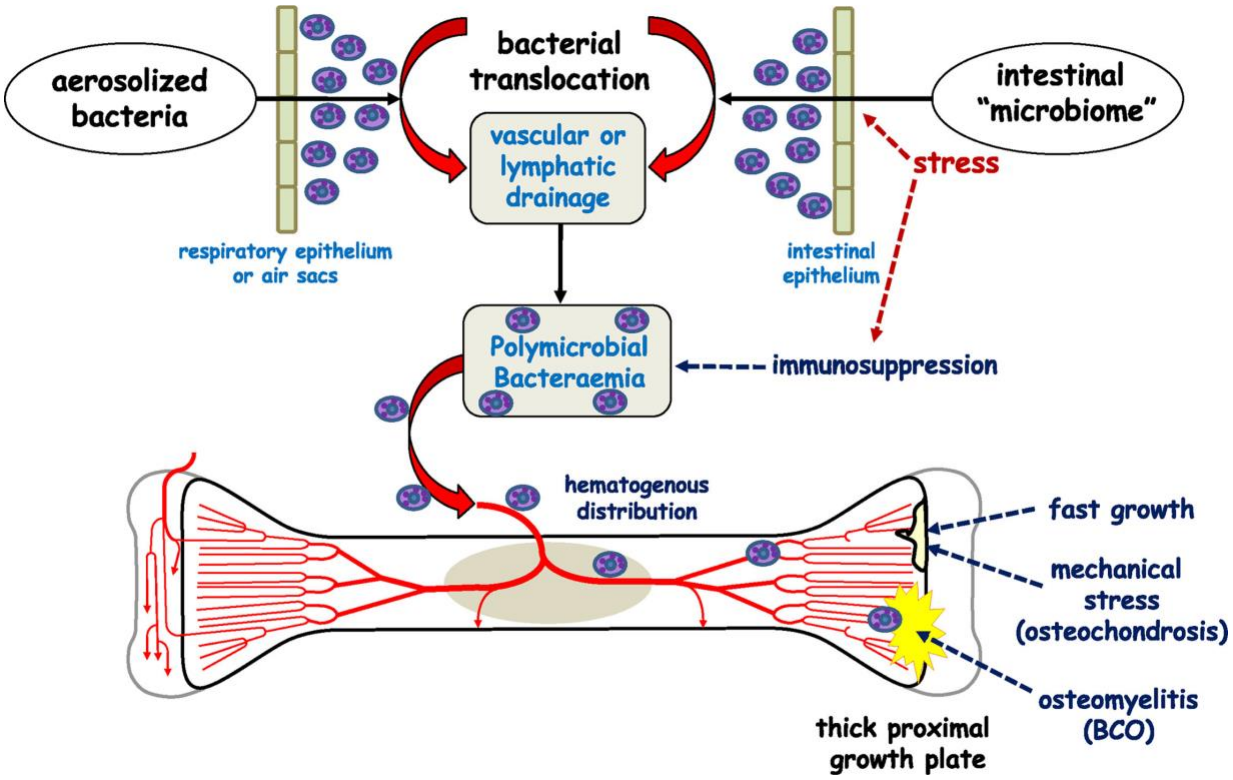

Figure 1. 5: Infectious pathways contributing to pathogenesis of bacterial chondronecrosis with osteomyelitis (BCO). This figure illustrates bacterial translocation through the respiratory system or gastrointestinal tract, their spread hematogenously to colonize the osteochondrotic clefts and zones of necrosis and forms bacterial foci within the fast-growing growth plates and adjacent metaphysis. These clefts are initiated because of the poorly mineralized layer of chondrocytes which become more sensitive to develop microfractures in response to mechanical stress. Image reproduced from (Wideman et al., 2016).

Macroscopically, BCO can be represented by focal regions of yellow caseous exudate or lytic areas that cause affected bones to be fragile and vulnerable (Skeeles, 1997). Lesions associated with BCO vary from small pale areas adjacent to the growth plate to more extensive zones of yellow tissue expanding from growth plate to the medullary cavity (Figure 1.6) (McNamee and

Smyth, 2000; Wideman et al., 2012). The proximal femorae and tibiae can be categorized according to macroscopic features of lesions into the following 6 diagnostic categories: normal tibia head (Normal Tibia); tibial head necrosis (THN); tibial head necrosis severe or caseous (THNsc); normal femoral head (Normal Femur); femoral head separation or epiphyseolysis (FHS); femoral head necrosis (FHN). These categories emphasize the progressive severity of the BCO lesions (Figure 1.7) (Jiang et al., 2015). Microscopically, BCO lesions appear to have clumps of basophilic bacteria in the epiphyseal or metaphyseal blood vessels which are surrounded by poorly stained cartilaginous matrix due to the generalized necrotic abscesses and voids (Bradshaw et al., 2002; Wideman et al., 2012; Wideman et al., 2014).

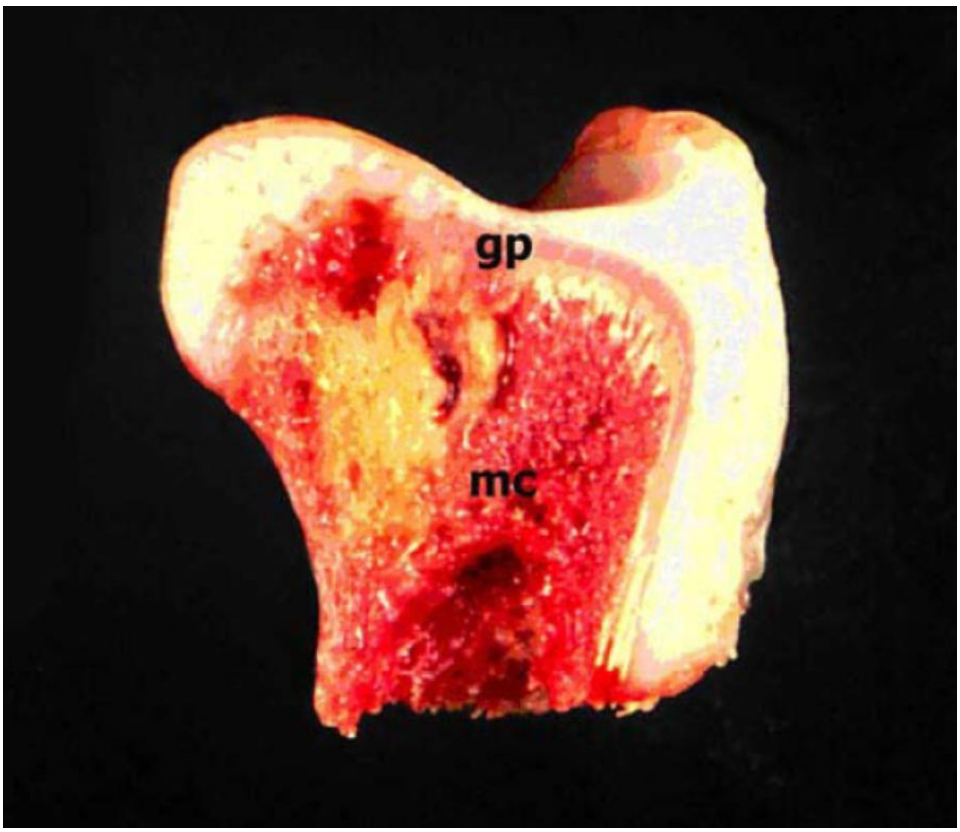

Figure 1. 6: Proximal end of the femur. The midline frontal section of a 28 days old lame commercial bird with BCO showed a macroscopically visible lesion of yellow tissue extending from the growth plate (gp) to the medullary cavity (mc). Photo reproduced from (McNamee and Smyth, 2000)

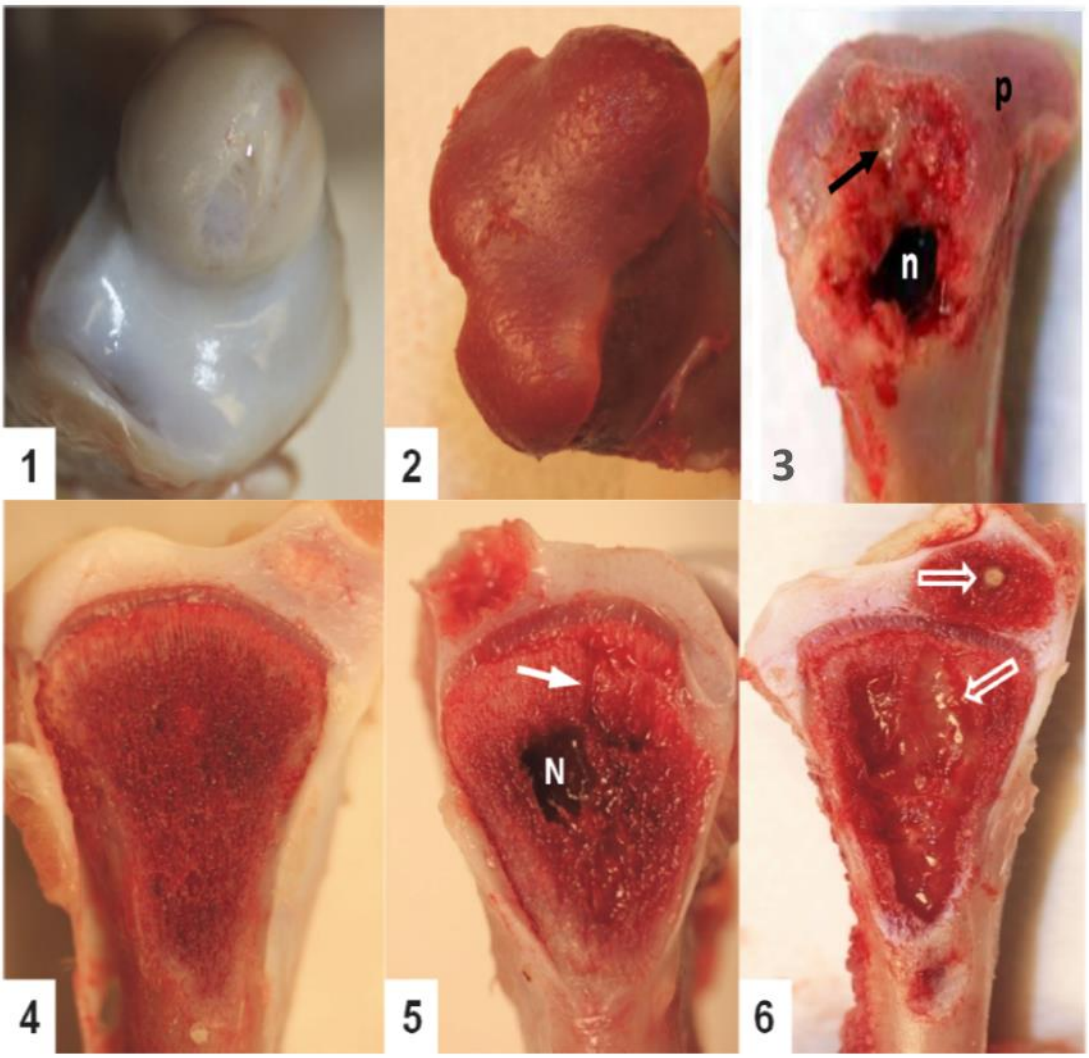

Figure 1. 7: Stages of proximal femoral (upper row) or tibial (lower row) head degeneration leading progressively to bacterial chondronecrosis with osteomyelitis (BCO): (1) Normal proximal femoral head; (2) Femoral head separation (FHS or epiphyseolysis); (3) Disarticulation of femur showing the underlying surface of growth plate or physis (p) with necrotic voids (n) and fibrinonecrotic exudate (black arrow) which display typical characteristics of femoral head necrosis (FHN) with osteomyelitis; (4) Normal proximal tibial head with spicules of trabecular bone in the metaphyseal zone which provide a supportive scaffold for the growth plate; (5) tibial head necrosis (THN) showing necrotic voids (n) in the metaphyseal zone which results in microfractures of the growth plate, lytic channel (white arrow) penetrate from necrotic voids into the growth plate; (6) Tibial head necrosis severe or caseous (THNsc) showing regions of caseous bacterial infiltration and sequestrate (open arrows) which indicate macroscopic evidence of osteomyelitis. Photo reproduced from (Jiang et al., 2015).

1. 3. 4 Microbiology of BCO lameness

A wide range of opportunistic bacteria have been reported to be involved in the development of BCO (Nairn and Watson, 1972; Thorp et al., 1993; Wideman, 2012; Wijesurendra et al., 2017). Bacterial species which have been recovered from BCO lesions include: Staphylococcus spp., Staphylococcus aureus, Escherichia coli, frequently in mixed culture with other bacteria such as Salmonella spp. In addition to that pure cultures of Enterococcus cecorum was isolated from osteomyelitis lesions in broilers in the Netherlands, Scotland, and in broiler breeders in the US (Armour et al., 2011). The poorly described pathogen, Staphylococcus agnetis, has been found to be overrepresented in BCO lesions from lame broilers at the University of Arkansas poultry research farm (Al-Rubaye, 2013). These bacterial strains were recovered from 81 samples (87%) out of 93. This finding was not surprising; however, it was unexpected to find S. agnetis which has been reported to be associated with mastitis in cows and had not been associated with broiler chickens.

1. 3. 4. 1 Staphylococcus spp.

Bacteria that belong to the genus Staphylococcus are the prominent causative agents of lameness attributable to BCO (Bradshaw et al., 2002). They are widely distributed in the environment including the air, soil, and water (Marek et al., 2016). Also, members of this genus are recovered from different animal species including poultry. Currently, more than 70 species belong to this genus. For instance, S. aureus, the most pathogenic staphylococcal species, is a causative agent of community and hospital acquired infections in humans (Das, et al., 2008). It is responsible for a significant mortality and morbidity due to a wide range of acute and chronic infections in both human and domesticated animals. In animals, S. aureus is frequently associated with mastitis or intramammary infection in dairy cows (Martinez-Pulgarint, et al., 2009). In poultry, serious

losses occur, most often due to infections which are related to invasion by S. aureus (Stępień-Pyśniak, et al., 2016). S. aureus-related infections include osteomyelitis, bumble foot, arthritis/synovitis, omphalitis, and acute septicemia. The most commonly septicaemic pathogen associated with BCO infection is S. aureus (Emslie and Nade, 1983; McNamee et al., 1999; McNamee and Smyth, 2000; Wijesurendra et al., 2017). S. aureus has been routinely recovered from BCO lesions over the course of several decades and across wide-ranging geographic distribution (Bradshaw et al., 2002; Wijesurendra et al., 2017).

S. aureus was used to experimentally induce BCO in chickens by administrating bacteria by intravenous injection (Emslie and Nade, 1983; McNamee et al., 1999). In this model, S. aureus is primarily deposited in the growing ends of the metaphyseal blood vessels. Moreover, exposing broiler chickens to a suspension of S. aureus by aerosol has been evidenced to successfully induce BCO, with higher incidence in birds exposed to S. aureus and inoculated with chicken anaemia virus (CAV) and infectious bursal disease virus (IBDV) (McNamee et al., 1999). However, other Staphylococcus species have also been reported in association with poultry infections such as S. hyicus which has been reported to be associated with osteomyelitis in turkey (Stępień-Pyśniak, et al., 2016), and S. simulans with endocarditis in broiler chickens (Stępień-Pyśniak, et al., 2016), and BCO lesions (Bradshaw et al., 2002). S. xylosus, S. cohnii, S. lentus are the other most frequently occurring species identified in poultry (Stępień-Pyśniak, et al., 2016). Recently, S. agnetis has emerged as the prominent septicaemic pathogen causing BCO of the proximal femora and tibiae in broilers (Al-Rubaye, 2013; Al-Rubaye et al., 2015). S. agnetis has been known to be associated with bovine subclinical and mild clinical mastitis (Taponen et al., 2012; Adkins et al., 2018; Thakur et al., 2018; Chávez et al., 2019). It is a common pathogen isolated from the cow's mammary glands. In addition to be associated with

lameness in broiler chickens, S. agnetis is also reported to be recovered from cases of endocarditis and septicemia in broilers (Brüggemann et al., 2019). S. agnetis was among other coccal species such as S. aureus, S. lentus, S. simulanse, Enterococcus faecalis, and Enterococcus hirae that were isolated from the blood of broiler breeders with footpad lesions or footpad dermatitis and found to be associated with fatal infections (Thøfner et al., 2019). S. agnetis was also identified as part of the microbiota of pigs, particularly the ventral skin surface microbiota which is rich in S. agnetis as compared to other sites including nose, ear and dorsal skin surface (Strube et al., 2018).

1. 3. 4. 2 Pathogenesis of S. agnetis.

Staphylococcus spp. are found to be the main species that are involved in causing lameness in broilers, particularly S. agnetis (Al-Rubaye, 2013; Al-Rubaye et al., 2015). Knowing the exact pathogen and the mechanisms by which this pathogen is associated with causing the disease will help to develop procedures for mitigating BCO and eventually minimizing the economic loss (Bradshaw et al.,2002). Modes of transmission by which S. agnetis switched from cows to broilers include birds, flies, feed, personnel in the hatcheries, and air (Al-Rubaye, 2013; Al-Rubaye et al., 2015. Also, bacteremia or the presence of bacteria in the blood of the lame and apparently healthy broilers has been reported. Isolation of S. agnetis from blood samples suggests that, this bacterial species has already disseminated to the majority of tissues and body organs. A study was conducted at the University of Arkansas research farm to investigate the prevalence and pathogenesis of the chicken isolate S. agnetis 908, obtained from BCO lesions (Al-Rubaye et al., 2017). Bacteria were administered in the drinking water to broilers. The results of this study are consistent with what has previously reported by Al-Rubaye, in 2013 regarding S. agnetis as the predominant cause of lameness attributed to BCO. Moreover, the

results showed that S. agnetis 908 is likely to colonize some areas of the gut mucosa and continue to survive until later stages when it translocates from gut into bloodstream and distributes to the rapidly growing growth plate of the proximal leg bones. Furthermore, the results of this study revealed that challenging birds with S. agnetis 908 to induce BCO is communicable to pen-mates. This suggests that spreading to pen mates may occur through direct contact, nipple waterers contamination by exposed broilers, or shedding from birds that are suffering early bacteremia developed during the advanced stages of the disease.

A study was conducted to investigate the potential ability of mastitis-associated S. agnetis to internalize into bovine mammary epithelium cells bMEC (Chávez et al., 2019). The results revealed that besides being resistant to different antibiotics including methicillin, S. agnetis showed ability to be internalized into bMEC. The process of internalization into these non-professional phagocytes is through receptor-mediated endocytosis (zipper mechanism) which enable this microorganism to invade host cells. Also, isolation of S. agnetis from the blood of broiler breeders with footpad dermatitis lesions has suggested that these lesions may serve as predisposing factor for bacterial infection (Thøfner et al., 2019). Based on the fact that Staphylococci are commonly found as part of the skin flora of vertebrates, including chickens, therefore, damaged epithelial lining of the footpads allow these gram-positive cocci to invade host cells and cause many pathological disorders such as salpingitis, peritonitis, arthritis, septicaemia, amyloidosis, endocarditis and pododermatitis.

1. 3. 5 Identification of bacterial communities associated with BCO

In the past, clinical microbiology laboratories relied overwhelmingly on the traditional culture-based assays to detect and identify clinically important bacteria followed by conducting biochemical tests utilizing semi-automated platforms (Petrosino et al., 2009). These platforms

are more subjective because it depends on phenotyping identification leading to misidentification of bacterial species or subspecies (Cheng et al., 2006; Ajitkumar et al., 2013; Schmidt et al., 2018). Traditional detection techniques are generally laborious and time consuming (Soumet et al., 1999; Ajitkumar et al., 2013). Therefore, molecular techniques, particularly DNA-based amplification are increasingly being utilized for diagnosis of infectious agents because they provide the potential for more specificity and sensitivity in detecting infectious agents in a much shorter period as compared to using classical culture-based approaches (Saeidabadi et al., 2017). Culture-independent assays to detect and identify microbes such as using universal primer pairs targeting specific conserved sequences of a particular bacterial gene such as 16S rRNA offers a powerful tool for comprehensively investigating the structure and diversity of microbial communities (Hjelmsø et al., 2014), and as tool to differentiate bacterial species from one another (Cheng et al., 2006; Robertson et al., 2009). The 16S rRNA gene sequences have hyper-variable regions which can provide valuable analysis regarding bacterial communities including diversity and composition (Kullen et al., 2000; Bartram et al., 2011; Ajitkumar et al., 2013). V1 and V2 regions of 16S rRNA gene sequences were mostly used for genus identification of coagulase-negative Staphylococci (CNS) species from bovine milk (Ajitkumar et al., 2013), differentiation of Lactobacillus acidophilus complex which includes 6 closely related species (Kullen et al., 2000).

Many studies were performed to identify the economically important pathogens of poultry worldwide such as S. aureus (Butterworth et al., 2001), Salmonella spp. (Ren et al., 2017; Saeidabadia et al., 2017), Campylobacter spp. (Ricke et al., 2019), Enterococcus sp., and Escherichia coli (Song et al., 2019). As BCO has been recognized as a prominent cause of lameness in commercial broilers with noticeable economic and welfare concerns among poultry

producers (McNamee et al., 1999; Wideman et al., 2012), rapid and accurate identification of microbial pathogens is of critical importance for the prevention of the progression of BCO (Song et al., 2019). Many molecular techniques were used for molecular typing of bacterial pathogens associated with BCO such as phage typing, multilocus enzyme electrophoresis, pulse-field gel electrophoresis (PFGE), bacterial restriction endonuclease digest analysis, random amplification of polymorphic DNA (RAPD) (Butterworth et al., 2001), and high resolution melt analysis (HRMA) (Al-Rubaye et al., 2017).

HRMA is a screening tool for DNA analysis that was adopted in 2002 by a collaboration between industry (Idaho Technology. UT, USA) and academics (University of Utah, UT, USA) (Reed et al., 2007). This technique can detect nucleotide sequence variations and is therefore ideal for genotyping, species determination, sequence matching and mutation scanning (Steer et al., 2009). HRMA has been used for detection and discrimination of microbial pathogens including viruses (Hewson et al., 2009; Steer et al., 2009), bacteria (Ajitkumar, 2013; Ren et al., 2017; Saeidabadia et al., 2017), mycoplasma (Jeffery et al., 2007), nematodes, and fungi (Ren et al., 2017). HRMA represents a post-PCR assay for DNA fingerprinting by measuring changes in fluorescent level of the melting DNA amplicon (Papavasileiou et al., 2016). HRMA has been adapted to real time PCR (RT-PCR). Prior to HRMA, the region of interest is amplified using qPCR in the presence of a saturating intercalating fluorescent dye that does not inhibit the amplification reaction and fluoresces when bound to DNA. After the qPCR, the DNA amplicon is gradually heated, and the dsDNA binding dye fluoresces brightly at low temperatures and drop in level of fluorescent as the DNA denatures as the temperature is increased (Reed et al., 2007). The fluorescence will decrease slowly at the very beginning of heating process, and then at a characteristic temperature, the fluorescence rapidly drops reflecting the release of the dye upon

the melting of the DNA into single strands and this results in a characteristic melting profile of the DNA amplicon. HRMA-curve analysis of PCR amplicons has been used as a routine diagnostic tool because it provides a rapid and cost effective assay to the direct analysis of the DNA sequences, especially when large numbers of samples are to be examined and also it does not require any further separation and additional processing after PCR (Hewson et al., 2009; Jeffery et al., 2007).

1. 4 Intracellular surviving in macrophages:

Chronic and persistent infection caused by microbial pathogens is due to their ability to produce multiple virulence factors which impair the host defenses (Garzoni and Kelley, 2009). Virulence factors expressed by pathogens facilitate their attachment to the surface of host cells, colonization, and invasion. One strategy used by pathogens to survive and trigger infection is using defensive pathways that enable them to avoid the killing mechanisms used by phagocytes (Das et al., 2008).

How pathogens circulate in the blood of apparently healthy birds, their ability to avoid being eliminated by the host's defense mechanisms, and the consequent persistence in the site of infections without producing an inflammatory response need to be addressed (Wideman, 2016). This suggests focusing on understanding the essential role of the immune system during the pathogenesis of BCO.

1. 4. 1 The macrophage

Like neutrophils and dendritic cells, macrophage are professional phagocytes which are equipped with antimicrobial substances and therefore represent an essential component of the innate immunity (Garg et al., 2011; Roche and Furuta, 2015). Moreover, they can shape adaptive immunity by acting as antigen presenting cells after phagocytosis of microbes. Therefore,

avoidance of macrophage-dependent killing is required for successful establishment and persistent infection (Flannagan et al., 2015). Macrophage consist of heterogeneous populations of cells that can be either derived from circulating monocytes in response to inflammatory stimuli or can be self-renewing tissue resident cells (Serbina and Pamer, 2006; Perdiguero et al., 2015). These immune cells can be polarized to acquire functional plasticity, and this can be determined by the presence of signals such as cytokines released in the cell's local environment (Sica and Mantovani, 2012; Zhou et al., 2014). Therefore, macrophage play an important role as microbicidal cells by recognizing, engulfing, destroying, and clearing microorganisms. Also, they provide functions associated with tissue remodeling and repair.

1. 4. 2 Phagosome formation and maturation

The process of bacterial entry, proliferation, or resistance to phagocytosis rely on host-pathogen interactions (Kummari et al., 2015). Phagocytosis is a receptor-mediated process to capture and ingest foreign particles larger than 0.5 μm, including pathogenic microorganism and cellular debris and thus help maintaining tissue homeostasis (Flannagan et al., 2015). Ingestion of the target particle occurs through a membrane bound vacuole termed the phagosome (Araki et al., 1996; Flannagan et al., 2012). Phagosome formation is not a microbicidal event by itself because the lumen of newly formed vacuole is a reflection of the fluid phase in the extracellular compartment surrounding the macrophage while the phagosomal membrane that covers the internalized microbe is derived directly from the cell membrane (Flannagan et al., 2009). However, the nascent phagosome quickly develops the characteristics of the early, intermediate, and then late phagosome (Flannagan et al., 2009; Flannagan et al., 2015; Uribe-Querol and Rosales, 2017). This progression to the phagolysosome can be defined by the presence of specific surface markers which define the stages of phagosome maturation. Phagosome

maturation is a dynamic process composed of sequential fusion and fission events between the nascent phagosome and subcompartments of the endocytic pathway which culminates with the formation of the phagolysosome at the end of maturation process through fusion with lysosomes. Consequently, membrane remodeling, phagosome acidification through a marked drop in the internal pH, and creation of a degradative milieu with robust microbicidal properties occurs to eliminate and degrade the ingested microbe.

1. 4. 3 Microbicidal activity of the phagosome

During the course of maturation, phagosomes are equipped with a full arsenal of microbicidal factors (Figure 1.8 a). The major destructive strategies include:

1. 4. 3. 1 Phagosome acidification

Acidification of the phagosomal lumen occurs via the action of vacuolar ATPases (V-ATPase) that hydrolyze ATP and exploit the released energy to translocate H^+ across the membrane bilayer into the phagosome lumen (Figure 1.8 a) (Tougard et al., 1985; Hackam et al., 1997; Flannagan et al., 2009). Phagosomal acidification creates a harsh environment that blocks microbial growth (Jabado et al., 2000; Uribe-Querol and Rosales, 2017). The low pH disrupts the normal metabolism pathways of some bacteria and prevents the use of several essential nutrients by the presence of special capture molecules or by transporters. Furthermore, phagosome acidification is intertwined with the function of other antimicrobial mechanisms (Deurs et al., 1996; Flannagan et al., 2009). For example, luminal acidification favors the activation of many phagosomal hydrolytic enzymes that work optimally in an acidic pH. In addition, the V-ATPase pump also favors the generation of superoxide O_2^- by counteracting the negative charges generated by oxidation reactions and by combining O_2^- with H^+ to generate more complex ROS (Deurs et al., 1996; Babior, 2004; Uribe-Querol and Rosales, 2017).

1. 4. 3. 2 Reactive oxygen and nitrogen species

Professional phagocytes can concentrate reactive oxygen species (Alders et al.) to intoxicate the ingested microorganisms in the phagolysosome (Pandary et al., 2015; Uribe-Querol and Rosales, 2017). Direct or indirect generation of ROS is achieved by NADPH oxidase (NOX2) which is a critical innate immune mechanism. The active enzyme catalyzes the formation of highly unstable superoxide O_2^- in the phagosomal lumen (Minakami and Sumimotoa, 2006; Flannagan et al., 2009). In the acidic condition of the phagosome, O_2^- will quickly dismutate into H_2O_2 which will in turn react with O_2^- and release hydroxyl radicals (OH^-) and singlet oxygen. Also, myeloperoxidase catalyzes the conversion of H_2O_2 into hypochlorous acid and chloramines (Figure 1.8 a). Collectively, these toxic ROS efficiently destroy intraphagosomal microbes by targeting their proteins, lipids, and DNA converting these intact molecules into damaged ones by the action of oxidization (Reeves et al., 2002; Flannagan et al., 2009). Moreover, O_2^- can also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), both of which have potent cell damaging action (Figure 1.8 a) (Lam et al., 2010; Flannagan et al., 2009; Uribe-Querol and Rosales, 2017).

Similarly, to ROS, phagocytes can also produce nitrogen-based radicals or reactive nitrogen species derived from nitric oxide (NO•) (Stuehr, 1999; Flannagan et al., 2009). RNS are also important antimicrobial agents found prominently in macrophage and contribute to microbial destruction. The enzyme inducible nitric oxide synthase 2 (iNOS or NOS2) catalyzes the conversion of L-arginine and oxygen into L-citrulline and nitric oxide radicals (NO•) (Figure 1.8 a). This enzyme does not exist in the resting macrophage and it is only expressed in response to proinflammatory agonists (Weinberg et al., 1995). Unlike superoxide, NO• is produced on the cytosolic side of the phagosome, but it is able to diffuse across the membranes and accumulate

inside the phagosomal lumen where it can react with superoxide to produce the highly toxic peroxynitrite ($ONOO^-$) which can modify proteins and DNA of the ingested microbes (Flannagan et al., 2015; Grosser et al., 2016; Uribe-Querol and Rosales, 2017). Also, upon NO• diffusion, it can directly impair microbial enzymes and consequently inhibit their growth.

1. 4. 3. 3 Nutrient capture or nutritional immunity

Besides the low pH and oxidative conditions, microbial growth can be inhibited by minimizing the amount of essential nutrient inside the phagosome (Uribe-Querol and Rosales, 2017). Sequestration of nutrients by the host can be accomplished by special capture molecules, also called scavengers, delivered into the phagosome or by transporters located on the phagosomal membrane which have bacteriostatic activity. Lactoferrin is an example of a nutrient capture molecule that is released into the phagosomal lumen (Flannagan et al., 2009). Lactoferrin captures the host Fe^{2+} which is important for the growth of some bacteria (Figure 1.8 a). Mn^{2+} is another important metal required for bacterial growth (Searle et al., 1998; Flannagan et al., 2015; Uribe-Querol and Rosales, 2017). Therefore, upon its maturation, the phagosome becomes increasingly depleted of both Fe^{2+} and Mn^{2+} by the action of an integral membrane glycoprotein located on the phagolysosome called natural resistance-associated macrophage protein 1 (NRAMP-1). This membrane glycoprotein requires H^+ ions to transport divalent cations out of the phagolysosome, thus this H^+-dependent transporter is functioning more efficiently in the acidic environment (Figure 1.8 a) (Jabado et al., 2000; Flannagan et al., 2009; Flannagan et al., 2015).

1. 4. 3. 4 Antimicrobial proteins and peptide

A set of enzymes and peptides that are involved in the direct bacterial killing mechanisms are delivered to the phagosome upon its maturation (Flannagan et al., 2009). These protein molecules have potent antimicrobial effects by disrupting the integrity of pathogens. Antimicrobial peptides are small, approximately 10 kDa, cationic and amphipathic polypeptides (Ganz, 2004; Uribe-Querol and Rosales, 2017). They can be classified based on their structure into defensins (disulfide-bridged peptides) and cathelicidins (α-helical or extended peptides). These antimicrobial peptides attach to the negatively charged molecules on the surface of microbes causing membrane permeabilization on both Gram-positive and Gram-negative bacteria (Figure 1.8 a) (Lehrer et al., 1993; Zanetti, 2005; Flannagan et al., 2009).

Cathepsins are the most extensively described group of lysosomal proteases among the many degradative enzymes (Flannagan et al., 2009). These are cysteine proteases that display an important role in the direct killing of ingested microbes by triggering the disruption of bacterial membranes (Figure 1.8 a). For instance, cathepsin L and cathepsin K were reported executors of non-oxidative killing of phagocytosed S. aureus (Mueller et al., 2014; Flannagan et al., 2015). In contrast, cathepsin D has been found to be associated with controlling Listeria monocytogenes intracellular growth, probably by degrading the secreted bacterial pore-forming toxin listeriolysin O of L. monocytogenes and thus thwarting bacterial escape from the phagosome (Cerro-Vadillo et al., 2006; Flannagan et al., 2015).

Phagosomes are also equipped with many lysosomal hydrolases which are involved in the destruction of ingested pathogens (Miyauchi et al., 1985; Kockx et al., 2014; Flannagan et al., 2015). Lysozyme is one important example of an antimicrobial protein that can be expressed and secreted by different cell types. Lysozyme displays bactericidal effects by disrupting the

peptidoglycan layer, the primary component of the bacterial cell wall. Furthermore, other hydrolase enzymes are delivered to the phagosome to complete the lysis of bacterial cells such as phospholipase A2 which targets phospholipid components of bacterial membranes (Weinrauch et al., 1996; Flannagan et al., 2015), in addition to other hydrolases, such as α-hexosaminidase, and β-glucuronidase, that target carbohydrates (Flannagan et al., 2009). Therefore, antimicrobial peptides and degradative enzymes are delivered to the phagosomal lumen creating a hostile environment that completely degrades most phagocytosed microbes (Figure 1.8 a).

1. 4. 4 Bacterial resistance to phagocyte killing

Many pathogens, which include bacteria, fungi, and viruses, can survive inside the host cell and they do so by evolving multiple strategies that enable them to avoid destruction during the process of phagocytosis (Kubica et al., 2008; Gilbert et al., 2017). Some pathogens are able to block phagocytosis by either inhibiting or even degrading opsonic complement or antibody receptors (Prasadarao et al., 2002; Rooijakkers et al., 2005a), or by directly impairing the process of bacterial internalization exerted by phagocytes (Garrity-Ryan et al.,2000; Grosdent et al., 2002). Other pathogens have evolved strategies to resist one or more of the antimicrobial agents exerted by phagocytes (Figure 1.8 b) (Flannagan et al., 2009). Some bacterial species have developed metabolic pathways to thwart acidification of phagosomal lumen or have expressed uniquely acid tolerant proteins to endure the low pH value (Park et al., 1996; Vandal et al., 2008). Furthermore, other bacteria are able to actively degrade (Schmidtchen et al., 2002) or shield (Peschel et al., 2001; Trent et al., 2001) themselves from host defense peptides and proteins produced by phagocytes. Some bacteria can secret detoxifying enzymes, such as catalase, that counteract ROS and/or RNS (St John et al., 2001; Ng et al., 2004). Alternatively, some bacterial species prevent oxidative damage by blocking recruitment of the protein complex

that mediate ROS and RNS synthesis (Mott et al., 2002; Davis et al., 2007). Another microbial defensive mechanism used by bacterial species to overcome the insufficient amount of iron by expressing specialized iron-scavenging molecules termed siderophores, that sequester the cation targets for bacterial use (Luo et al., 2005), or by synthesizing iron storage (Velayudhan et al., 2007) or transport proteins (Robey and Cianciotto, 2002) to maintain bacterial iron homeostasis. Many bacteria improve their intracellular survival capacity by developing a vigorous stress response to degrade and repair damaged proteins (Raivio, 2005).

Many studies have been conducted to investigate the intracellular survival of such pathogens inside phagocytes. Mycobacterium tuberculosis, L. monocytogens (Bayles et al., 1998), Salmonella enterica (Balan and Babu, 2017), and Brucella suis (Foulongne et al., 2000) are some facultative intracellular pathogens that are able to invade host cells. These pathogens must possess several mechanisms to be protected from the harsh intracellular environment (Bayles et al., 1998). These 'professional' intracellular pathogens parasitize host cells by either interfering with phagosome maturation, by escaping maturing phagosomes, or by resisting the microbicidal contents of the phagolysosome (Flannagan et al., 2009).

Although considered an extracellular pathogen, many studies confirmed that S. aureus is able to invade host cells and resist killing, so as to survive and proliferate inside phagocytes (Das et al., 2008; Bayles et al., 1998; Martinez-Pulgarint et al., 2009; Garzoni and Kelley, 2009; Kubica et al., 2008). A plethora of multiple virulence determinants have been identified that modulate host defense mechanisms to promote S. aureus survival (Kubica et al., 2008).

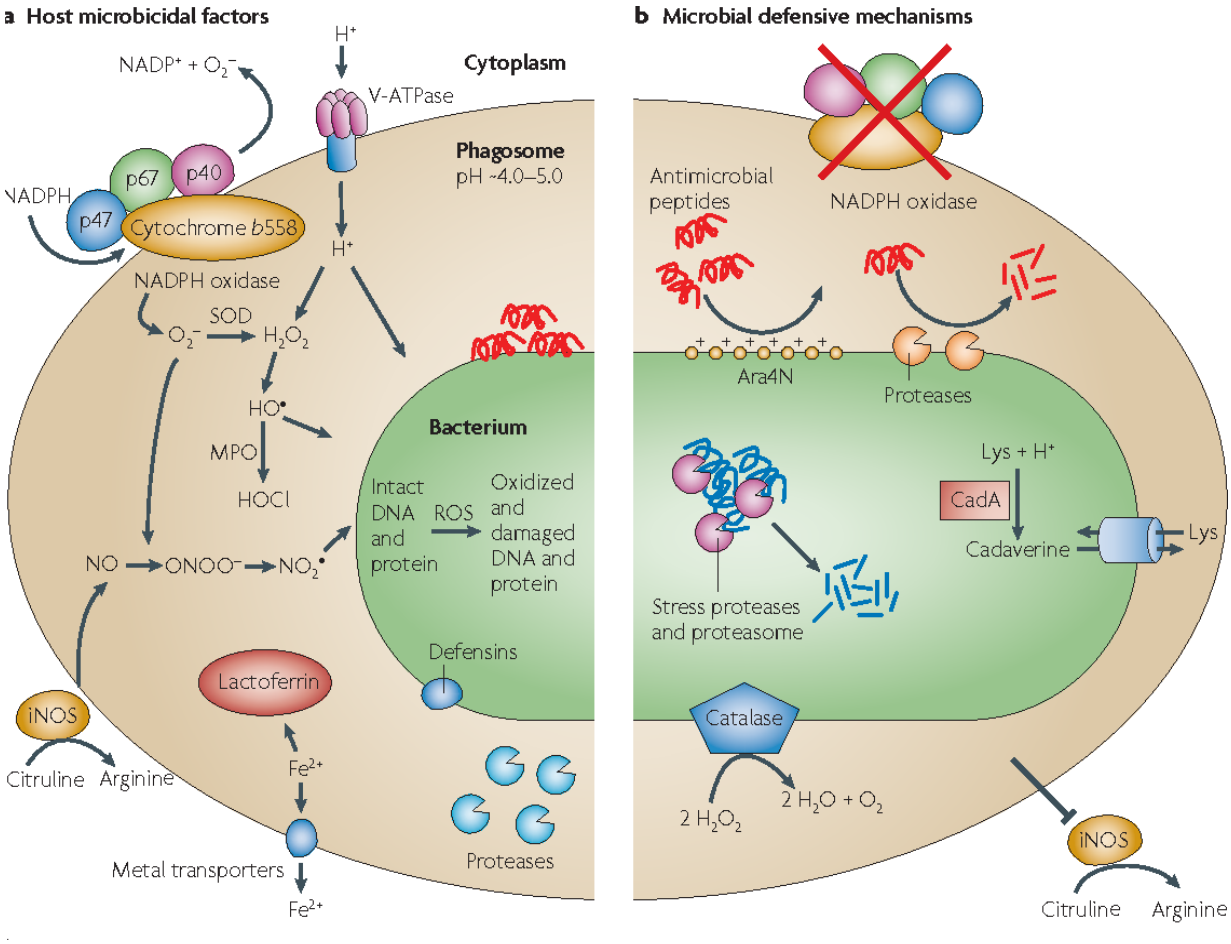

Figure 1. 8: The microbicidal activity of phagocytes versus the defensive strategies of the microorganisms. (a), illustrates host microbicidal factors which include the NADPH oxidase (NOX2), the inducible NO synthase (iNOS), lactoferrin and natural resistance-associated macrophage protein 1 (NRAMP1) which function as iron scavengers and transporters, respectively. In addition to these factors, antimicrobial peptides (APs) and proteins that compromises bacterial cell wall and cause cell lysis. In (b), microbial defensive mechanisms include alteration of the cell wall composition which confers resistance or degradation of APs. Also, expression of detoxifying enzymes such as catalase and superoxide dismutase (SOD) which catalyze the conversion of reactive species into less damaging components or blocking the recruitment of protein complexes that are responsible for the formation of ROS and RNS. reproduced from (Flannagan et al., 2009).

1. 4. 5 S. aureus immune evasion strategies against macrophage-mediated response

S. aureus possesses an impressive array of virulence factors to augment its pathogenic potential (Flannagan et al., 2015; Thammavongsa et al., 2015). The expression of these factors is controlled by complex gene regulatory pathways which are activated in response to multiple

environmental cues such as low pH, production of oxidant, metal depletion, cell density, and limitation of amino acids. The expression of these gene products will intoxicate phagocytes, block opsonization and phagocytosis, inhibit complement activation, resist antimicrobial protein attack, neutralize ROS and RNS, and extract essential nutrients that support bacterial growth.

1. 4. 5. 1 Extracellular intoxication of phagocytes

S. aureus can produce various membrane damaging toxins which evoke host cell lysis and death due to compromising the cytoplasmic membrane integrity of the affected cell (Uribe-Querol and Rosales, 2017). These toxins include the leukocidins and α-hemolysin (Hla) that are essential for the pathogenesis of S. aureus because of their ability to induce membrane permeability through the formation of pores upon their binding to specific receptors on the surface of the macrophage membrane (Figure 1.9) (Otto, 2014; Flannagan et al., 2015). This results in the efflux of vital molecules and metabolites, and therefore, they are cytolytic. These pore-forming toxins are produced to make the macrophage unable to phagocytose (Thurlow et al., 2011). Pore formation in the plasmalemma permits the efflux of cytosolic K+ outside the cell and consequently triggers inflammasome activation and production of proinflammatory cytokines (Melehani et al., 2015). Interestingly, ingested S. aureus has been reported to kill infected cells from within the phagosome by pore formation on the internal phagosome membrane that allows ion exchange between the phagosomal lumen and cytosol causing death of the infected cell during toxin-mediated attack (Muñoz-Planillo et al., 2013).

1. 4. 5. 2 Evasion of complement and opsono-phagocytosis

The process of phagocytosis is more efficient when microbes are opsonized by antibodies or complement (Rooijakkers et al., 2005b; Uribe-Querol and Rosales, 2017). Microorganisms have elaborate strategies to block opsonization. One mechanism presented by S. aureus to prevent

opsonization is simply destroy opsonins through staphylococcal protease-mediated attack, such as staphylokinase (Figure 1.9). This bacterial encoded plasminogen activator is responsible for the degradation of IgG and C3b molecules on the bacterial surface. Another strategy is to scavenge the opsonin, so that it does not recognize and bind to bacteria (Sulica et al., 1979; Uribe-Querol and Rosales, 2017). For example, protein A (SpA) is a well-characterized protein expressed by S. aureus on the bacterial cell wall that binds specifically to the Fc domain of IgG blocking the antibody from being engaged to activate the Fc receptor (FcγRs) on the surface of phagocytes. This results in an efficient inhibition of IgG-mediated phagocytosis (Figure 1.9). Another secreted protein from S. aureus is aureolysin which is involved in complement inactivation by functioning as a C3 convertase leaving C3a′ and C3b′ fragments (Laarman et al., 2011; Uribe-Querol and Rosales, 2017). C3b′ is a ligand for the opsonic receptor Mac-1 on the surface of phagocytes. Upon its deposition onto bacterial surface, C3b′ marks bacteria as targets for phagocytosis. The aureolysin-generated C3b′ is not functional and quickly degraded so that C3b′ deposition on the bacterial surface fails (Figure 1.9). In addition, the staphylococcal complement inhibitor (SCIN) is another secreted protein that can inhibit complement activation on the bacteria preventing phagocytosis and killing of staphylococci (Figure 1.9) (Rooijakkers et al., 2009; Uribe-Querol and Rosales, 2017). S. aureus also secretes the extracellular fibrinogen binding protein (Efb), that binds the serum protein fibrinogen (Ko et al., 2013; Kuipers et al., 2016; Uribe-Querol and Rosales, 2017). Accordingly, the bacterium is able to encase itself in a proteinaceous shield which masks bacterial-associated opsonin from phagocytic receptors (Figure 1.9).

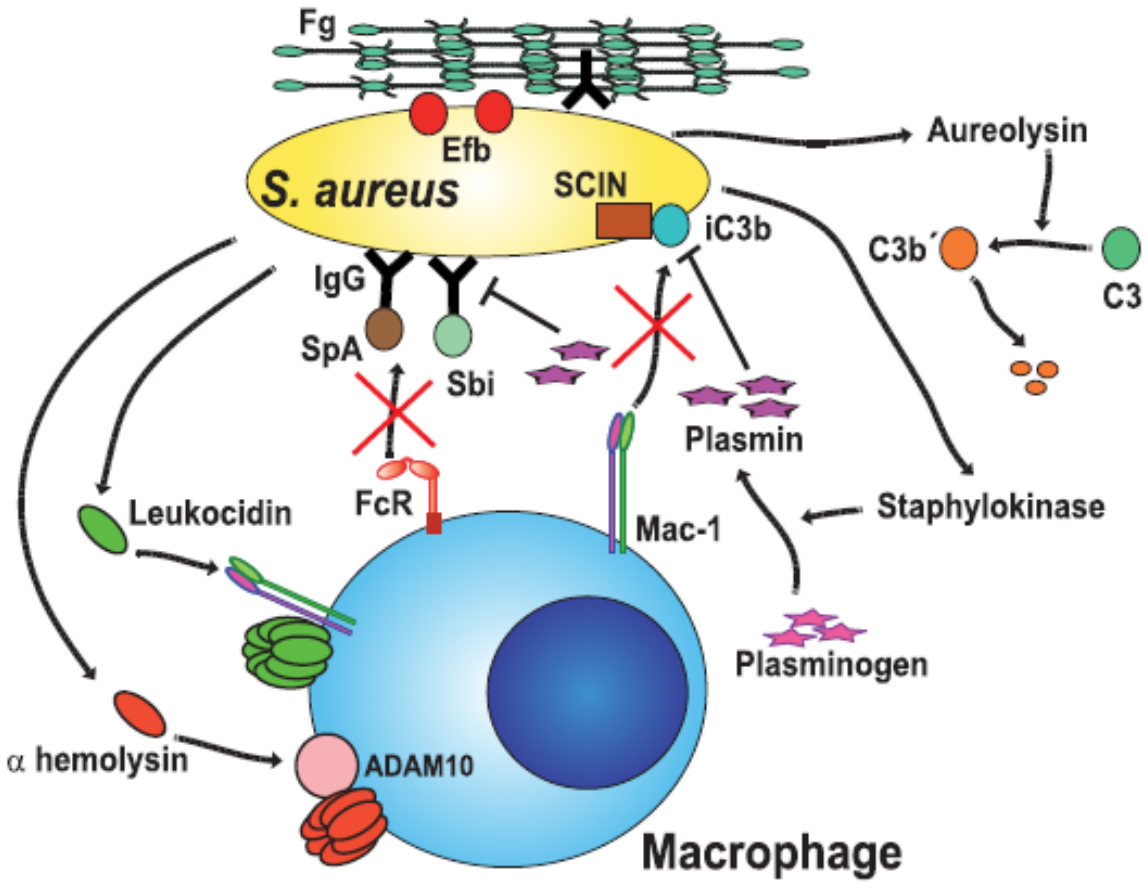

Figure 1. 9: Staphylococcus aureus inhibits opsonic phagocytosis. S. aureus-secreted toxins, leukocidins and α-hemolysin, promote membrane permeability via forming lytic pores on the infected cell membrane. For their activation, leukocidin A, for instance, engages the complement receptor Mac-1, while α-hemolysin recognizes and binds to protein ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10). Staphylokinase catalyzes the conversion of host plasminogen to the active serine protease plasmin, and activated plasmin induce the degradation of IgG or C3b on the bacterial surface. Protein A (SpA) binds IgG (Sbi) protein through the Fc domain of IgG, inhibiting Fc receptor (FcγRs) binding and activation. Aureolysin functions as a C3 convertase, leaving non-functional C3b′ fragments. Complement inhibitor (SCIN) prevents complement activation on the bacteria. Secreted S. aureus effector protein Efb (extracellular fibrinogen binding protein) promotes binding of the host fibrinogen (Fg), creating a proteinaceous shield that masks surface-bound opsonin. Image reproduced from (Uribe-Querol and Rosales, 2017).

1. 4. 5. 3 Resistance to phagosome content

In order for S. aureus to proliferate intracellularly, the bacteria must combat the hostile environment found in the phagolysosome lumen (Flannagan et al., 2015). It has been reported

that S. aureus is immune to the lytic action of lysozyme on the cell wall peptidoglycan (PGN). This bacterial species produces acetylated peptidoglycan which is catalyzed by the enzyme O-acetyltransferase A (OatA) (Bera et al., 2006; Uribe-Querol and Rosales, 2017). Acetylation modification renders PGN resistant to lysozyme (Figure 1.10). S. aureus can also repel host antimicrobial peptide (AMP) attack by the enzyme staphylokinase which directly binds to α-defensins and consequently blocks their antibacterial effect (Figure 1.10) (Jin et al., 2004; Uribe-Querol and Rosales, 2017). Another strategy to resist AMPs is modification of bacterial membrane composition which to reduce the negative surface charge of the membrane. Modification of phosphatidylglycerol with positively charged L-lysine residues along with addition of positively charged D-alanine to teichoic and lipoteichoic acids in the bacterial cell wall reduces the susceptibility of S. aureus to cationic AMPs (Peschel et al., 1999; Peschel et al., 2001; Uribe-Querol and Rosales, 2017). By making the surface less anionic, bacteria become less accessible to the AMPs attack through the diminished electrostatic interactions with the cationic AMPs (Figure 1.10). Furthermore, cathelicidin LL-37, an antimicrobial peptide with robust activity against staphylococci, can also be degraded by the secreted metalloprotease aureolysin (Figure 1.9) (Sieprawska-Lupa et al., 2004; Uribe-Querol and Rosales, 2017). Moreover, phagocytosed S. aureus can afford protection against the extreme acidity of the phagosomal lumen by expressing staphylococcal urease which is used to catalyzes the hydrolysis of urea to produce ammonia (Bore et al., 2007; Uribe-Querol and Rosales, 2017). This results in intraphagosomal pH neutralization (Figure 1.10).

1. 4. 5. 4 S. aureus Resistance to Oxidative and Nitrosative Killing

The oxidative environment of the phagosome has a significant killing potency against most microorganisms (Cosgrove et al., 2007; Ballal and Manna, 2009; Uribe-Querol and Rosales,

2017). In order to maintain viability, S. aureus has evolved strategies to counter ROS and RNS-mediated killing. This can be achieved by producing detoxifying enzymes such as super oxide dismutases, sodA and sodM, and catalase (KatA) which function in the sequential conversion of O_2^- to H_2O_2 to H_2O and O_2, respectively to confer resistance against the microbicidal effects of ROS (Figure 1.10). Another mechanism used by S. aureus to counter the effect of reactive species is the production of antioxidants such as the golden pigment staphyloxanthin (Sx) which gives the bacteria its golden pigmentation and prevents damage caused by peroxide (Figure 1.10) (Liu et al., 2005; Uribe-Querol and Rosales, 2017). Also, the recently characterized SOK protein (surface virulent factor which promotes resistance to oxidative killing), which is produced on the bacterial surface to block the effect of ROS (Malachowa et al., 2011 a). Moreover, ingested S. aureus has also to inhibit the effects of iNOS-derived RNS to promote bacterial surviving and growth (Gonçalves et al., 2006; Kinkel et al., 2013; Uribe-Querol and Rosales, 2017). This can be accomplished by using the two-component system SsrAB which controls the expression of flavohemoglobin that acts as an NO· scavenger (Figure 1.10).

1. 4. 5. 5 Resistance to nutrient capture

The phagosome is a compartment where essential microbial nutrients are sequestered from invading microbes to arrest their growth (Cellier et al., 2007). The divalent cations such as Fe^{2+} and Mn^{2+} are actively exported out of the phagosome. In response to Fe^{2+} depletion, S. aureus has mechanisms by which expression of Fe-acquisition gene is enhanced post phagocytosis (Malachowa et al., 2011 b). One strategy relies on the production of iron-binding molecules, termed siderophores which have high affinity to bind to iron and remove it from host proteins such as hemoglobin, and transferrin (Beasley et al., 2011). S. aureus synthesizes two distinct citrate-based siderophores, staphyloferrin A (SA) and staphyloferrin B (Oldenbroek & van der

Waaij) (Figure 1.10) (Beasley et al., 2009; Cheung et al., 2009; Uribe-Querol and Rosales, 2017).

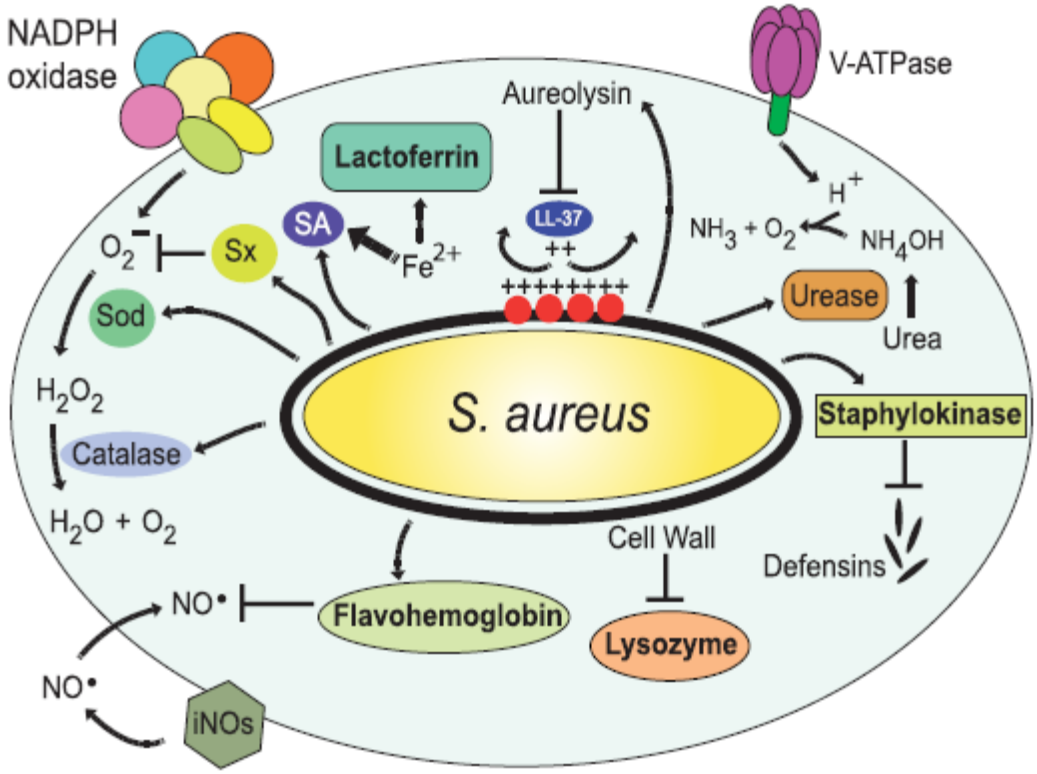

Figure 1.10: Defensive mechanisms implemented by S. aureus to resist bactericidal effects exerted by phagocytes. Resistance to phagosomal killing and host antimicrobial proteins such as lysozyme by modifying the composition of the bacterial cell wall and also altering the composition of its membrane by incorporating L-lysine and lipoteichoic acid to create less anionic surface thereby reducing the electrostatic interaction with cationic antimicrobial peptides, such as the cathelicidin LL-37. Secreted aureolysin and staphylokinase inhibit the action of LL-37 and α-defensins, respectively. The carotenoid pigment staphyloxanthin (Sx) functions as an antioxidant, catalase, and two superoxide dismutases (Sod) that work together to afford protection against ROS attack. Secreted flavohemoglobin works as an NO· scavenger. Urease production catalyzes the formation of ammonia for pH neutralization. Siderophores, such as staphyloferrin A (SA), trap iron to circumvent host-imposed nutrient restriction. Image reproduced from (Uribe-Querol and Rosales, 2017).

2. CHAPTER 2 Molecular identification of bacteria associated with chondronecrosis with osteomyelitis in broilers using litter to wire bacteraemia model to predict BCO predisposition.

2. 1 Introduction

Success in the commercial poultry industry relies to a great extent on reducing the frequency of disease occurrence, and eradication of conditions that are responsible for producing stress to the birds, that consequently result in considerable economic loss (Manohar et al., 2015). The production potential of commercial poultry has showed a dramatic increase through the application of genetic improvement programs which were accompanied with negative consequences (Cheema et al., 2003). Fast-growing broiler flocks display high mortality due to increased susceptibility to infectious or metabolic diseases. Lameness attributable to bacterial chondronecrosis with osteomyelitis (BCO) is a substantial animal welfare issue leading to losses of millions of dollars in the broiler industry annually (Wideman and Prisby, 2013; Al-Rubaye et al., 2017).

A wide range of opportunistic bacteria have been found to be involved in causing BCO in broiler flocks (Nairn and Watson, 1972; Thorp et al., 1993; McNamee et al., 1998; Dinev, 2009). Staphylococcus aureus and Escherichia coli are the most frequently isolated pathogens from BCO lesions. Also, they were reported to be isolated mostly in mixed culture with other bacteria such as Salmonella spp. In order to investigate the specific bacterial strains that we are able to culture and identify, more reliable species identification of these pathogens needs to be developed (Schmidt et al., 2018). Phenotypic differentiation requires a large number of biochemical tests which lack accuracy, are time consuming and can be expensive. The development of DNA-based approaches for the classification and differentiation of bacterial species have been applied as a high discriminatory tool with a significant reproducibility and

precision than traditional phenotypic approaches. DNA sequencing has been applied as a common facility in research projects due to its increased availability and relatively reduced cost (Hjelmsø et al., 2014). However, the costs of the combined analysis of nucleotide sequencing and the following bioinformatics still represent an excessive expense in many research budgets especially if they are used to identify numerous isolates. Therefore, an affordable and efficient preliminary screening of samples should precede sequencing to differentiate samples and thus potentially minimize expenses by concentrating the efforts on the most interesting samples. Gel-based methods have been extensively applied to investigate diversity changes in complex environmental samples (Muyzer et al., 1993; Siqueira et al., 2017). For example, terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE) were previously used for this purpose but they require considerable technical experience, and they are not yet economically viable. Therefore, we investigated using high-resolution melting (HRM)-analysis as a rapid, reliable, and cost-effective tool for the detection and identification of bacterial isolates from different BCO lesions.

HRM analysis has been used as an alternative tool for the identification of bacteria, viruses, nematodes, and fungi by combining real time PCR assay with HRM analysis (Ren et al., 2017). HRM analysis of the 16S rRNA gene, in addition to other highly conserved housekeeping genes has been successfully utilized to identify microbial isolates such as Staphylococcus, Mycoplasma, Lactobacillus, Salmonella, Brucella, and Listeria to the species or strain level (Miller et al., 2015).

BCO samples were obtained from broilers reared on wire flooring system at the University of Arkansas research farm. Broilers reared in cages with wire flooring have shown higher incidence of lameness when compared to those that were reared on litter (Wideman, 2016). Therefore, this

wire flooring system was used to reliably induce BCO through sustained footing instability which imposes excessive pressure/stress on the susceptible leg joints. This leads to the formation of osteochondrotic micro-fractures within the growth plates of the mechanically fragile proximal heads of the femora and tibiae as well as thoracic vertebrae. This sustained physiological stress and barrier function failure facilitate bacterial translocation into the bloodstream (Wideman et al., 2012). Translocated bacteria then persist in the circulation causing bacteraemia, apparently, due to immunosuppression or a compromised innate immune system. Bacteria can exit the bloodstream via the fenestrated endothelium and accumulate in osteochondrotic clefts or wound sites within growth plates that are subjected to excessive torque and shear stress (Emslie and Nade, 1983; Wideman et al., 2012). Bacteraemia appears to be essential for BCO development and progression and promoting broilers' resistance to bacteraemia certainly will improve productive performance and aid mitigating the systemic bacterial infection. Strategies must be elaborated to identify broilers that are resistant to bacteraemia. We hypothesized that, when exposed to suitable challenge, BCO-susceptible broilers will develop a significant bacteraemia while BCO-resistant broilers will not.

As a consequence of housing broilers on wire flooring, bacterial translocation and bacteraemia are enhanced due to the elevated stress hormone levels leading to chronic immunosuppression (Wideman and Prisby, 2013). Therefore, this study was designed to evaluate the incidence or intensity of bacteraemia preceding or following the transfer of the challenged broilers from litter to wire flooring. In the present study, broilers were challenged by rearing them on, or transferring them to, a flat wire flooring system, which consistently induces significant incidence of BCO even without exposing the challenged broilers to known pathogens.

This chapter includes two conducted studies. The first study developed a qPCR-HRM analysis technique for rapid, high throughput post-PCR analysis to detect and discriminate different bacterial species recovered from BCO lesions by amplifying different variable regions of 16S rRNA gene and using different fluorescent dyes for best analysis of produced melt curves of the different bacterial isolates. In the second study, we used litter to wire challenge model to (i) investigate the relationship between susceptibility to bacteraemia and susceptibility to BCO, (ii) compare the magnitude and persistence of the bacteraemic response and subsequent lameness, (iii) identify the major bacterial groups in blood and BCO lesions that are uniquely associated with BCO lameness by the developed PCR-HRM curve assay.

2. 2 Materials and Methods:

2. 2. 1 Development of qPCR-HRM as a discriminatory tool for identification of the major species recovered from blood and BCO lesion of the lame bird.

2. 2. 1. 1 Bacterial DNA samples

The DNAs of previously diagnosed bacterial samples, stored at -20° C, were used as templates in this study to develop qPCR-HRM curve analysis for more distinctive discrimination of the major Staphylococcus spp. strains isolated from blood and BCO lesions.

2. 2. 1. 2 Amplification of 16S rRNA using qPCR-HRM curve analysis

Twenty microliter PCR reaction were performed using 96 well plate with the CFX thermal cycler (BioRad, Hercules, CA). The PCR mixture included 2 µl of 10X buffer, 1 µl of 10x fluorescent dye SYBR Green (Lonza Group Ltd, Basel, Switzerland), EvaGreen (Biotium Inc., Fremont, CA, or LCGreen (BioFire Defense, Murray, UT), 0.2 µl of 20 mM of $MgCl_2$, 0.2 µl of 20 mM dNTP, 0.2 µl of 20U Taq polymerase, 0.4 µl of 50µM forward and reverse primers, and 1 µl of bacterial DNA. The cycling conditions were as follows: initial denaturing at 90 °C for 30 s, 5

cycles of denaturation at 90 °C for 15 s, annealing at 71.5° C for 15 s, and extension at 72 °C for 60 s, followed by 30 cycles using the same parameters with a plate read after each cycle. Afterwards, High Resolution Melt followed consisting of 72° C for 180 s, then samples were heated to 95 °C for 60 s, and cooled down to 65 °C for 180 s, followed by measuring fluorescence during temperature increase from 75 °C to 90 °C with 0.1 °C/ 5 s increments and plate read. The reproducibility of the assay was verified for the reference bacterial strain by determining the differences in melt profiles of the extracted DNAs from two independent experiments of the same tested species. The obtained melt curves were analyzed with Bio-Rad CFX manager software. To exclude contamination in the reaction mixture, a negative control with no DNA template was added to each experiment.

2. 2. 2 Litter to wire bacteraemia model for predicting BCO susceptibility

2. 2. 2. 1 Animal Housing and Care:

Animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol #11002). The experiment was conducted using 24 pens at the University of Arkansas Poultry Research Farm. Twelve pens were set up with clean wood shaving litter flooring and twelve pens were set up with flat wire flooring (Wideman et al., 2012). This experiment was designed to have three treatment groups of chicks. Chicks raised on litter and continued on litter for 56 days are designated (L1-56). Chicks raised on wire and continued on wire for 56 days are designated (W1-56). Chicks started on litter until day 35 are designated (L1-35), and on day 35 they were transferred to wire flooring pens. In each pen, tube feeders were positioned at the front, and nipple waterers were placed at the back of the pen to force chicks to cross the length of the floor to eat and drink and thereby to induce lameness as suggested by (Wideman et al., 2012).

2. 2. 2. 2 Clinical and post-mortem diagnosis

Beginning at day 15, all birds were observed to detect the onset of lameness by walking them around inside each pen daily. Affected birds had difficulty standing, showed an obvious limping gait while dipping one or both wing tips, and if not removed from the flock, they became completely immobilized within 48h. Clinically lame birds were humanely euthanized with CO_2 gas or by cervical dislocation, and post-mortem lameness diagnosis was conducted by Dr. Adnan Alrubaye/ University of Arkansas. Lame birds with BCO die quickly because they have difficulty accessing water and food. Therefore, all birds that were found dead or that who had developed clinical lameness also were necropsied to determine the cause of death and assess BCO lesions distributions. During necropsies proximal femoral and tibial head BCO lesions were assessed and the birds were assigned to one of the diagnostic categories as illustrated by (Wideman et al., 2012; Wideman and Pevzner, 2012). Only birds that showed specific lesions were reported as lame. The percentage of cumulative lameness per pen was calculated beginning on d 15 as (total lame from pen) / (number of birds in each pen). The percentage of cumulative lameness for each treatment group was determined from d 15 as (total lame from a treatment)/ (total birds in each treatment group).

2. 2. 2. 3 Sample collection:

Blood samples (1 ml) were collected aseptically from a wing vein using EDTA vacutainers. Blood samples were collected from five apparently healthy birds per pen on days 14, 31, 41, and 49. Additional blood samples were collected from birds succumbing to BCO, and from lame and healthy birds on day 56. The euthanized birds were necropsied, and all dissection utensils were sterilized by placing them in 95% alcohol. Before making the incision, the external skin was sterilized with 95% alcohol. The initial visual diagnosis of the BCO lesions was recorded. Then,

proximal femoral and tibial heads were collected and transferred into sterile plastic tubes. Bone and blood sample tubes were placed on ice and then transported to Dr. Douglas Rhoads' laboratory in the Daniel Ferritor building, on the campus of the University of Arkansas.

2. 2. 2. 4 Bacterial media preparations and culturing of viable bacteria

Preliminary microbiological investigation for bone and blood samples involved culturing the collected samples on chromogenic media both differential, and selective agar plates. The media used included CHROMagar Orientation, and CHROMagar Staphylococcus (DRG International Inc, Springfield, NJ). CHROMagar Orientation is highly diagnostic and used to obtain a large spectrum of differentiation between bacterial species based on the colony color patterns. CHROMagar Staphylococcus was used for isolation and direct differentiation of Staphylococcus spp. Media were prepared according to the instructions of the manufacturer, sterilized, if needed, by autoclave under (121 °C for 20 min). Solidified plates were stored at 4 °C.

Blood samples (0.1 ml) were spread on both CHROMagar Orientation, and CHROMagar staphylococcus media. For bone samples, a sterile cotton swab was used to rub the bone lesions then streaked onto the above-mentioned media. Plates were incubated for 24 h at 37 °C, and growth assessed for number of colonies and colony color(s).

2. 2. 2. 5 DNA extraction

Individual colonies were sampled with a sterile toothpick into 50 µl of sterile water in 200 µl PCR plates, sealed and lysed by heating to 100 °C for 10 minutes, and then spun for 1-2 minutes then subjected to qPCR-HRM assay.

2. 2. 2. 6 Amplification of 16S rRNA using qPCR-HRM curve analysis

For bacterial species screening, DNAs extracted from blood and BCO lesion samples were used as a template to amplify the V2 region of the16S rRNA gene product using custom primers: Bac16Sv2 F 5′-GCGRACGGSTGAGTAACACGTG-3′ and Bac16Sv2 R 5′-CGATCRCCCTYTCAGGTCGGCT-3′. PCR master mixture preparations, amplification conditions, and HRM analysis were achieved as previously described using EvaGreen as the fluorescent dye. This assay was used for rapid and reliable detection of the prominent bacterial species which was found to be associated with BCO lameness in our poultry research farm using wire flooring system. Staphylococcus agnetis 908 strain was reported as the main bacterial species from BCO lesions. Therefore, a positive control of S. agnetis (908) DNA (2 ng/ µl) was added to each reaction plate in addition to the negative control with no DNA which was added to eliminate any possible contamination.

2. 2. 2. 7 Polymerase Chain Reaction and 16S rDNA sequencing

Isolates that were not identified as S. agnetis based on colony color or qPCR-HRM were identified to species level by PCR sequencing of the entire 16S rRNA V1-V5 regions using previously published broad-range bacterial primers, Bact-8F (5'-AGAGTTTGATCCTGGCTC AG-3') and Bact-936 R (5'-GTGCGGGCCCCCGTCAATTC-3') (Baker et al., 2003). Forty microliter PCR mixture was conducted using 96 well plates with Eppendorf Mastercycler gradient using the following: 4 µl of 10X Taq buffer, 0.4 µl of 20 Mm of $MgCl_2$, 0.4 µl of 20 Mm dNTP, 0.8 µl of 50µM forward and reverse primers, 0.4 µl of 20U Taq polymerase, 2 µl of bacterial DNA, and bring to the volume by adding diH2O. The following PCR conditions were applied: 90 °C for the initial denaturation for 30seconds followed by 30 cycles of 90 °C denaturation for 15 seconds, 60 °C annealing for 30 seconds, 72 °C elongation for 90 seconds,

and final elongation for 3 minutes at 72°C. Samples of the PCR products (10 µl) were resolved on standard 1.5% agarose gels to evaluate the quality of the PCR. Gels were visualized using a Typhoon FLA9500 (GE Health Care), and gel images were analyzed using ImageQuant software.

PCR products were cleaned using RapidTips (Diffinity Genomics, West Chester, Pennsylvania), and quantified using a TKO 100 Fluorometer (Hoefer, Rochester, NY). The cleaned product was mixed with the Bact-8F primer, and submitted for sequencing (Eurofins Genomics Company, Louisville, KY). Sequences were aligned using SeqMan Pro (DNASTAR, Madison, WI), and then compared with reference 16S rDNAs using the NCBI blast tool, and Ribosomal Database Project (RDP) sequence match tool to identify bacterial species for each sample.

2. 3 Results

2. 3. 1 Development of qPCR-HRM curve analysis

For development of a rapid qPCR-HRM assay we targeted different variable regions of the 16S rRNA, V2, V3 and V4, for amplification. We also tested three different DNA-incorporating fluorescent dyes for the quality of the HRM curves. These modifications were applied to see if we can obtain specific melt profile which is diagnostic to the species level. In the amplification reactions, three different sets of primers were added to each reaction tube. Bac16Sv2F+R. Bac16Sv3F+R, and Bac16Sv4F+R were used to amplify V2, V3, and V4 regions, respectively (Table 2.1). Also, we extended the targeted PCR fragment by making a combination of Bac16Sv2F+ Bac16Sv3R.We evaluated the suitability of this assay to discriminate between different bacterial species by testing different bacterial strains obtained from BCO lesions at our poultry research farm as reference isolates. The reference bacterial species included 14 isolates identified as Staphylococcus agnetis, 2 were Staphylococcus hyicus, 4 were Staphylococcus

xylosus, and 2 were Staphylococcus aureus. These isolates were previously identified to species level by sequencing the 16S rDNA gene (Al-Rubaye, 2013). Three different fluorescent dyes including SYBRGreen (SG), EvaGreen (EG), and LCGreen (LCG) were used to analyze the distinct HRM profile of each reference isolate. The results of comparing the DNA-binding profiles of the three dyes showed that both EG, and LCG gave more precise examination of the melting profile than SYBRGreen does, with EvaGreen showing even more distinctive diagnostic profile (Figure 2.1). In this comparative study, all three dyes do not display interference with the PCR at the concentration we used. The DNAs of reference bacteria were all amplified, and we got distinctive melt curve profiles with each intercalating dye. The melting profiles of all isolates resulted in a single peak representing one specific bacterial species. With using SG, 86% of the identified bacteria matched those in the reference isolates' data sheet. While with using both EG and LCG dyes, we got 91% match with reference species. The results of HRM analysis came out as 16 isolates of S. agnetis, 3 isolates of S. xylosus, and 2 were S. aureus based on using SG. While with using both EG and LCG, all results matched the reference except for identification of the 2 isolates of S. hyicus which was grouped with the closely related S. agnetis isolates.

Moreover, besides amplifying the V2 region of the 16S rDNA gene, we also amplified the V3, and V4 regions. We expanded the amplified fragment by using two primer combination sets between all three variable regions in order to investigate whether we can get more distinctive melt behavior to get 100% match with the reference data or prove that V2 is the best option to differentiate between different species of Staphylococcus. Previously published universal primers (Table 2.1) were used to amplify the V2, and V3 regions, and a combination between V2 and V3 were also used. The results of this comparative study showed that neither V3 nor V4 amplification was successful to precisely discriminate between the different Staphylococcus spp.

(Figure 2.1). In the case of using Bac16S V3F+R primer to amplify the V3 region, the amplification reaction was successful, but the melting profile was not as distinctive as the one produced by amplifying the V2 region. On the other hand, using Bac16Sv4 F+R primer failed to amplify the reference DNAs of the different Staphylococcus sp. (Figure 2.1). Combining V2 and V3 regions by using Bac16Sv2F+v3R amplified the reference DNAs, but this combined region was not specific to differentiate between the four different Staphylococcus sp. and the melt behavior of these isolates was shifted towards higher melting temperatures as compared to the amplification and melt profile of the V2 region (Figure 2.2 and 2.3). Therefore, using Bac16Sv2F+R primer to amplify the V2 region was the most reliable assay to specifically distinguish between Staphylococcus sp.

Table 2.1: All PCR primer pairs with their name, sequences, Forward (F), Reverse R

Primers	Sequence
Bac16Sv2 F	5′-GCGRACGGSTGAGTAACACGTG-3′
Bac16Sv2 R	5′-CGATCRCCCTYTCAGGTCGGCT-3′
Bac16Sv3 F	5′-GAGCAACGCCGCGTGAGTGA-3′
Bac16Sv3 R	5′-CCTACGTATTACCGCGGCTGCT-3′
Bac16Sv4 F	5′-AYTGGGYDTAAAGNG-3′
Bac16Sv4 R	5′-TACNVGGGTATCTAATCC-3′

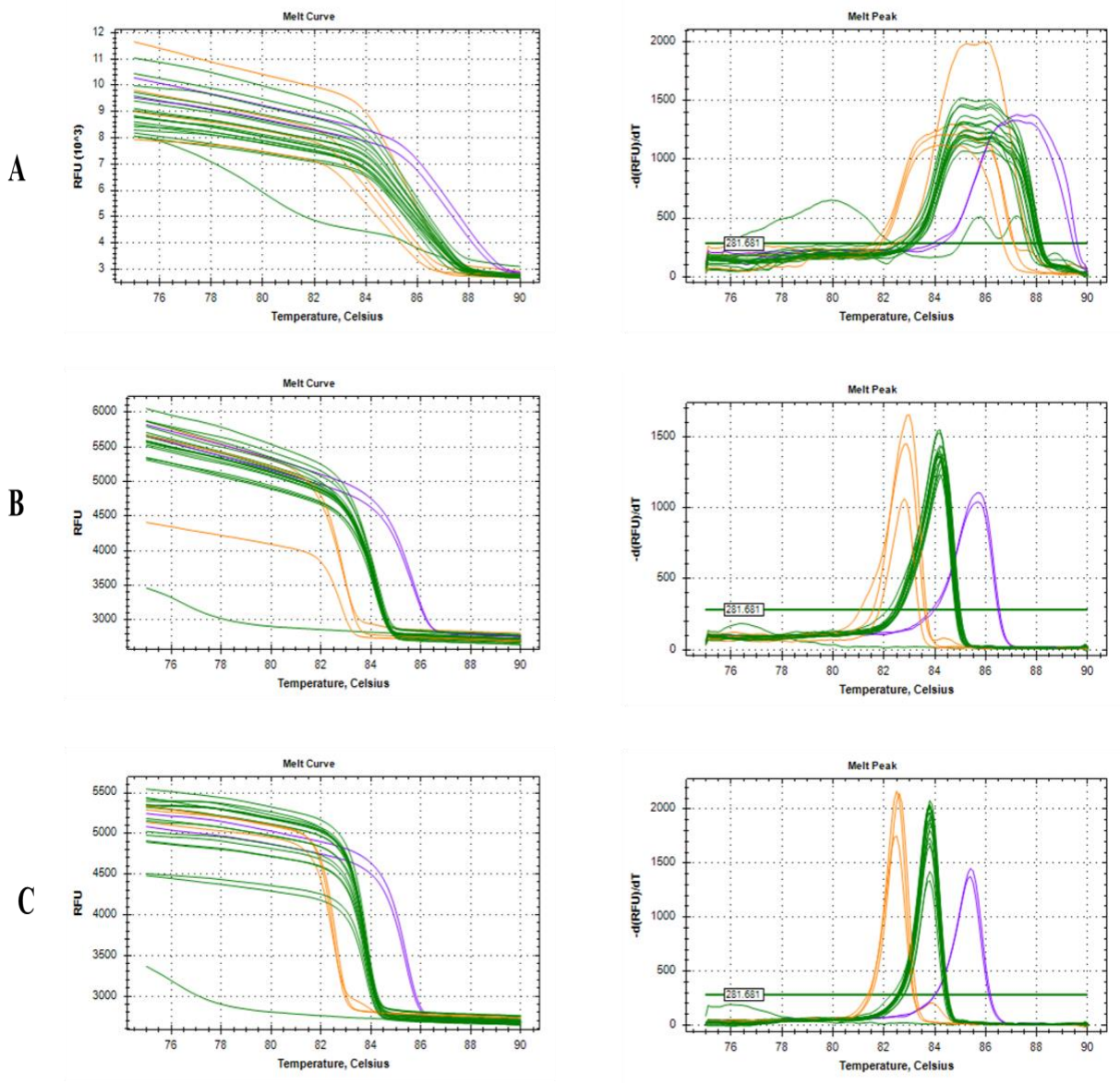

Figure 2.1: Comparative analysis of high resolution melting curves of reference DNA samples (n = 22), amplifying the V2 region of the 16S rDNA using three different fluorescent dyes. (A) using SG in the amplification reaction. (B) LCG, and (C) EG. Staphylococcal species. S. xylosus (orange), S. agnetis (green), and S. aureus (purple).

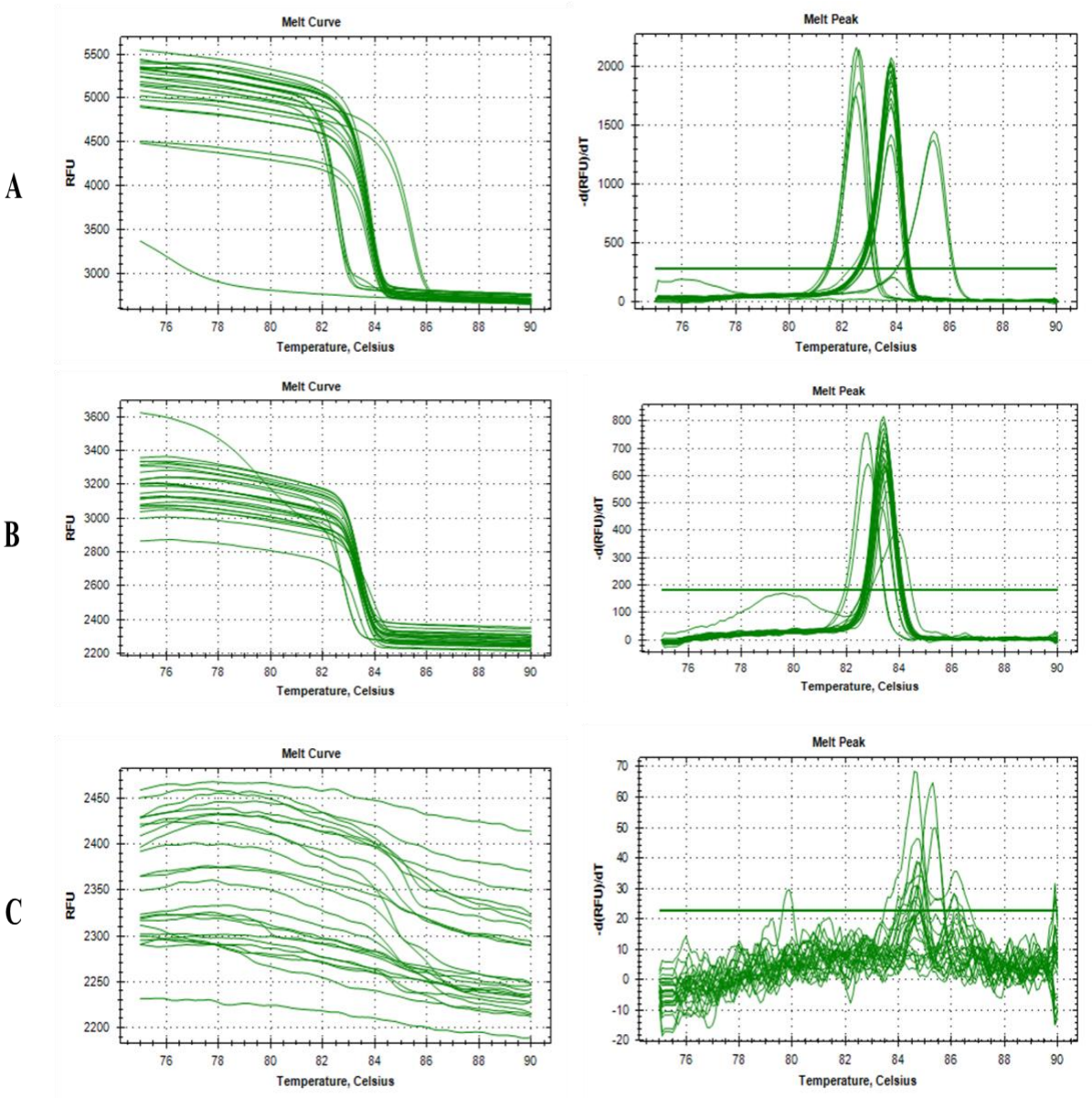

Figure 2.2: Comparative analysis of high resolution melting curves of reference DNA samples (n = 22) by amplifying three different variable regions of the 16S rDNA gene. (A) amplification of V2 region. (B) amplification of V3 region. (C) amplification of V4 region.

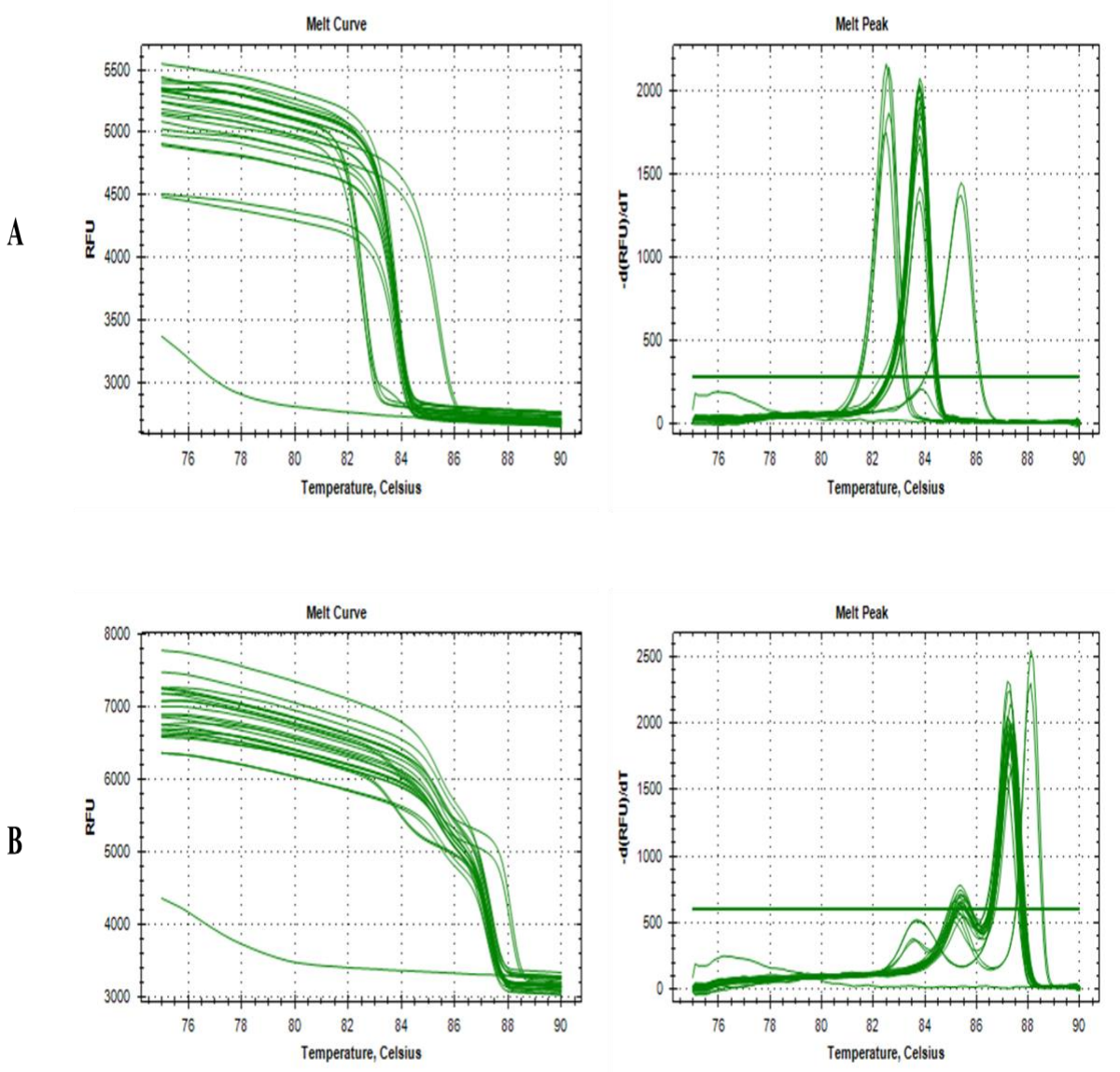

Figure 2.3: Comparative analysis of high resolution melting curves of reference DNA samples (n = 22) by amplifying two different variable regions of the 16S rDNA gene. (A) represents the amplification of V2 region. (B) amplification of the combined region of V2-V3. Results showed that V2 region amplification is more distinctive to distinguish between the different Staphylococcal sp.

2. 3. 2 Assessment of bacteraemia and bacterial species identification using litter to wire model to evaluate BCO susceptibility and subsequent lameness:

It has been proposed that using wire flooring system would successfully induce lameness attributable to BCO (Wideman, 2016; Al-Rubaye et al., 2013). Raising broilers on wire flooring was reported to result in bacterial translocation into bloodstream, and subsequent bacterial colonization of the growth plate of the susceptible, fast growing proximal femora and tibiae. Therefore, we designed this study to evaluate whether we could detect and identify particular bacterial species in the blood at different time course for broilers reared from d1-d56 either on litter (L1-56), on wire (W1-56), or those that reared on litter through d35 and then transitioned to wire flooring through d56 (L35W).

Beginning at d1, each pen has 80 birds, and they were culled to 60 per pen on d14. Between d14 and d56, the birds were monitored to investigate any signs of lameness. Postmortem diagnosis was conducted to investigate lame and non-lame mortality causes in the dead birds. Accordingly, total lameness exclusively attributable to BCO, with cumulative BCO incidences through d 56 was totaling 2.5%, 59.2% and 44.0% for the Litter, Wire, and Litter-to-Wire floor treatment groups, respectively (Table 2.2). The BCO incidences for individual pens are shown in (Figure 2.4). These incidences were reasonably consistent within each floor treatment group, with the obvious exception of the markedly low incidence in pen 23 when compared with the other pens in the same treatment group (Litter-to-Wire). Figure 2.5 illustrates the time course of cumulative percent lameness for broilers in the Litter, Wire, and Litter-to-Wire floor treatment groups (all pens combined per treatment group). The results showed that BCO levels were significantly developed after day 44 in the Wire and Litter-to-Wire groups. Beginning on d49 through d56, the percentage of BCO lameness was significantly higher in the Wire flooring treatment group

(71%) than in the Litter-to-Wire group (52%), and both of those groups had consistently higher percentage of lameness relative to that on litter flooring group (2.5%) (Figure 2.5). Blood samples were taken aseptically from apparently healthy birds and any lame birds found on days 14, 30, 41, and 49. Five birds randomly selected from each of four pens for both litter and wire treatment group, and eight pens for litter to wire treatment group as summarized in Table 2.3. These collected birds were marked so that they were sampled for only one-time point. The blood (0.1ml) was plated onto CHROMagar media for total counts and colony color so that we can differentiate between the different bacterial species based on the colony color profile of each species (Figure 2.6). Colonies were initially tested using our developed qPCR-HRM assay where we can distinguish the specific melt curve for S. agnetis as this bacterial species was reported to be the predominant isolate recovered from blood and BCO lesions from clinically healthy and lame birds (Al-Rubaye, 2013, Al-Rubaye et al., 2015). The average number of colonies, as well as the percent of samples positive for S. agnetis and percent of samples positive for isolates other than S. agnetis were plotted (Figure 2.7). For healthy birds, results showed that there were few detectable bacteria through d41. However, the colony counts in blood increased in all three treatment groups by d49 with the average colony counts for W56 and L35W nearly 10 times that of L56 treatment group. Furthermore, none of the collected samples had S. agnetis until d41 in any treatment and then only detected in the W56 samples. At d49, results showed that half of the apparently healthy birds were positive for bacteria in their blood, and the percentage of species distribution for both wire treatments (L35W and W56) was approximately equal for being positive for S. agnetis and non-agnetis species, while 70% of the 20 tested birds from L56 produced few detectable bacteria in their blood and were all identified as non-agnetis. The same analyses were applied to lame birds sampled at d14 through d 56. There were no

detectable signs of lameness until beginning on d41 as summarized in Figure 2.8. In general, the average colony counts were higher than that of the healthy birds. Also, the results revealed that there were more colonies in L35W blood samples than either other treatment groups. As this compared to the results we obtained for healthy birds (Figure 2.7), we also found that L35W blood samples had higher average colony counts than W56 samples. This suggests that litter to wire treatment may contribute to earlier bacteraemia in the blood of susceptible birds. By d49, the reported average colony counts of both L35W and W56 treatment group increases. There was only one lame bird in L56 with no detectable colonies in its blood. On d56, the colony counts for L56 started to increase while the colony counts for both wire treatments stayed elevated. Although the L35W sample colony average dropped from 117 on d 49 to 57 on d 56, whether this is statistically significant would require a much larger sample set.

The distribution of the identified species (agnetis vs non-agnetis) for all treatment groups showed that S. agnetis is the main species isolated from the blood samples of the lame birds in W56. For the lame birds in L35W, the blood analysis showed a presence of bacterial species other than S. agnetis at d41, but later on at d49 through d 56, the majority of lame birds have S. agnetis as the predominant species circulating in their blood. Even though we have only 7 lame birds in L56 treatment group, three blood samples were positive for S. agnetis and one was identified as non-agnetis at d56 with average colony count of 11.7 which is much less when compared to those of 57.3 for L35W and 94.5 for W56.

Moreover, colony color profile of different bacterial isolates on CHROMagar media also support the evolution of bacterial species in the circulation of the tested birds, and enhance our view of the differences between the litter and wire treatment groups as represented by Figure 2.9. The results revealed that in all three treatment groups, the main observed colony color pattern was

pink to purple at d 30. However, the total number of blood samples that were positive for bacteria and number of colonies was low on d 30 as illustrated in Figure 2.7. By d41, we observed an increase in the number of blue/ green and white colonies recovered from blood of the challenged birds on wire treatment while the pink/ purple colonies still recorded high in the birds on litter. For d49, the number of white colonies continue to increase as compared to the blue/ green colonies which drop back to lower numbers in the birds on wire group. At the same time the pink/ purple colonies were still high in the samples of the birds on litter. On d56, we sampled only the lame birds in all three treatment groups and the results showed that almost all of the recovered colonies were white in all of them.

We used the qPCR-HRM curve analysis to verify that S. agnetis was the main bacterial species for the white colonies on CHROMagar Orientation. Based on the sequence results of the 16S rDNA gene, pink/ purple color was determined to represent other Staphylococcus spp. Including S. lugdunensis, S. haemolyticus, and S. saprophyticus. The blue / green colonies were identified as S. pseudintermedius. Mixed colony colors were also identified when we sampled blood from healthy and lame birds. We also encountered two isolates of E. coli with pink colony colors which can be differentiated from the other not-agnetis Staphylococcus sp. with the same colony colors by plating them on CHROMagar Staphylococcus medium that selectively permits the growth of only Staphylococcus sp. We also detected some unusual bacterial species in a few blood samples from healthy birds. Delftia tsuruhatensis was the best match for three samples from d30, two from birds on litter and one from wire. This species has been found to be associated with rare cases of sepsis and biofilms on catheters in human nosocomial infections. Variovorax paradoxus was the best match for one mixed colony type sample from L35W on d41, and one sample producing 15 colonies on d49 from W56. This is not reported as a pathogen and

is associated with biodegradative processes. Both of these species are Gram negative members of the Comamonadaceae. We had one sample at d49 from L35W that produced 14 pink colonies where analyses showed a best sequence match with Acinetobacter calcoaceticus. This species is a soil-borne, Gram negative that is reported as normal human intestinal flora. However, these latter bacterial species were rarely seen and comprise only few colonies and could represent contaminations while sampling in less than ideal conditions.

2. 4 Discussion:

The first study was designed to develop a fast and reliable molecular diagnostic tool for the detection and identification of the major bacterial species recovered from blood and BCO lesions from the affected birds. Once colonies are identified the results can be available in approximately two hours. The results of the comparative analysis of the of the high resolution melting (HRM) profiles of the different Staphylococcal species by using three different DNA-binding dyes have shown that using EvaGreen (EG) and LCGreen (LCG) in the amplification reaction were the most suitable dye for HRM curve analysis when compared to SYBRGreen I, with EG being more distinctive (higher and sharper overlapped peaks).

Using proper DNA-binding dye is a critical factor in the HRM analysis because it provides detailed information on the melting behavior of the amplified target region. We used three different classes of dsDNA-binding fluorescent dyes and compared them based on the best diagnostic melt profile. We did not get ideal performance when we used SG. This dsDNA intercalating dye belongs to non-saturating dye class (Varga and James, 2006; Eischeid, 2011). Although it has a very strong fluorescent signal, it is less preferable to be used in the HRM analysis. Using lower concentration of this dye results in complicated analysis of melt curve profiles because of the dye's effect on melting temperature (Tm) and the dye's redistribution

which takes place during the melting process. Accordingly, lower reproducibility and poor base-difference discrimination are mostly associated with using SG in low concentration (Giglio et al., 2003). Adding high concentration of SG leads to DNA stabilization and this results in artificially shifting the Tm or inhibiting the PCR reaction. Also, SG shows sequence binding preference when compared to the other intercalating fluorescent dyes.

Both LCG and EG performed better than SG. LCG belongs to the saturating class of dyes. As compared to SG, LCG gives more accurate results because when added to the target DNA, complete intercalation between the dye and the amplicon occurs. Therefore, this dye cannot redistribute during melting since the dsDNA is saturated. The best results were obtained with adding EG to the amplification reaction. EG belongs to the release-on-demand class of dyes (Sang and Ren, 2006; Mao et al., 2007). The highly sensitive HRM analysis that we got using EG suggests that this intercalating dye is more stable and reliable, and this may be related to its unique mechanism of action. This dye is essentially not fluorescent (inactive) by itself or when it is free in the solution. Upon binding to dsDNA, the dye becomes active and emits high fluorescent signal. Therefore, this dye may be applied at a relatively high concentration in the qPCR to exclude the problem of dye redistribution and also to maximize the fluorescence signal which provides strong and sharp DNA melt peaks so that it is less likely to cause nonspecific amplification. In addition, EG has excitation and emission spectra very close to those of SG and fluorescein (FAM) which makes it compatible with existing instruments. EG is a safe dye because it is nonmutagenic, nontoxic by being completely impermeable to cell membrane unlike SG which is highly mutagenic because of its ability to enter cells and enhance mutations. These features make EG a promising dye for qPCR and other related application.

In this study we amplified different variable regions of the 16S rRNA gene (V2, V3, and V4), and a combination of V2 and V3 to see which variable region(s) can be used as a best amplification target to distinguish between different Staphylococcal species. The result of amplifying V2 region has shown that this region was the best diagnostic target for species-specific identification when compared to the other aforementioned regions. This suggests that V2 has a considerable sequence diversity among different Staphylococcal sp. In specific words, V2 contains the maximum SNP variations amongst these species except for distinguishing between S. agnetis and S. hyicus. It was reported that S. hyicus is closely related species to S. agnetis. S. hyicus is the causative agent of swine exudative epidermitis, and in birds it is considered a commensal organism of the skin and a secondary pathogen in the preexisting dermatoses. It was found to be associated with folliculitis and dermatitis in a line of white leghorn laying chickens (Chénier and Lallier, 2012). Moreover, both S. agnetis and S. hyicus were found to be associated with subclinical and mild clinical mastitis in cows and isolated from bovine milk. Because of their similarities they can be difficult to differentiate (Adkins et al., 2018).

According to Chakravorty et al., (2007), V2 has a length of 106 nucleotides (nucleotides 137-242) which gives a relatively larger target region to be amplified with universal primers specific to the conserved flanking sequences. While V3 has a length of 65 nucleotides (nucleotides 433-497) which gives shorter DNA sequences than V2. Therefore, we thought that using shorter DNA sequences as an amplification target for species identification may be more conclusive so that we can differentiate between S. agnetis and S. hyicus. On the other hand, amplification of V4 (nucleotides 576-682) was less suitable for species identification which suggests that this region has higher degree of sequence conservation when compared to V2 and V3. We also amplified larger target region by combining V2 and V3 to provide sufficient sequence diversity

to identify all the four types of Staphylococcus to species levels. The results indicate that species-level discrimination was able to differentiate all the four reference bacterial species, but they were not matching the sequencing results of these references DNA. According to the melt profile some of the S. agnetis melting curves were overlapping with S. xylosus which makes this assay less sensitive. The melt profile of the three identified reference species by amplifying this region was shifted towards a higher Tm when compared to Tm of V2 amplification. This indicates that amplifying larger target sequences may include higher GC content and this requires higher temperatures to convert dsDNA into ssDNA during the melting process.

The results of the second experiment confirms that raising young birds on suspended wire flooring has been proved to successfully induce lameness attributable to BCO as reported (Wideman et al., 2012, 2013, 2014, Wideman and Prisby, 2013; Al-Rubaye et al., 2015). This proposed model suggests that flooring instability results in microfractures in the growth plates of rapidly dividing leg bones which consequently end up with being colonized by hematogenous bacteria. Bacterial colonization is facilitated due to the increased bacterial leakage from their main route of entry which can be either the gut or respiratory system into the circulation of the stressed wire-reared birds. It has been previously reported that S. agnetis is the main bacterial species which is found to be associated with BCO lameness in the wire-reared birds at our poultry research farm. The results of this experiment showed that the stressed apparently healthy birds that were raised on wire flooring model are susceptible to bacteraemia, with few detectable levels of bacteria in their blood at day 14 through day 49. Furthermore, birds raised on litter flooring have significantly lower bacterial levels in their blood than those of the birds raised on wire. Moreover, our data revealed that the initial bacterial population composed of primarily Staphylococcus species with a few other genera detected on some occasions. The number of

viable bacteria increased with age of susceptible birds. By d49, we were able to culture bacteria from blood samples from 70% of apparently healthy litter-reared birds. However, the number of colony counts was low. Healthy birds on both wire treatments (W56 and L35W) showed an equal overall frequency of blood being positive for viable bacteria with average colony counts of 10 folds higher. The signs of BCO lameness did not develop until after 44 days of age in the wire group when birds gain more body weight with the sustainable footing instability that create insufficiently strong legs to support their heavy bodies.

Bacterial species distribution at d49 begins to shift and by d56 we primarily detected S. agnetis. This bacterial species was rarely detected in healthy birds until d49 and was the predominant species in the circulation of the lame birds at d56. These results suggest that S. agnetis has a competitive advantage in both colonization and immune evasion. This bacterial strain is well-equipped for the attachment to the exposed bone tissues and leaking capillary system which is formed within the growth plates of the susceptible wire-reared birds. This adaptation is based on the surface receptors on the bacterium since S. agnetis targets the damaged regions and colonizes the damaged tissues by forming microcolonies and then biofilms which permit for immune avoidance while leading to necrosis of the surrounding tissues of the affected bones. At later stages of the disease, S. agnetis is released in large quantities from the necrotizing tissues into the bloodstream again and this explain why lameness is chronic and persistent disease. This proposed pathogenesis model resembles the one used by S. aureus associated with osteomyelitis in human.

Table 2.2: Necropsy categories for broilers that died or became lame in the litter flooring, wire flooring, or litter to wire flooring treatment groups: incidences (% of number beginning on day 14) within each diagnostic category[1] for male broilers.

Floor Treatment[2]	PHS %	SDS %	KB %	TW %	TD %	LAME UNK %	ALL BCO Lame %	Total Lame %
Litter	0	0.8 (2/240)	0	0	0	0	2.5 (6/240)[c]	2.5 (6/240)[c]
Wire	0	1.3 (3/240)	0	0	0	0	59.2 (142/240)[a]	59.2 (142/240)[a]
Litter to Wire	0	2.1 (10/480)	0	0	0	0	44.0 (211/480)[b]	44.0 (211/480)[b]

[1] PHS: pulmonary hypertension syndrome (ascites); SDS: sudden death syndrome; KB: kinky back or spondylolisthesis; TW: twisted leg, perosis; TD: tibial dyschondroplasia; LAME UNK: cause of lameness could not be determined = unknown; ALL BCO Lame = lameness was attributed to proximal femoral or tibial heads with BCO lesions; Total Lame = KB + TE + TD + Lame UNK + All BCO.
[2]Litter = wood shavings litter days 1 to 56; Wire = flat wire flooring days 1 to 56; Litter to Wire = litter days 1 to 34 then wire days 35 to 56.
[a,b,c] Values with different superscripts within a category differed significantly at $P \leq 0.05$; z-tests were used to compare proportions.

Table 2.3: Number of birds for which blood samples were taken for each treatment group, day, and clinical diagnosis.

	Healthy					Lame				
Day	14	30	41	49	56	14	30	41	49	56
L56	20	20	20	20	0	0	0	0	1	6
L35W	41	40	40	39	0	0	0	4	7	33
W56	19	20	20	20	0	0	0	1	8	16

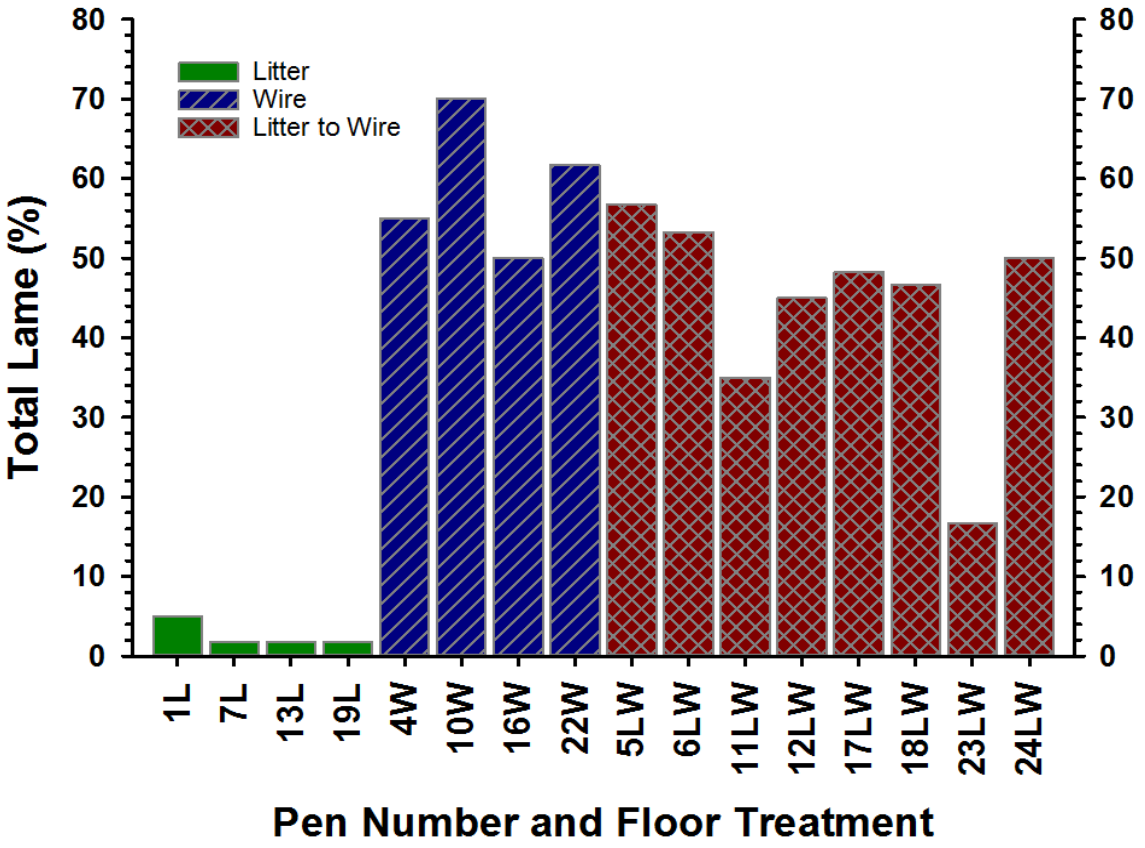

Figure 2.4. Total percentage lameness per pen for broilers that were reared on litter flooring from d1 through d56 (1L, 7L, 13L, 19L), on wire flooring from d 1 through d 56 (4W, 10W, 16W, 22W), or on litter flooring from d 1 to d 35 and thereafter on wire flooring through d 56 (5LW, 6LW, 11LW, 12LW, 17LW, 18LW, 23LW, 24LW). Values are calculated as the cumulative lameness percentage through d 56 based on the initial number of birds per pen on d 14.

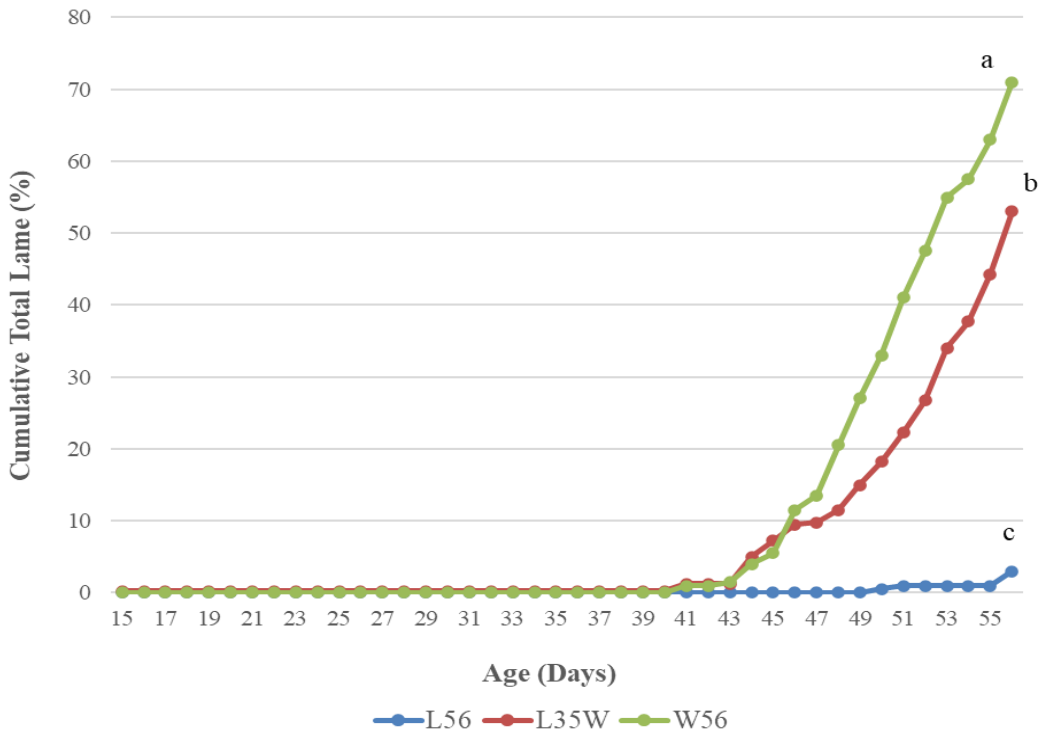

Figure 2.5: Time course of cumulative percentage lameness for broilers reared on litter flooring from d1 through d56 (a), on litter flooring from d1 to 35 and thereafter on wire flooring through d56 (b), on wire flooring from d1 through d56 (c). Values are calculated as the percentage of the total number of birds per floor treatment starting on d14.

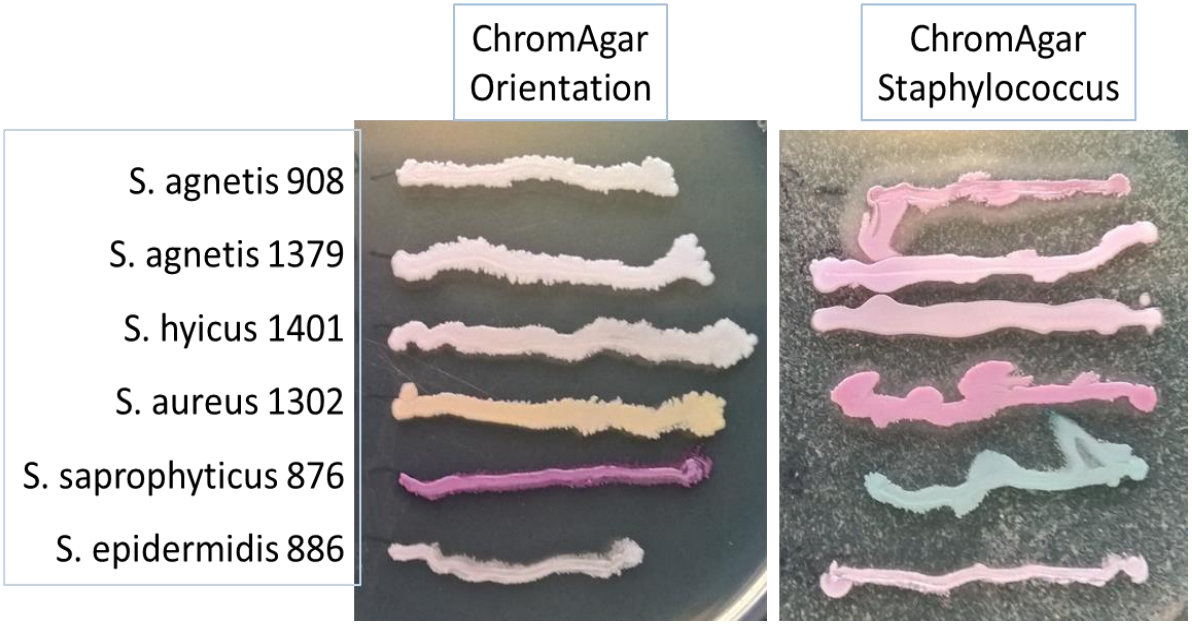

Figure 2.6: Colony color of different Staphylococcus species isolated from blood and BCO lesions, plated on two different CHROMagar media.

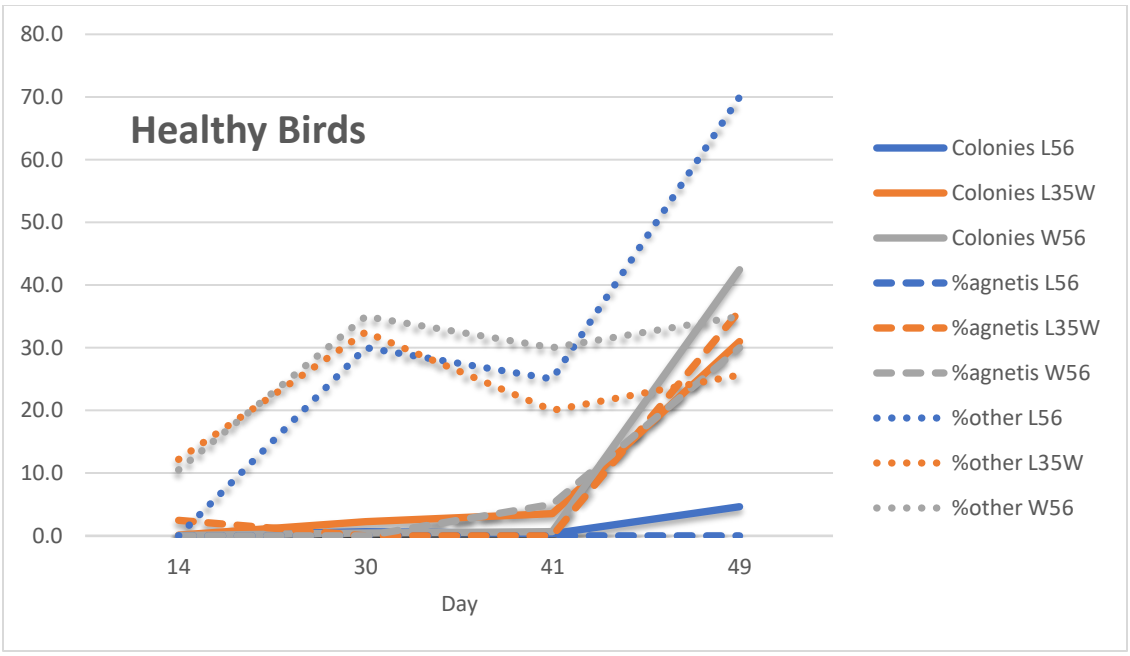

Figure 2.7. Bacteremia data for healthy birds for the three treatment groups for days 14 through 49. Solid lines indicate the average number of colonies per blood sample. Dashed lines show the percent of blood samples diagnosed as S. agnetis and dotted lines are the percent of blood samples diagnosed as other than S. agnetis. Treatment groups litter (L56), litter to wire (L35W), and wire (W56).

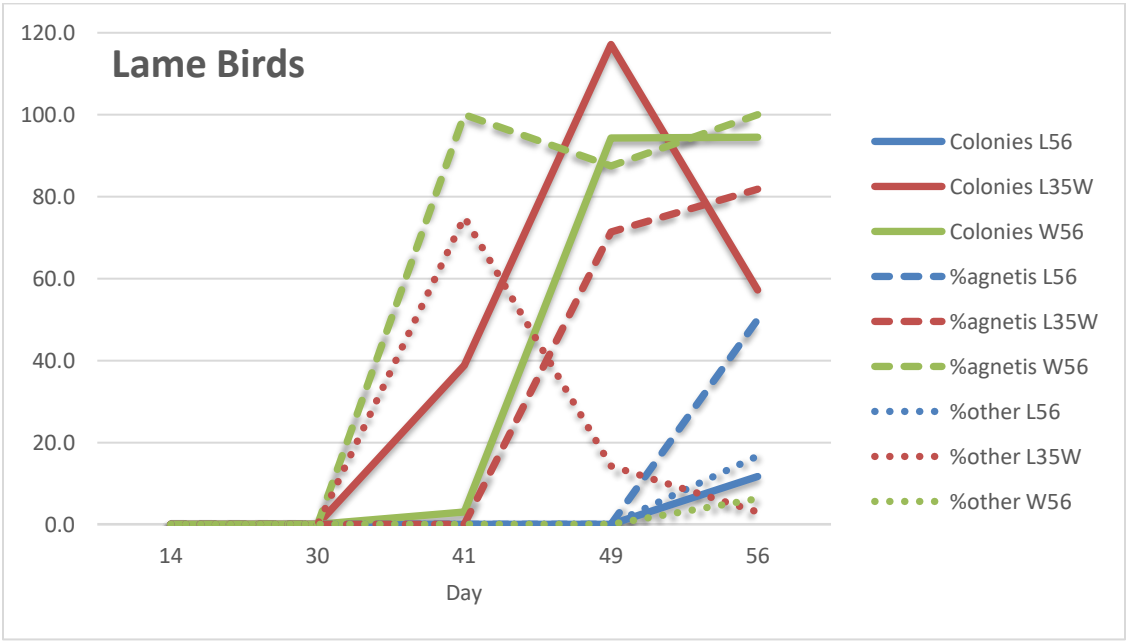

Figure 2.8. Bacteremia data for lame birds for the three treatment groups for days 14 through 56. Details are as for Figure 2.7.

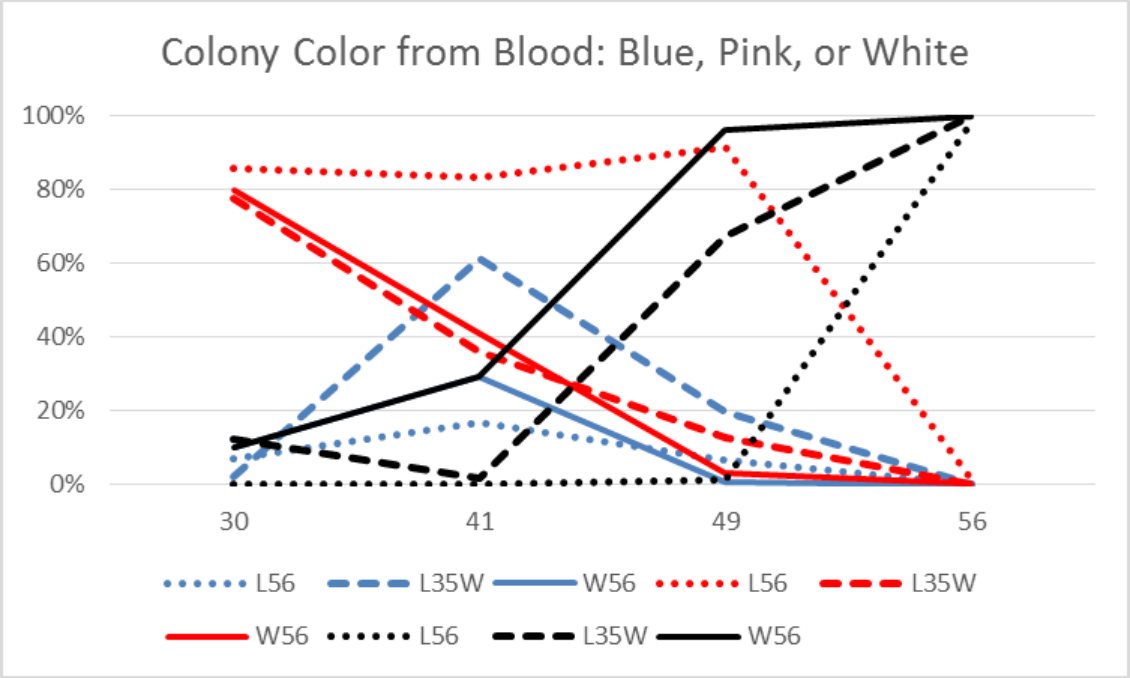

Figure 2.9: Colony colors for blood samples for birds from the three treatment groups. The graphs are the percentage of samples showing a given colony color for all samples showing any colony growth; colony colors of Blue/BlueGreen (blue lines), pink/purple (red lines), or white (black lines). Percent of birds is plotted for d30 through d56. Treatment groups litter (L56), litter to wire (L35W), and wire (W56).

CHAPTER 3 Staphylococcus agnetis: intracellular survival in chicken macrophage of lame broiler

3. 1 Introduction

One consequence of the intense genetic selection for growth rate and high meat production is an increased risk of lameness and gait problems (Gocsik et al., 2017). Therefore, research must be initiated to reduce serious welfare implications for broiler chickens due to impaired walking ability. Leg disorders are one of the most significant causes of morbidity and mortality in broiler chickens worldwide. Due to the adverse effect on the performance and well-being of individual animals, lameness constantly causes serious production losses in the broiler industry (Bradshaw et al., 2002). This negative impact on economy can be improved by determining specific risk factors and adopting effective management practices that affect the overall level of leg problems and consequently reduce lameness in broilers (Knowles et al., 2008). Gait abnormality associated with infectious agents, primarily bacteria, is the most chronic and frequent form of lameness in rapidly growing broilers (Wideman and Prisby, 2013). A wide range of opportunistic bacteria can cause bone and skeleton system dysbiosis in poultry leading to lameness attributable to bacterial chondronecrosis with osteomyelitis (BCO).

Bacteria in the genus Staphylococcus are a prominent causative agent of lameness attributable to BCO (McNamee et al., 1998, McNamee et al., 1999, Bradshow et al., 2002, and Kierończyk et al., 2017). Members of this genus are recovered from different animal species including poultry (Marek et al., 2016). Currently, more than 70 species belong to this genus. For instance, Staphylococcus aureus is a causative agent of community and hospital acquired infections in humans (Das et al., 2008). It is responsible for a significant mortality and morbidity due to a wide range of acute and chronic infections in both human and domesticated animals. In animals,

S. aureus is frequently associated with mastitis or intramammary infection in dairy cows (Martinez-Pulgarint et al., 2009). In poultry, serious losses occur, most often due to infections which are related to invasion by S. aureus. Staphylococcus spp. -related infections include Staphylococcus hyicus which has been reported to be associated with osteomyelitis in turkey (Stępień-Pyśniak, et al., 2016). Moreover, Staphylococcus simulans has been found to cause endocarditis in broiler chickens. Staphylococcus xylosus, Staphylococcus cohnii, Staphylococcus lentus are the other most frequently occurring species identified in poultry. Beside Staphylococcus spp., infections with Escherichia. coli (Dinev, 2009; Wideman, 2016), Enterococcus cecorum (Kense and Landman, 2011; Wideman, 2016), and Salmonella spp. (Padron, 1990; Wideman, 2016) have been isolated from BCO lesions. Recently, Staphylococcus agnetis has emerged to be strongly associated with BCO of the proximal head of femora and tibiae in broilers (Al-Rubaye, 2013). Staphylococcus agnetis was reported to be overrepresented in BCO lesions from lame broilers at the University of Arkansas poultry research farm. This bacterium was recovered from 81 samples (87%) out of 93. S. agnetis was previously not known to be involved in poultry infection (Al-Rubaye, 2013; Al-Rubaye et al., 2015). S. agnetis was reported to be associated with cattle mastitis (Taponen, et al., 2012), and in the gut of sheep scab mite (Hogg and Lehane, 1999). Possible modes of transmission by which this bacterial species switched from cows to broilers include air, birds, flies, feed, and personnel in the hatcheries (Al-Rubaye, 2013). The presence of bacteria (Bacteremia) in the blood of the lame and apparently healthy broilers has been reported (Al-Rubaye et al., 2017). Isolation of S. agnetis from blood samples suggests that, this bacterial species has already disseminated to the majority of tissues and body organs.

Chronic and persistent infection caused by microbial pathogens is due to their ability to produce multiple virulence factors which results in the impairment of the host defenses (Garzoni and Kelley, 2009). Virulence factors exerted by pathogens facilitate the attachment process to the surface of host cells, colonization, and invasion. One strategy used by pathogens to survive and produce infections is using defense pathways that enable them to avoid the killing mechanisms exerted by phagocytes (Das et al., 2008). Macrophages, as one of the phagocytic cells, are an essential component of the innate immunity which play an important role by recognizing, engulfing, destroying, and clearing microorganisms (Gilbert et al., 2017). Some types of bacterial pathogens are able to evade killing mechanisms applied by phagocytes. Many studies have been conducted to investigate the intracellular survival of multiple pathogens inside phagocytes such as Mycobacterium tuberculosis, Listeria monocytogenes (Bayles et al., 1998), Salmonella enterica (Balan and Babu, 2017), and Brucella suis (Foulongne, et al., 2000) are some facultative intracellular pathogens that are able to invade host cells. Bayles and his colleagues stated that bacteria survive inside host cells to shield themselves from humoral immune functions such as secreted antibodies (Bayles et al., 1998). However, these pathogens must possess particular mechanisms that confer protection against the harsh intracellular environment. Although considered an extracellular pathogen, many studies confirmed that S. aureus is able to invade host cells and resist killing, survive or proliferate inside phagocytes (Das et al., 2008; Bayles et al., 1998; Martinez-Pulgarint et al., 2009; Garzoni and Kelley, 2009; Kubica et al., 2008). Survival is due to possessing a plethora of multiple virulence factors that are responsible for the initiation of host-pathogen interactions (Kubica et al., 2008). Therefore, a comprehensive understanding of how pathogens circulate in the blood of apparently healthy birds, the bacterial determinants to avoid being eliminated by the host's defense mechanisms,

and the consequent persistence in the site of infections without producing an inflammatory response need to be addressed (Wideman, 2016). This suggests focusing on understanding the essential role of the immune system during the pathogenesis of BCO. This chapter investigates whether S. agnetis isolated from chickens can persist inside macrophage and affect the viability of these cells. Monocyte-derived macrophages cell line or HTC cells will be used as a model to understand the macrophage function in poultry. The work will compare the pathogenesis of S. agnetis 908 from chicken and S. agnetis 1379 from cattle

3. 2 Materials and Methods:

3. 2. 1 Cell cultures

Chicken macrophage cell line (HTC) was kindly provided by Dr. N. Rath, (poultry Science Center, University of Arkansas, Fayetteville, AR). Murine macrophages cell line (RAW cells) was kindly provided from Dr. Jeannine Durdik lab, (Biological Science, University of Arkansas, Fayetteville, AR). Chicken monocytes were isolated from peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated from chicken blood using Ficoll-paque^{TM} plus density gradient (Sigma, St. Louis, MO). Samples were centrifuged for 35 min (400× g, 23^{o} C). Mononuclear cells were retrieved through collecting the opaque interface. Isolated cells were washed twice in 5 mL phosphate-buffered saline (PBS) for 10 min (400× g, 23^{o} C), counted, and viability confirmed based on the exclusion of 0.1% blue dye (≥90%). Then PBMCs were resuspended to a final concentration of 5×10^5 cells/well. PBMCs were incubated at 37 ^{o} C and 5% CO_2 for 1-2 days to allow monocyte adherence. Warm PBS used to remove other non-adherent cells. Monocytes are cultured for 5-6 days to allow cell maturation and differentiation into macrophages with medium changes every other day. All cell lines were cultured in RPMI-1640) with L-glutamine (VWR Scientific Products) supplemented with 50 µg/ml gentamycin, 1

x concentration of antibiotic-antimycotic solution, 1 mM Na-pyruvate, 50 μM β-mercaptoethanol, and 10% normal and heat inactivated fetal bovine sera FBS (v/v). Cultures were maintained at a density equal to 10^6 cells/ml

3. 2. 2 Bacterial strain, preparation, storage and growth conditions:

Different bacterial strains were used to infect macrophage cell lines. S. agnetis 908 was isolated from BCO lesions of lame broilers at the University of Arkansas poultry research farm. S. agnetis 1379 (MU50.56) was obtained from a subclinical mastitis sample and provided by Dr. J. Middleton from Department of Veterinary Medicine and Surgery, University of Missouri, Columbia. S. hyicus 1401, was isolated from BCO lesions from lame broilers at the University of Arkansas research farm, and Staphylococcus agnetis 1416, isolated from a BCO lesions from lame broilers at a commercial broiler farm. Bacterial stocks were maintained at -80 °C in TSB medium containing 40% glycerol. Culture were inoculated from stocks into 2 ml medium. Bacterial strains were grown overnight under constant rotation (120 RPM) to stationary growth phase at 37 °C. Bacterial cells were collected by centrifugation (800× g, 8 min), washed twice with PBS and resuspended in RPMI-1640 to be ready for cell quantification by measuring the OD_{650nm}. To get more accurate preparations of bacterial samples for phagocytosis assay, the CFU/ml count was routinely done to verify the desired number of bacteria in each sample by plating dilutions on agar plates and counting the colonies.

3. 2. 3 Phagocytosis assay and sample preparations:

For macrophage infection, a suspension of bacteria in RPMI-1640 was added to 3×10^5 cultured macrophages in 24-well culture plate to the final amount of 3×10^5 CFU, 1.5×10^6 CFU, 3×10^6 CFU, 7.5×10^6 CFU and 3×10^6 CFU (the ratio bacteria : macrophages) from 1:1 to 100:1, multiplicity of infection, MOI=1, 5, 10, 25, and 100, respectively). Macrophage cell lines and

bacterial strains were incubated for 4-6 h at 37 ºC in a humidified atmosphere containing 5% CO. Stopping phagocytosis was conducted by placing the plates on ice for 10-20 min and washing the macrophages twice with ice-cold PBS to eliminate the non-phagocytosed bacteria. Any residual extracellular bacteria were killed by overnight culturing macrophages in RPMI-1640 supplemented with 50 µg/ml gentamycin. Then, cells were washed again, and antibiotic free medium was added. Cultures were maintained for 2-3 days (in one experiment for 6 days) with medium changes almost every day based on changes in the color of medium. Consecutive samples were taken every day until bacteria escape into the medium. Subsequently, macrophage cells were lysed for 5-10 min with sterilized milliQ distilled water as assessed by microscopic inspection. The cell lysates and tissue culture supernatants were plated at serial dilutions on TSB agar plates. Plates were incubated overnight at 37 º C and the number of colonies (CFU) was determined. Intracellular killing activity was expressed by the reduction of the initial number of viable phagocytosed bacteria. All phagocytosis assays were conducted in triplicates.

3. 3 Results

3. 3. 1 Detection of post infection time required to stop phagocytosis

Various interval times (2, 8, 6, and 24 h) were used to determine the time of post phagocytosis of both S. agnetis isolates, the chicken 908 and the cattle 1379 isolates, which are used to infect the HTC chicken macrophage line. Three different MOIs were used in this assay (10:1, 25:1, and 100:1). Figure 3.1 shows the results of phagocytosis pattern of the two isolates 908 (panel A and B) and 1379 (Panel C and D) in both cell suspension and lysate, respectively. It indicates that with longer time points and higher MOIs, bacterial growth represented by the number of CFU/ml was increased. In other words, enhanced intracellular viable bacteria inside chicken macrophages and bacterial growth in the outside environment (extracellular growth) increase with the time of

co-culturing and dose of bacteria being added to infect macrophages. We chose the time in between the first (2h) and second (8h) intervals, which is 4-6 h, as the time required to stop phagocytosis. Figure 3.2 shows that both isolates were internalized into the chicken macrophage cells almost at the same rate as represented by panels (E) and (F) of cell suspension and lysate, respectively. Bacterial growth rate represented by log of CFU/ml showed reduction in the number of CFU/ml as compared to the original number of bacteria This indicates that a certain number of bacteria were phagocytosed. In Figure 3.2 E, the presence of bacteria represents the extracellular bacterial growth, while Figure 3.2 F, the bacterial growth represents the number of viable intracellular bacteria which are able to form colonies on solid medium when recovered from HTC cell line after doing the cell lysis step. Bacterial growth increased in number when we exposed macrophages to bacteria for longer times, and doubled the number of bacteria being added to infect macrophages about 2.5 and 10 times to reach almost the same as the original growth rate as it is shown, especially, with the highest MOI 100:1 in Figure 3.2 E and F.

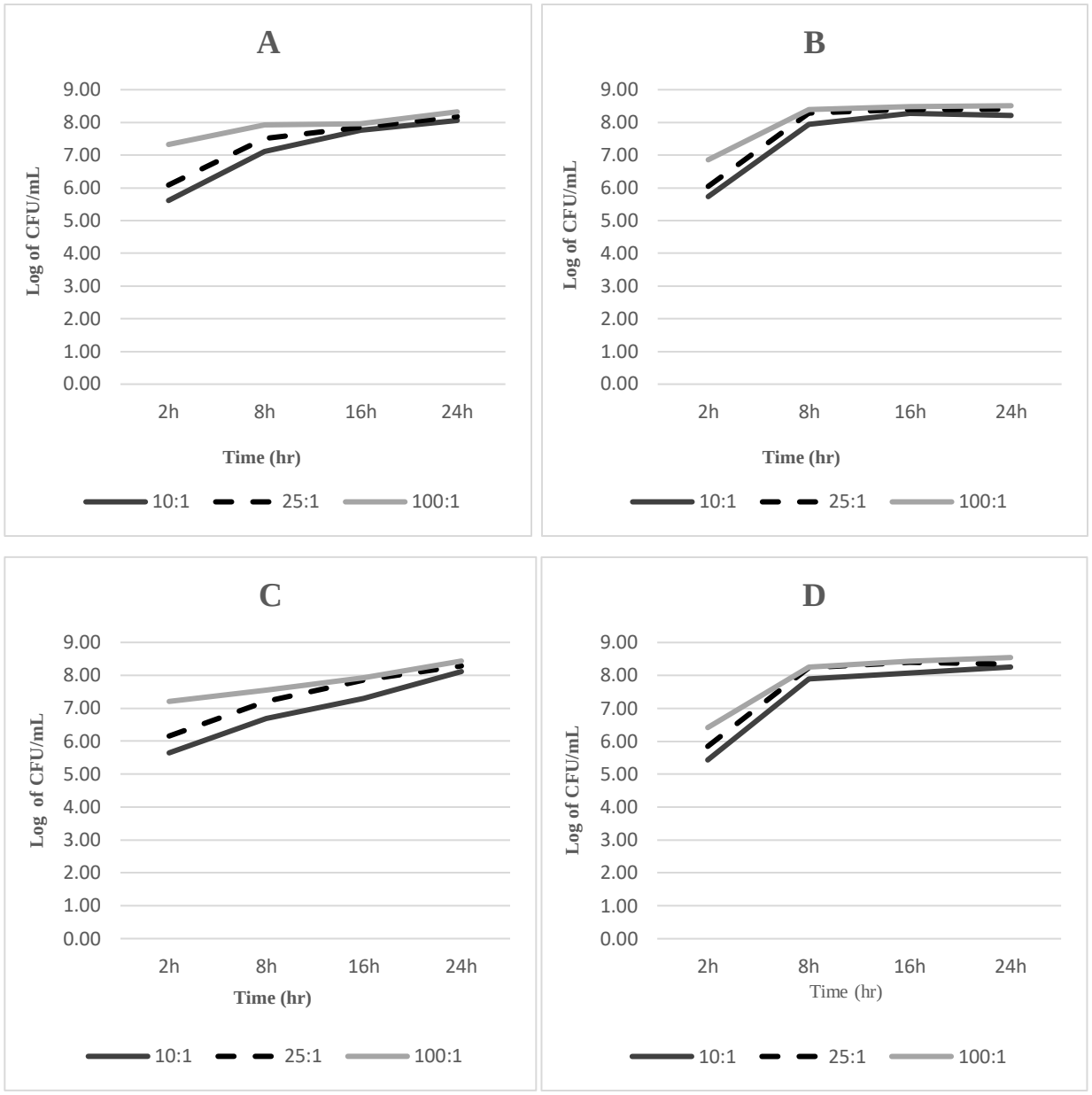

Figure 3.1: Phagocytosis assay of S. agnetis 908 and 1379 isolates at different time intervals and different MOIs. Panels (A) and (B) show the growth rate of 908 in cell suspension and lysate, respectively. While panels (C) and (D) show the growth rate of 1379 in cell suspension and lysate, respectively. Bacterial growth rate is represented by the log of CFU/mL. Enhanced bacterial growth in and outside chicken macrophages is dependent on both time and dose of bacteria that are used in in vitro infection.

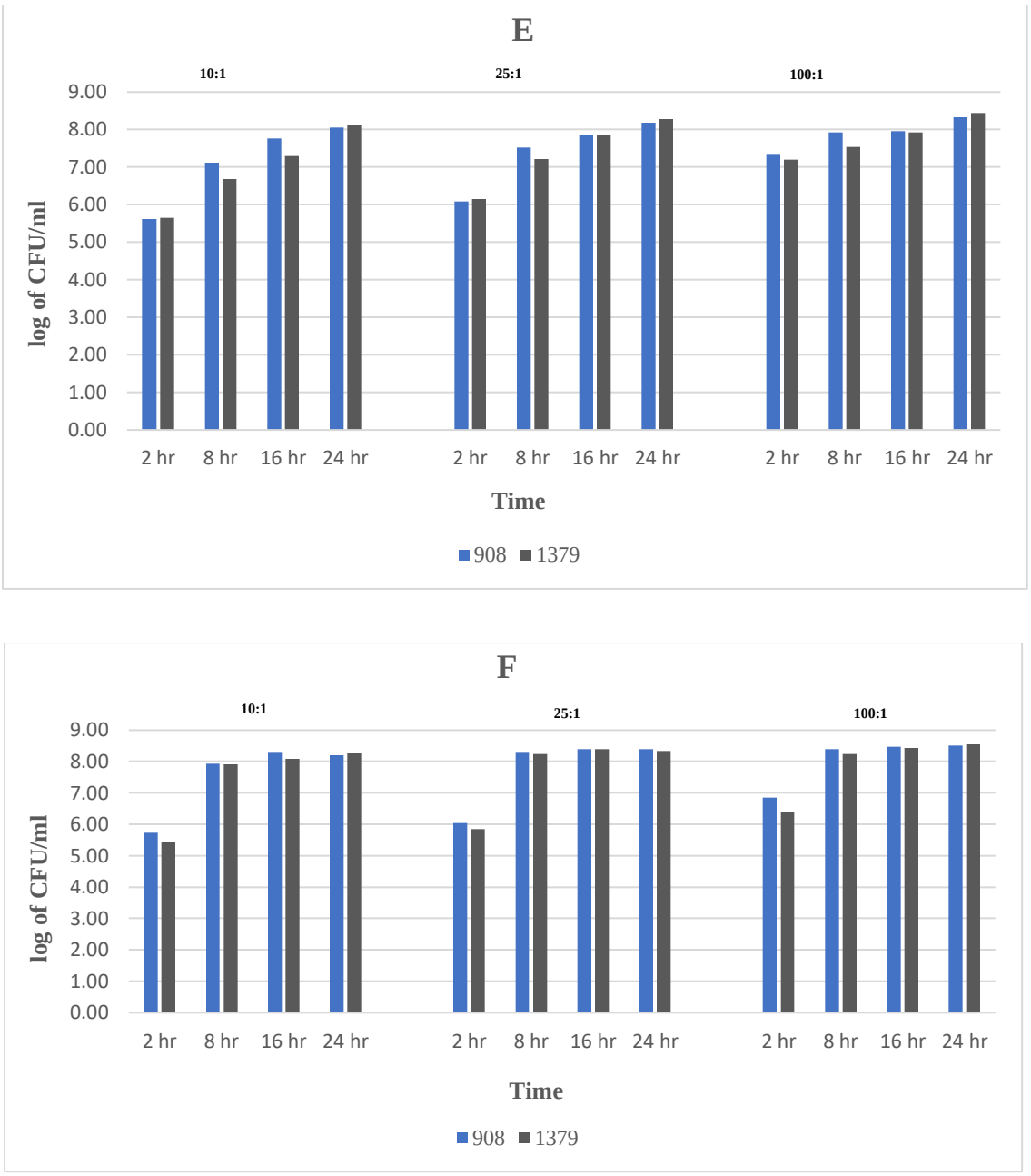

Figure 3.2: Phagocytosis pattern of S. agnetis 908 and 1379 isolates by chicken macrophages. Both isolates displayed almost a same pattern of bacterial internalization in cell suspension (E), and lysate (F). Bacterial growth rate represented by log of CFU/ml. Stopping phagocytosis after longer time periods lead to more bacterial growth in the supernatant (E) and an increase in the number of phagocytosed bacteria in the cell lysate (F) as compared to the original number of CFU/ml.

3. 3. 2 MOI comparisons for survival activity of both S. agnetis 908 and 1379.

It is important to establish the optimal bacterial load to be used to infect the cell line in order to assess how internalization and invasion are affected by the multiplicity of infection (MOI: number of bacteria per cell). Three different MOIs (1:1, 5:1, and 10:1) were used to determine the optimal bacterial load that can be used to compare between S. agnetis 908 and 1379 survival rates. Figure 3.3 represents the survival activity of 908 and 1379 strains in HTC cells using different MOIs. The results show that, from day 3 post phagocytosis we observed a clear difference between the two strains' behavior. There were significant differences between the CFU counts from cell supernatants and lysates of both strains. The most dramatic difference was observed at MOI of 1:1 for both cell lysate and suspension between 908 and 1379, as compared with the other MOIs especially. At higher MOI there was increased survival of 1379 intracellularly. 1379 showed more resistance to the killing mechanism of the macrophage cells when bacterial loads are increased 5, and 10 folds compared to the lowest MOI of 1. Also, the changes in the color of culture medium from pink to yellow as illustrated in (Figure 3.4), was considered to be an indication of intracellular survival/phagocyte-killing activity of bacteria. Usually, color of culture medium changes can be observed at day 2. Therefore, we expect to see less bacteria in the culture medium and more intact HTC cells when medium color remains pink. While in the case of 908-infected HTC cells, the color of the medium changed to yellow, and we observe a burst of bacterial growth in the culture media and fewer intact HTC cells. These observations were confirmed by inverted microscopic examination as illustrated in (Figure 3.5).

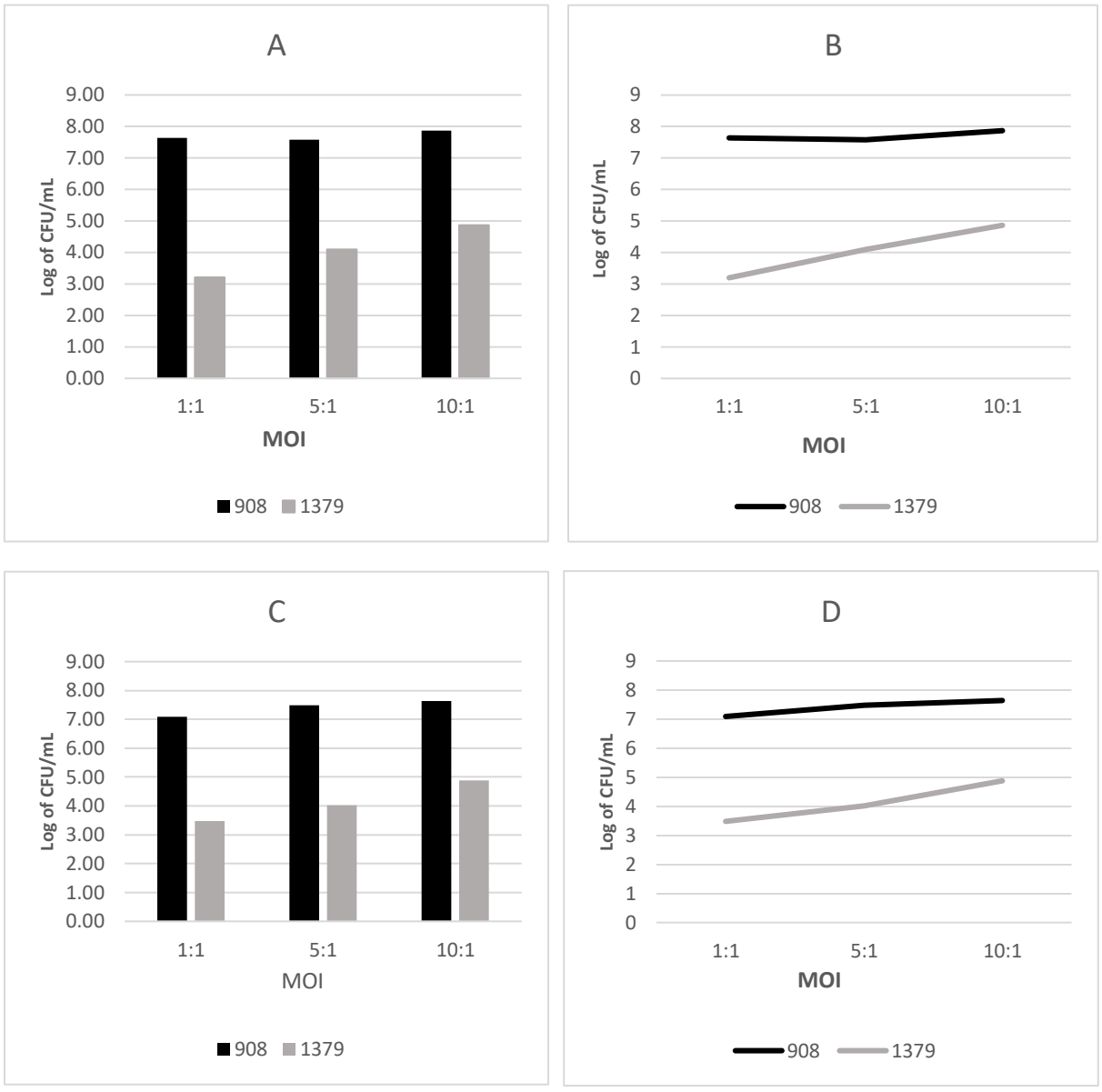

Figure 3.3: The survival activity of S. agnetis 908 and 1379 using different MOIs at day 3 postinfection. Panel A, and B in cell suspension. Panel (C) and (D) in cell lysate. The figure displays that 1379 isolate is dose dependent, and that intracellular survival/ bacterial killing activity increases with higher MOIs. On the other hand, 908 isolate is dose independent because it shows the same pattern of killing activity against HTC cells in the all tested MOIs.

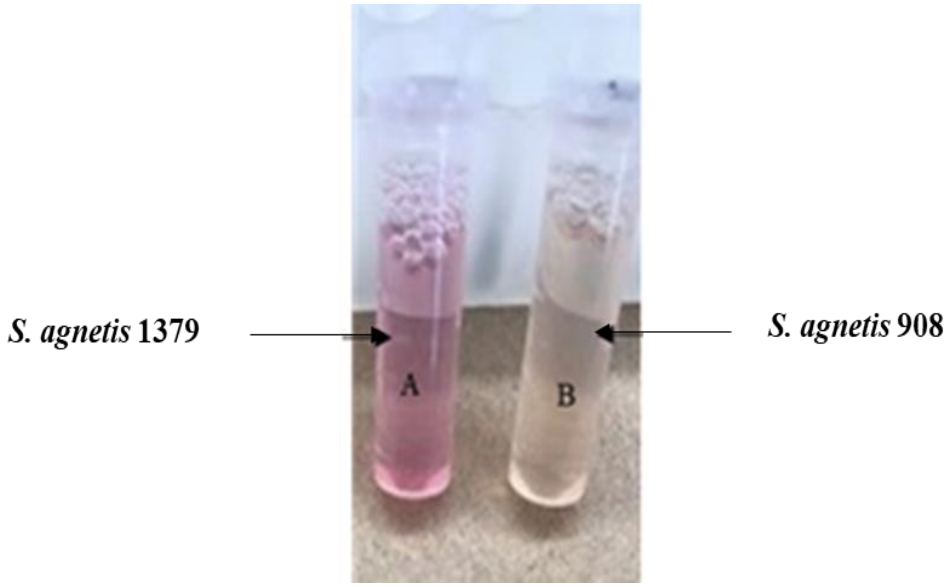

Figure 3.4: Color change of the medium at day 2. (A) 1379-infected HTC cells, less bacteria in the medium (pink) and more intact HTC cells' count. (B) 908-infected HTC cells, burst of bacterial growth in the medium (yellow) and less intact HTC cells' count.

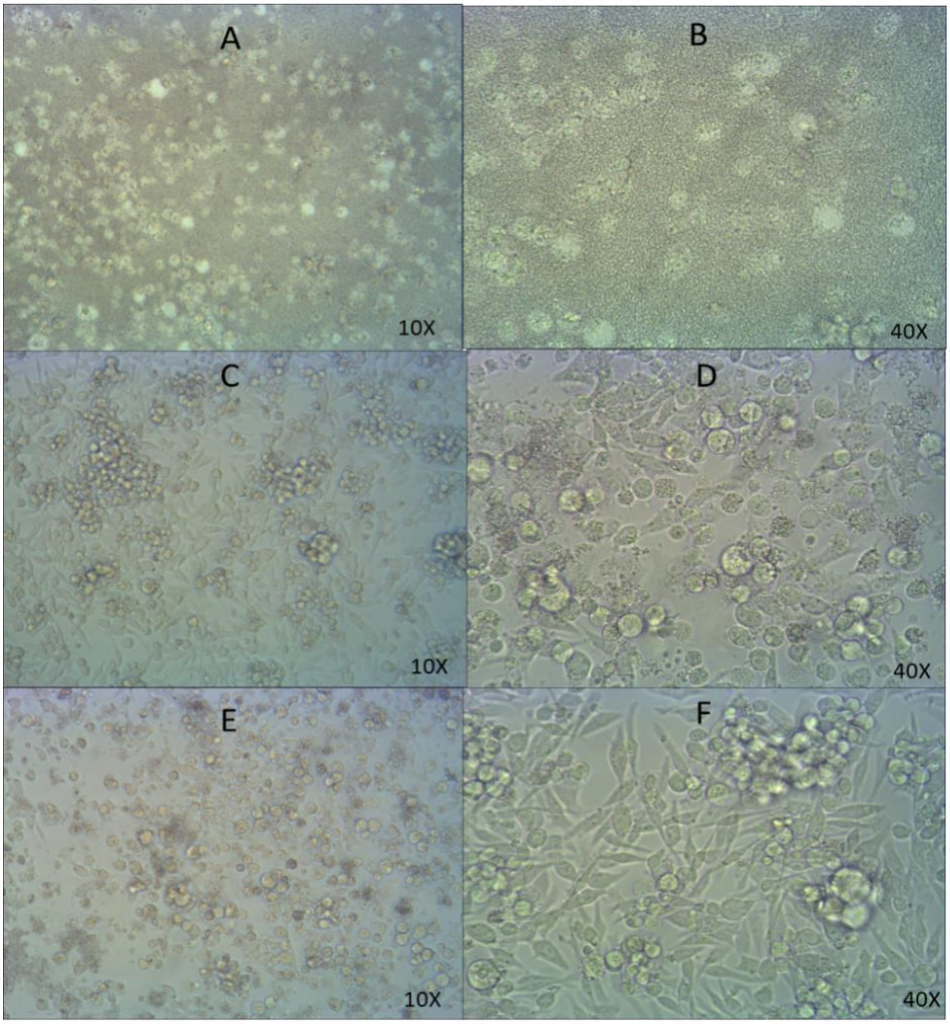

Figure 3.5: Images of infected Chicken macrophages line (HTC) with bacteria, two days post-phagocytosis using inverted microscope. (A) and (B) cells infected with 908 isolate. (C) and (D) cell infected with 1379 isolate. (E)and (F) Control (HTC cells only). In the case of 908-infected HTC cells, bacteria survive the killing mechanisms exerted by chicken macrophage and bacteria cause a rapid host cell killing indicated by lower intact cell count and the presence of more escaped bacteria from dead cells into the culture medium when compared with the 1379 infected HTC cells and control of HTC cells only. 1379-infected HTC cells show less resistance against bactericidal agents of infected chicken macrophage.

3. 3. 3 Detection of the survival rates of S. agnetis 908, 1379, and 1416, and S. hyicus 1401.

Several earlier studies have documented the intracellular survival capability of S. aureus inside professional phagocytes (Gresham et al., 2000; Kubica et al., 2008; Martinez-Pulgarin et al., 2009;) and non-professional phagocytic cells (Alva-Murillo et al., 2014; Löffler et al., 2014;

Rollin et al., 2017). Therefore, this bacterium might be termed a facultative intracellular pathogen due to acquisition of resistance against the killing mechanisms exerted by phagocytes (Fraunholz and Sinha, 2012). Intracellular survival of bacteria in phagocytes can be manifested by either showing an extreme tendency to rapidly kill infected cells or they display persistence for long time periods inside phagocytes (Löffler et al., 2014; Jubrail, et al., 2016, Strobel, et al., 2016).

In this study, our goal was to look at the survival activity of S. agnetis 908 inside chicken macrophages line (HTC) and compare it with the survival activity of other strains: S. agnetis 1379 and 1416, and the closely related species S. hyicus 1401. S. agnetis 908 was isolated from BCO lesions, can survive in blood, and is primarily associated with the buffy coat. We decided to follow the behavior of these isolates after being internalized into HTC cell line to assess the sensitivity of these pathogenic strains to macrophage killing. Therefore, we adapted an overnight gentamicin protection assay to ensure elimination of all extracellular bacteria continuously escaping the intracellular confinement to culture medium during the lysis of the infected cells. Gentamicin kills Staphylococci, but it is not able to penetrate into the eukaryotic cells and consequently has no effect on the intracellular bacteria (Gresham et al., 2000; Imbuluzqueta et al., 2012; Rollin et al., 2017). We first compared the ability of S. agnetis 908 and 1379 to survive in HTC cell line for six consecutive days postinfection at multiplicity of infection (MOI) of 1. As illustrated in (Figure 3.6), panel A and B display the phagocytosis assay of S. agnetis 908 and 1379, respectively. There is a clear difference in the behavior of the two isolates, 908 is more aggressive as compared to 1379. The data displayed details of one representative experiment performed in triplicate for MOI of 1:1 at day 1. Substantial differences were observed between the number of intra and extracellular bacteria of 908 and 1379 (CFU/ml). During the time period

of 4-6h, the HTC cell line took up a high amount of both 908 and 1379 (1379 is slightly higher in number of internalized bacteria). In panel B, during day 2 until day 5, 1379 showed drastically reduced levels of intracellular bacteria as compared to their numbers in the first-time interval of 6h. Enhanced levels of intracellular growth were observed during day 6, and bacteria start escaping from the dead, infected cells in high numbers during day 6 postinfection. While in the case of 908, this bacterium seems to be more aggressive as represented by panel A, it resists the killing activity exerted by chicken macrophage line and causes host cell killing starting from day 2, and bacteria escape to the culture media and proliferate in cell suspension. In day 1, there is no indicated bacterial growth in cell suspension in both panels because of the gentamicin action against bacteria. The 908 isolate can survive the killing mechanisms of the chicken macrophage, and it has also killed most of the macrophage and escapes the macrophage, as indicated by the number of the viable extracellular bacteria. While for 1379 it appears that this isolate is more sensitive to the killing activity of macrophage cell line. Both isolates were internalized by chicken macrophage as indicated by detecting viable bacteria in the cell lysate. In cell suspension of the cattle isolate, fewer numbers of escaped bacteria were detected until day 6 as we observed the number of escaped bacteria has reached very considerable amounts compared to the previous five days. Therefore, S. agnetis 1379 can persist intracellularly for longer times post infection before starting to considerably kills chicken macrophage (Figure 3.6).

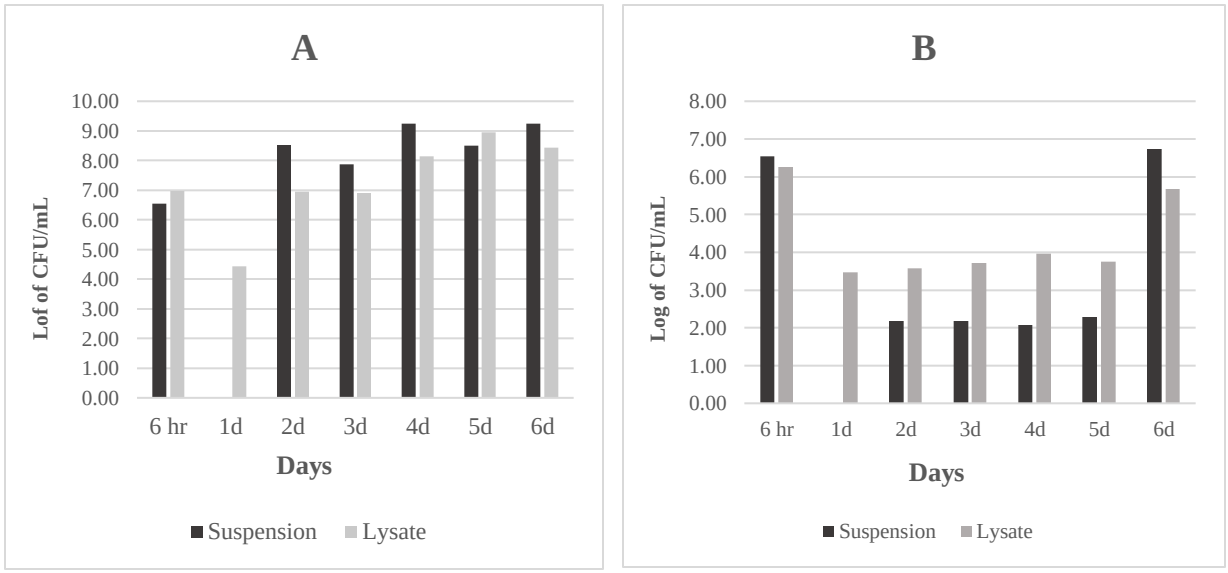

Figure 3.6: Phagocytosis of 908 (A) and 1379 (B) for 6 days. Macrophages were allowed to engulf both S. agnetis strains at a MOI of 1:1 for 4-6 h, washed, and extracellular bacteria were killed by adding gentamicin. Macrophage cells were cultured in antibiotic free medium. At consecutive days post phagocytosis, media was aspirated, and macrophage cells were lysed. Both cell supernatant and lysate were cultured on TSA to determine the CFU counts.

The same phagocytosis assay was conducted to investigate the survival activity of S. hyicus 1401 isolated from BCO lesions of lame broilers at the University of Arkansas research farm and S. agnetis 1416 from BCO lesions of the lame broiler at a commercial broiler farm. The survival/ killing activity of both isolates was compared with the hyper virulent S. agnetis 908 isolates as displayed in (Figure 3.7). We observed that in the case of Staphylococci 1401 and 1416, the numbers of viable bacteria in both cell supernatant and lysate decreased at day 3 by approximately five order of magnitude compared to 6 hour postinfection time. The data displayed details one representative experiment performed in triplicate. Both Staphylococci 1401, 1416 are more susceptible to the killing mechanisms by HTC cells, while S. agnetis 908 is able to not only survive the killing mechanisms, it also kills the HTC cells more efficiently and escapes to the culture medium (908 S). The CFU count of the resistant S. agnetis 908 in cell lysate and supernatant increased by approximately 5 orders of magnitude compared to chicken

BCO isolate of the closely related species S. hyicus 1401 and the other BCO isolate from another farm S. agnetis1416 which are killed by the HTC cells at the same time span. This suggests that macrophages are more efficient in killing the phagocytosed Staphylococci 1401 and 1416, and fewer numbers of the survived bacteria escaped into the culture medium or cell supernatant. Compared to S. agnetis 908 which are successfully survived intracellularly and killed HTC cells as indicted by the high numbers of CFU count in both cell suspension and lysate at day 3. Based on these results, we have hypo and hyper virulent strains of Staphylococci that show a great discrepancy in sensitivity to macrophages killing.

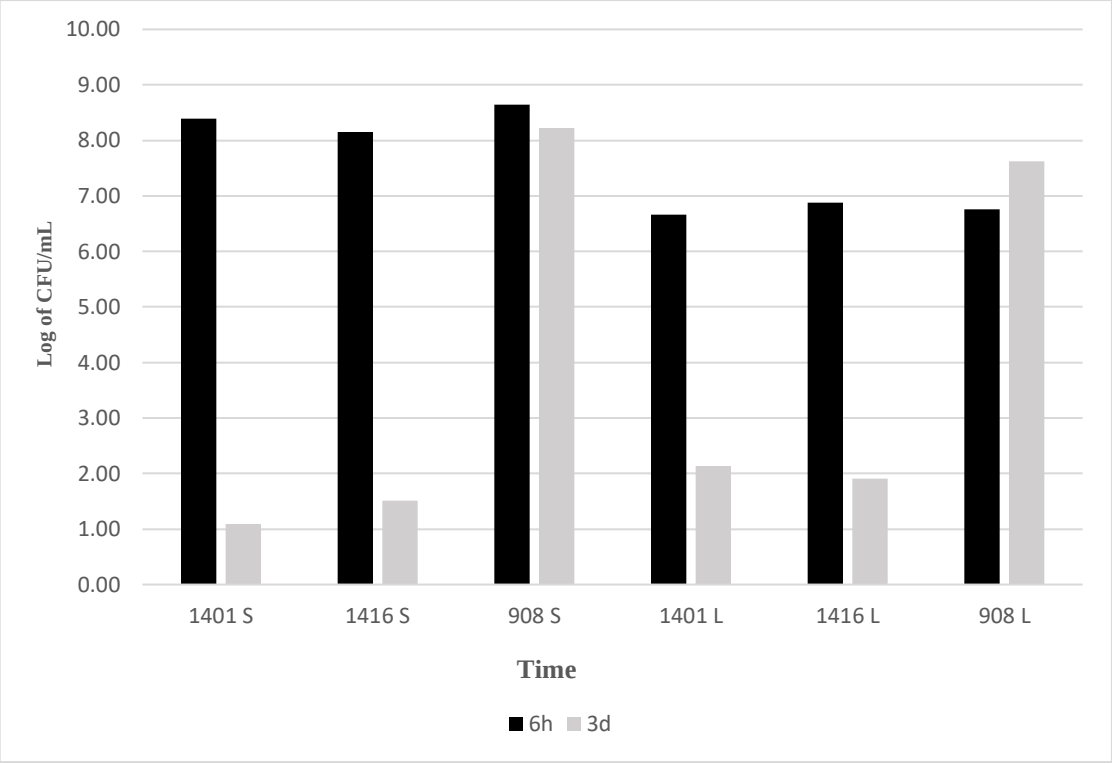

Figure 3.7: Comparing the surviving activity of S. hyicus 1401, S. agnetis 1416, and 908 at 6 h and day 3 post infection. HTC cells were allowed to phagocytose three bacterial strains at a MOI of 1 for 6 h. infected cultures were washed, followed by overnight incubation with gentamicin to kill the extracellular bacteria. Infected macrophage cells were cultured in antibiotic free medium for two more days. At day 3 post phagocytosis media was aspired, and macrophage cells were lysed. Both cell supernatant (S) and lysate (L) were cultured on TSA to determine the CFU counts.

3. 3. 4 Comparative response of two macrophage cell lines, chicken (HTC) and mouse (RAW) cell lines to infection with S. agnetis isolates.

In this study we evaluated the intracellular survival rate of two chicken and one cattle S. agnetis using chicken HTC and mouse RAW macrophage cell lines. Same procedure was used to infect both cell lines. We compared the ability of S. agnetis 908 and 1379 to survive inside both cell line models for two days post infection at a MOI of 1:1 (Figure 3.8). Two day assays were used because during day two, a burst of live 908 isolate in the supernatant was detected by counting the number of viable bacteria that proliferate intracellularly, with large number of the survived bacterial cells being liberated from the lysed macrophage into the growth medium or supernatant changing the medium color from pink to yellow (Figure 3.4). Intracellular persistence of both isolates showed almost the same pattern as aforementioned. The data displayed details one representative experiment performed in triplicate. S. agnetis 908 is able survive the killing mechanisms, and also kills the macrophage of the two models more efficiently and escapes to the culture medium. While 1379 showed more susceptibility to be killed by both RAW and HTC cells with being more persistent inside RAW cells as indicated by the CFU count of the bacterial cells of both lysate (P= 0.004) and supernatant (P=0.06) compared to bacterial cell count of both suspension and lysate recovered from HTC cells. The ability of S. agnetis 908 to survive inside both cell line models was more aggressive in killing both types of macrophage compared to 1379 which showed less resistance to antimicrobial killing mechanisms exerted by both macrophage cell lines. Even though 1379 was more sensitive (has less survival rate) in HTC, this isolate showed more persistence inside RAW cells than inside HTC cells as indicated by the number of viable bacterial cells in both cell lysate and supernatant with displaying more significant

resistance to antimicrobial killing in the lysate than in the suspension (P= 0.004, P= 0.06, respectively) (Figure 3.8).

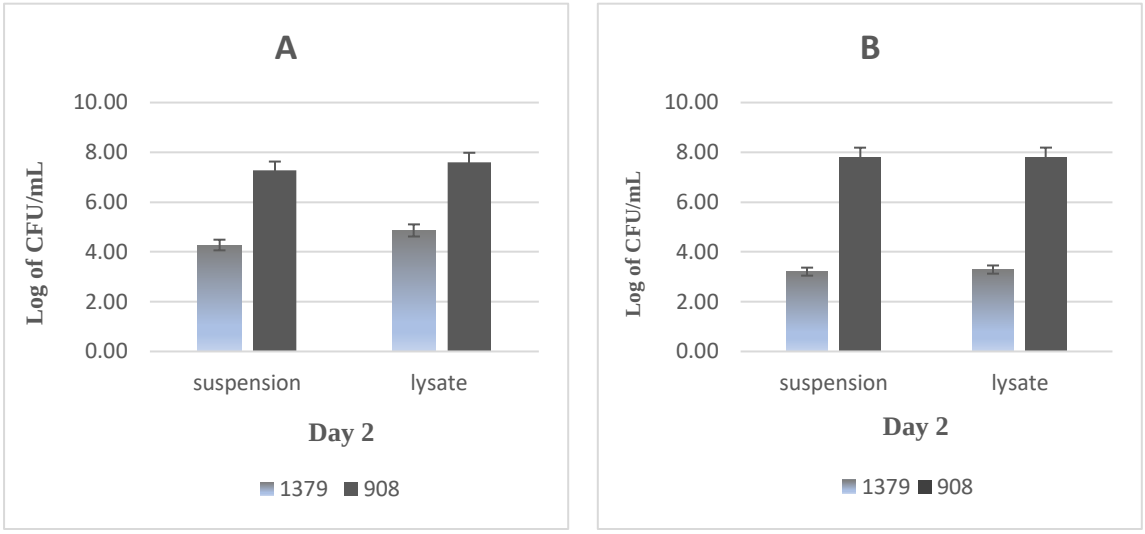

Figure 3.8: Comparative response to both S. agnetis chicken 908 and cattle 1379 isolates using two cell line models, RAW cells (panel A) and HTC cells (panel B). HTC cells were allowed to phagocytose the two bacterial strains at a MOI of 1 for 4 h. Infected cultures were washed, followed by overnight incubation with gentamicin to kill the extracellular bacteria. Infected macrophage cells were cultured in antibiotic free medium for one more day. At day 2 post phagocytosis media was aspired, and macrophage cells were lysed. Both cell suspension and lysate were cultured on TSA to determine the CFU counts.

3. 3. 5 Comparing the survival activity of both Staphylococci 908 and 1379 inside HTC cell line using normal (N) and heat inactivated (Paxton et al.) sera.

This experiment was conducted to investigate the uptake and survival activity of S. agnetis 908 and 1379 inside chicken macrophage cell line by growing both isolates in RPMI-1460 supplemented with normal and heat inactivated sera. Heat inactivation was done by heating the FBS for 30 min at 56°C with mixing to inactivate complement factors which are part of the immune response. Therefore, we decided to check if the intracellular persistence of both isolates can be affected by the presence or absence of the complement proteins in the growth medium (Figure 8). The data displayed show that S. agnetis 908 is able to survive the killing mechanisms,

kills HTC cells efficiently in both N and HI sera, and escapes to the culture supernatant. While 1379 showed more persistence to bactericidal mechanisms when incubated in the medium with heat inactivated FBS as indicated by the CFU count of the bacterial cells of both lysate (P= 0.001) and supernatant (P=0.0002) compared to bacterial cell count of both supernatant and lysate recovered from HTC cells in the medium supplemented with normal FBS. These results showed the importance of the receptors that mediate host-pathogen interactions and subsequent uptake, entry, and intracellular survival by differential modulation of gene expression involved in increased bacterial pathogenesis. Our data suggests that the internalization and intracellular survival of S. agnetis 908 inside chicken macrophage cell line were not affected by growing this isolate in normal and heat inactivated FB sera. On the other hand, the absence of complement and/or other heat labile proteins in the treated serum showed enhanced uptake and intracellular replication of S. agnetis 1379 represented by the number of CFU of the survived bacteria during day 2 postinfection. The number of viable nonopsonized 1379 in both cell supernatant and lysate has increased significantly (P= 0.0002 and P= 0.001, respectively). This difference in invasion of 1379 suggests that there was major impact of the presence of complement on bacterial uptake and intracellular survival in chicken macrophage cell line. Our data indicate that complement receptors (CRs) may play no role in the pathogenicity and virulence of 908. While in the case of 1379 strain, receptors other than CRs maybe involved in the downregulation of phagocytic pathways that induce bactericidal mechanisms exerted by macrophage cell line which explains the enhanced surviving intracellularly.

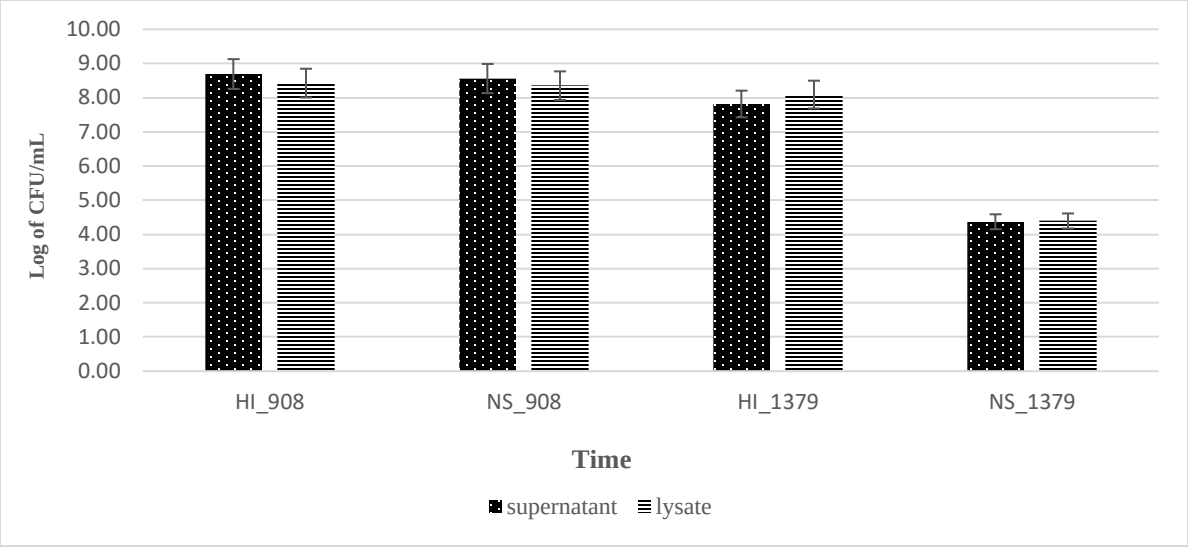

Figure 3.9: Comparative persistence in chicken macrophage cell line (HTC) following infection with S. agnetis chicken 908 and cattle 1379 isolates in RPMI-1640 supplemented with 10% normal (N) and heat inactivated (HI) FB sera. HTC cells were allowed to phagocytose the two bacterial strains at a MOI of 1 for 4 h. Infected cultures were washed, followed by overnight incubation with gentamicin. Infected macrophage cells were cultured in antibiotic free medium for one more day. At day 2 post phagocytosis media was aspired, and macrophage cells were lysed. Both cell supernatant and lysate were cultured on TSA to determine the CFU counts.

3. 3. 6 Directed genome evolution and in vitro selection of the survivors.

The rapid progress in genomics has led to paradigm shifts in microbial pathogenesis research (van Belkum et al., 2001). Directed genome evolution approach is applied as a powerful and high throughput tool to define specific genetic variations responsible for pathogen emergence, virulence, pathogenesis, and evolution (Wu et al., 2016). The whole genome sequence and annotation of S. agnetis 908 DNA was conducted to provide a profile of genetic information to explore S. agnetis 908 biological characteristics, the function of protein-coding sequences, and to identify virulence determinants (Al-Rubaye, et al., 2015). When complete genome sequences are accessible for multiple isolates of a single bacterial species, all genetic changes can be distinguished and catalogued. A system for Directed Genome Evolution (DGE) was adopted to identify which sp1ecific genes are involved in the survival/ killing activity of hyper-virulent

bacteria in chicken macrophages. Abdulkarim Shwani, a PhD student in our research group at the University of Arkansas, has been established an electroporation technique to transfer S. agnetis 908 DNAinto the cattle isolate S. agnetis 1379 (Shwani et al., manuscript in preparation). The transformed population (1437) was selected through three rounds of growth in chicken macrophage. This produced a resistant culture of S. agnetis 1437 that kills chicken macrophages (Figure 3.10). In passage/round #1 HTC cells were allowed to phagocytose the three different strains of S. agnetis at a MOI of 1:1 for 4-6 h, and infected cultures were washed, followed by overnight incubation with gentamicin to kill the extracellular bacteria. Infected macrophage cells were cultured in antibiotic free medium for two more days. At day 3 post phagocytosis (end of round #1) media was aspired, and macrophage cells were lysed. Both cell supernatant and lysate were cultured on TSA to determine the CFU counts. Then, the product of the mixed culture in round #1 was used to infect HTC cells to start the second round of selection of the survivors. Same steps were followed at each round of selection.

As indicated by this figure, there was an increase in the survival of the S. agnetis 1437 (transformed) compared to 1379 (wild) strain. This figure demonstrates that the supernatant and lysate cultures of both S. agnetis strains 908 and 1437 showed the same pattern of growth rate by the end of day 3 of round #3 represented by the log of CFU/ml. The presence of survivors at the end of round #3 indicates the successful transfer of one or more virulence genes to S. agnetis 1437. The transformant has received the required determinants from S. agnetis 908 strain that enable the transformed strain to effectively survive inside chicken macrophages. The transformant also showed the same ability to kill HTC cells within 3 days postinfection.

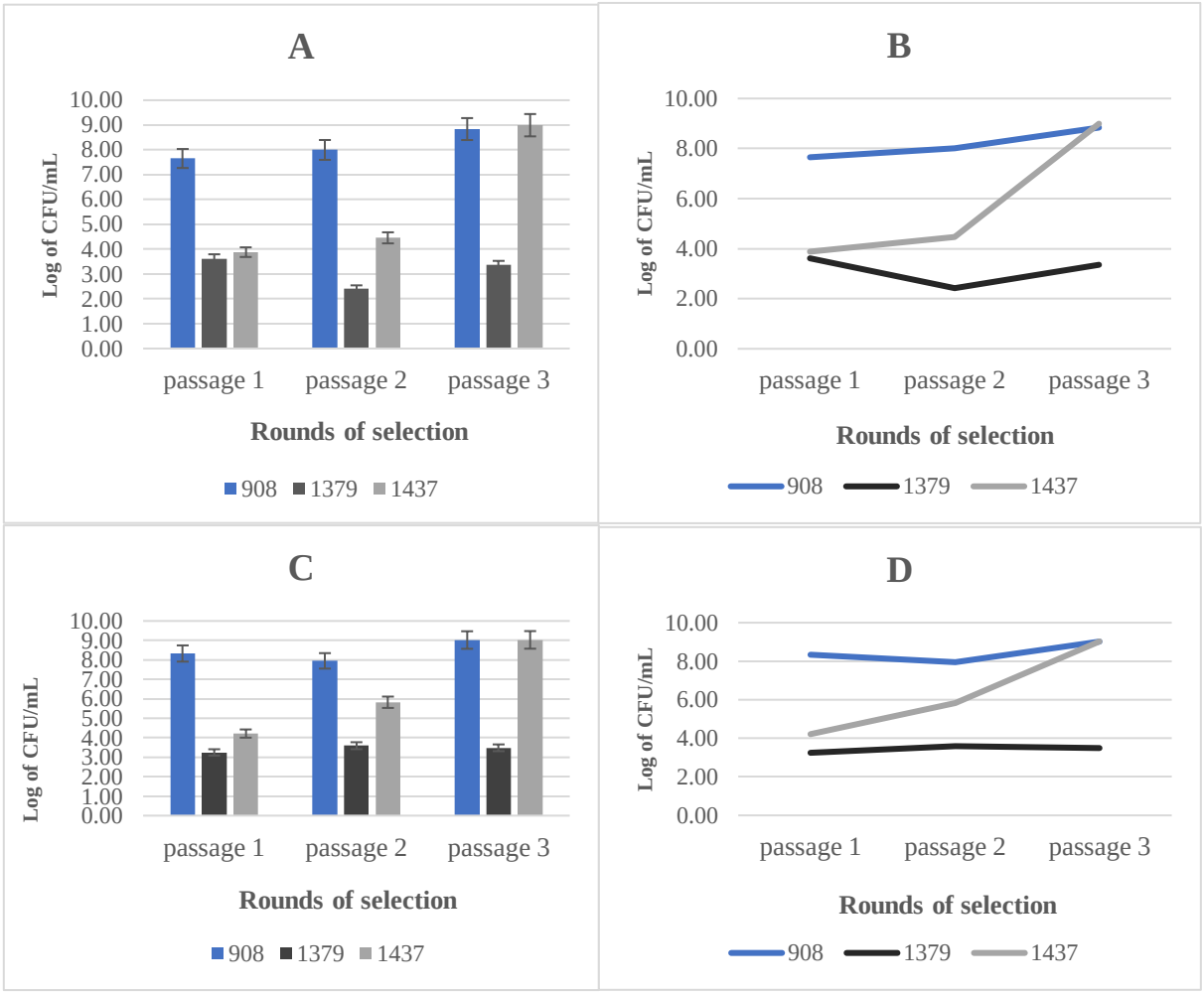

Figure 3.10: The survival/ killing activity of the of the transformed S. agnetis 1379 with total DNA preps, designated as 1437 at day 3 postinfection of each round. Panels (A) and (B) display the growth rate of S. agnetis 908, 1379, and 1437 in cell suspension. (C), and (D) represent the growth rate of 908, 1379, and 1437in cell lysate. The transformant (1437) showed the same ability to kill HTC cells within 3 days postinfection just like 908 (hyper virulent) when both were compared to 1379 (hypo virulent) strain.

We decided to repeat the DGE experiment with less total DNA and with purified plasmid preparations to further refine which genetic determinants are involved in macrophage killing (Figure 3.11). The products of the three rounds of selection of the transformed S. agnetis 1379 are designated as S. agnetis 1452 and 1453. The former was transformed with (330ng) genomic DNA (gDNA), and the later with (6 ng) plasmid DNA (pDNA). In passage/round #1 HTC cells were allowed to phagocytose the four different strains of S. agnetis at a MOI of 1 for 4-6 h, and

infected cultures were processed following the steps described earlier. Viable bacteria or CFU count in the cell supernatant and lysate was performed at day 3 post phagocytosis (end of round). The product of the mixed culture in round #1 was used to infect HTC cells to start the second round of selection of the survivors. Same steps were followed at each round of selection.

Figure 3.11 demonstrates that the supernatant and lysate cultures of both S. agnetis strains 908 and 1453 showed approximately same pattern of growth rate by the end of day 3 of round #3 represented by the log of CFU/ml. As indicated by this figure, there was an increase in the survival of the S. agnetis 1453 (transformed with pDNA) compared to 1452 (transformed with gDNA), and 1379 (wild) strains. The results showed that S. agnetis 1452 has higher killing activity against infected cell line compared to S. agnetis 1437tg which showed very less survival/ killing rate in both supernatant, (A, and B), and lysate (C, and D) as indicated by the number of survivors at day 3 post infection in each round. The results displayed that both 1379 (wild) and 1452 (transformed with 908 genomic DNA) showed a consistent pattern similar to 1379 of lower bacterial growth in both suspension and lysate culture in all of the three rounds of selection. Therefore, they are considered as hypo virulent strains. On the other hand, 908 and 1453 (transformed with 908 plasmids) which showed the same high killing activity at the end of round #3 in both cell suspension and lysate. Furthermore, figure 3.11 showed that, the survival/ killing activity of S. agnetis 1453 has increased as we carried out the second and third rounds of selection of the survivors in the cell lysate. We picked five random colonies from the final selection and repeated the phagocytosis assay to evaluate their killing activity. The results confirmed our finding that persistence of these transformants inside chicken macrophage occurred as a result of transformation with the 908 plasmid DNA preparation. We concluded that, the gene(s) necessary for survival in macrophages is located on the S. agnetis 908 plasmids.

S. agnetis 908 has 3 plasmids of 29, 3 and 2.2 kbp. We therefore used PCR primers for each of these plasmids to assess which plasmids had been transferred to 1453. However, we failed to amplify the expected fragment for any of these plasmids (data not shown).

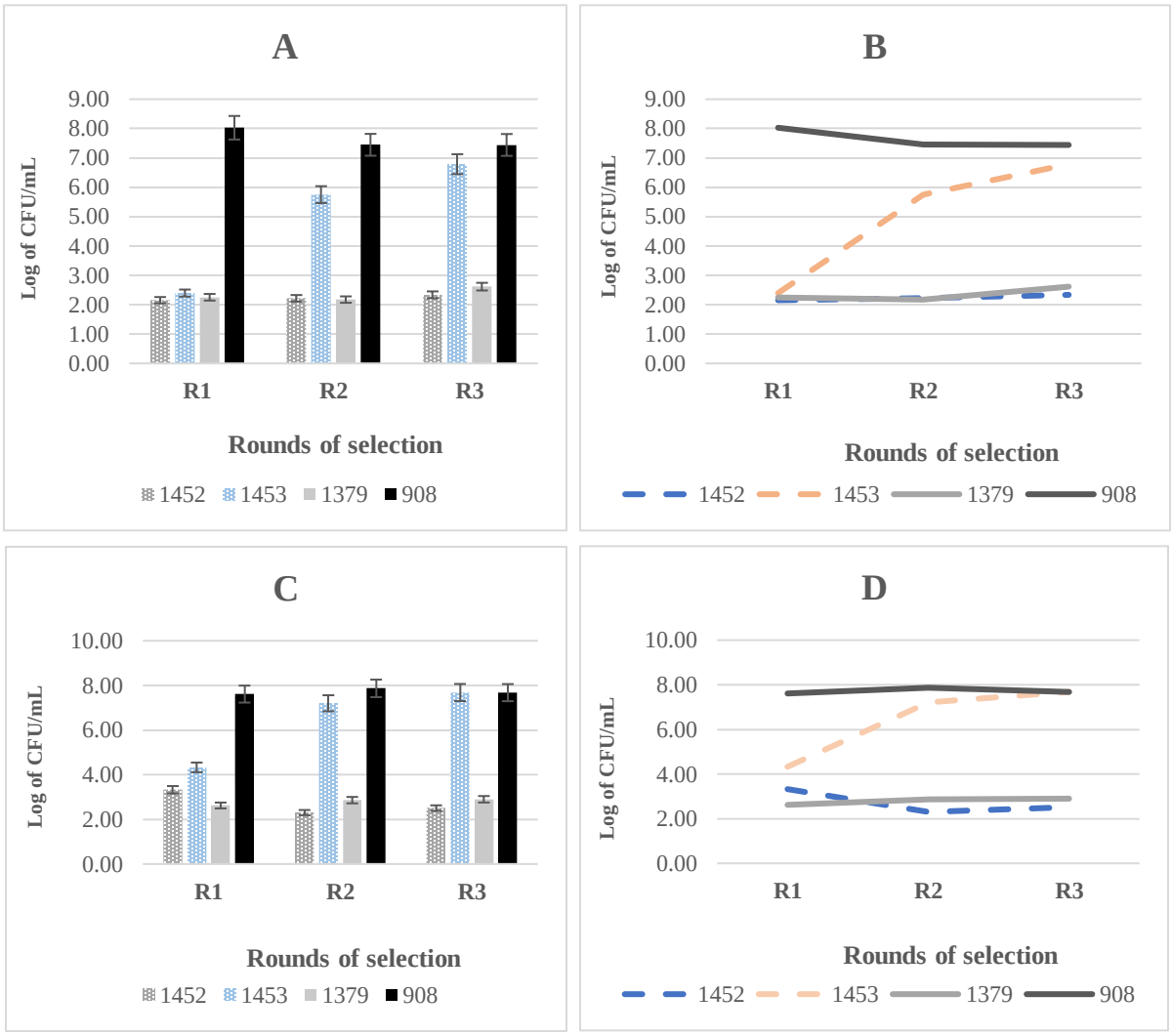

Figure 3.11: Survival/ killing activity of the transformed S. agnetis 1379, designated as S. agnetis 1452 (transformed with 908 gDNA), and S. agnetis 1453 (transformed with 908 pDNA). Panels (A) and (B) display the growth rate of S. agnetis 908, 1379, 1452 and 1453 in the cell suspension. (C), and (D) represent the growth rate of 908, 1379, 1452 and 1453 in cell lysate. S. agnetis 1379 (susceptible) was transformed with gDNA and pDNA from S. agnetis 908 isolate (resistant). Selection of the transformed mixture was performed through three rounds of growth in chicken macrophage, at MOI of 1. We concluded that, the gene(s) necessary for survival in macrophages is located on the S. agnetis 908 plasmids

3. 3. 7 The effect of trace elements supplementations on the peripheral blood monocytic cells (PBMCs)

Bird nutritional requirements have focused on the main dietary ingredients such as proteins and energy (Sirri et al., 2016). Besides that, trace mineral (TM) supplements of both organic and inorganic sources, have an essential role for a variety of animal metabolic functions. Trace minerals such as, manganese (Mn), copper (Cu), and zinc (Zn) are required to maintain the welfare and productivity in broiler chickens. The crucial role of mineral supplementation strategies in maintaining skeletal health has been well documented (Zhao et al., 2010). In order to obtain optimum bird growth and production, adequate trace minerals are routinely added to animal feeds.

The aim of this experiment was to evaluate the relationship between certain TM supplements and the performance of isolated chicken monocytes and consequently the incidence of BCO leading to lameness in broilers at the University of Arkansas research farm. We assessed the effectiveness of organic trace minerals (OTM) supplements by continuous administration of two different levels of OTM (high and low levels) into pelleted feed of broilers reared on litter flooring where the source of infection was birds infected with the chicken BCO hyper virulent strain S. agnetis 908. The chicken macrophage work has shown that this chicken BCO isolate is able to survive and kill chicken macrophage. Therefore, we evaluated the efficiency of incorporating OTM into broilers' feeds to see if these supplements have an impact on the performance of the isolated and differentiated monocytes (PBMC). Hence enhancing an immune response against lameness-inducing bacteria. Blood samples (2 mL) were collected from five randomly selected lame birds from three different pens raised on litter flooring at day 56. Isolated PBMCs were incubated at 37 ^{o} C and 5% CO_2 for 1-2 days to allow monocytes

adherence and then cultured for 5-6 days to allow cell maturation and differentiation into macrophages. Phagocytosis assay was performed by infecting cells with the hyper virulent strain S. agnetis 908 at MOI of 1.

Birds from one pen represent treatment one group (T1) which includes birds raised on standard feed (control). The other two pens represent birds of treatment two (T2) and three (T3) which were raised on feed supplemented with low (1000 mg/Kg), and high (1500 mg/Kg) levels of commercial OTM, respectively. Figure 3.12 illustrates the effect of adding high and low levels of commercial feed supplements (OTM) on the activity of the isolated chicken monocytes after in vitro challenge them with S. agnetis 908. The results at day 3 postinfection showed that the two doses of OTM influenced the isolated chicken monocytes and this was indicated by the enhanced killing activity of macrophages against S. agnetis 908 strain compared to the control or standard feed (T1). The results in figure 3.12 show that adding supplements to chickens' feed results in enhancing the immune response of the lame birds of both treatments (T2 and T3) as compared to the control or T1. Enhanced performance of the isolated monocytes was observed from the birds of T3 group ($P < 0.003$), in both culture suspension (Figure 3.12A) and lysate (Figure 3.12 B), respectively as compared to bacterial growth rate ($P = 0.028$) in cell supernatant (Figure 3.12 A) and ($P = 0.05$) in cell lysate (Figure 3.12 B) of the infected PBMC of the lame birds from T2 when compared to the control group. Reduced bacterial survival/ killing activity is represented by the log of CFU/ml. Bird# 3, from T2 group, showed no indicated bacterial growth in the culture supernatant of the infected macrophages. No considerable amount of bacterial growth was detected in the cell lysate (5-6 order of magnitude less compared to the other birds of the same group). Figure 3.12 C represents the comparison between both treatments, combining results from all five birds from the treatment. Although T2 appears to be better than T3, the T2

results are affected by bird #3 where the PBMCs were highly active against the bacteria. This one bird skewed the T2 group to have higher impact on the performance of the isolated monocytes compared to T3 group. Bird #3 in T2 may be an exceptional bird, or one that responded exceptionally well to the OTM treatment. Overall, low levels of OTM tended to significantly reduce bacterial survival/ killing activity represented by the log of CFU/ml ($P = 0.028$) in the cell supernatant (Figure 3.12 A) and reduce bacterial growth rate in the cell lysate (Figure 3.12 B) ($P = 0.05$). On the other hand, the high levels of OTM significantly reduced bacterial survival in both cell supernatant (Figure 3.12 A) and lysate (Figure 3.12 B) ($P = 0.001$) and ($P = 0.003$), respectively. These results suggest that incorporating supplements to chickens' feed can be adapted as one of the managements controls that lead to reduction of lameness incidence in broiler chickens.

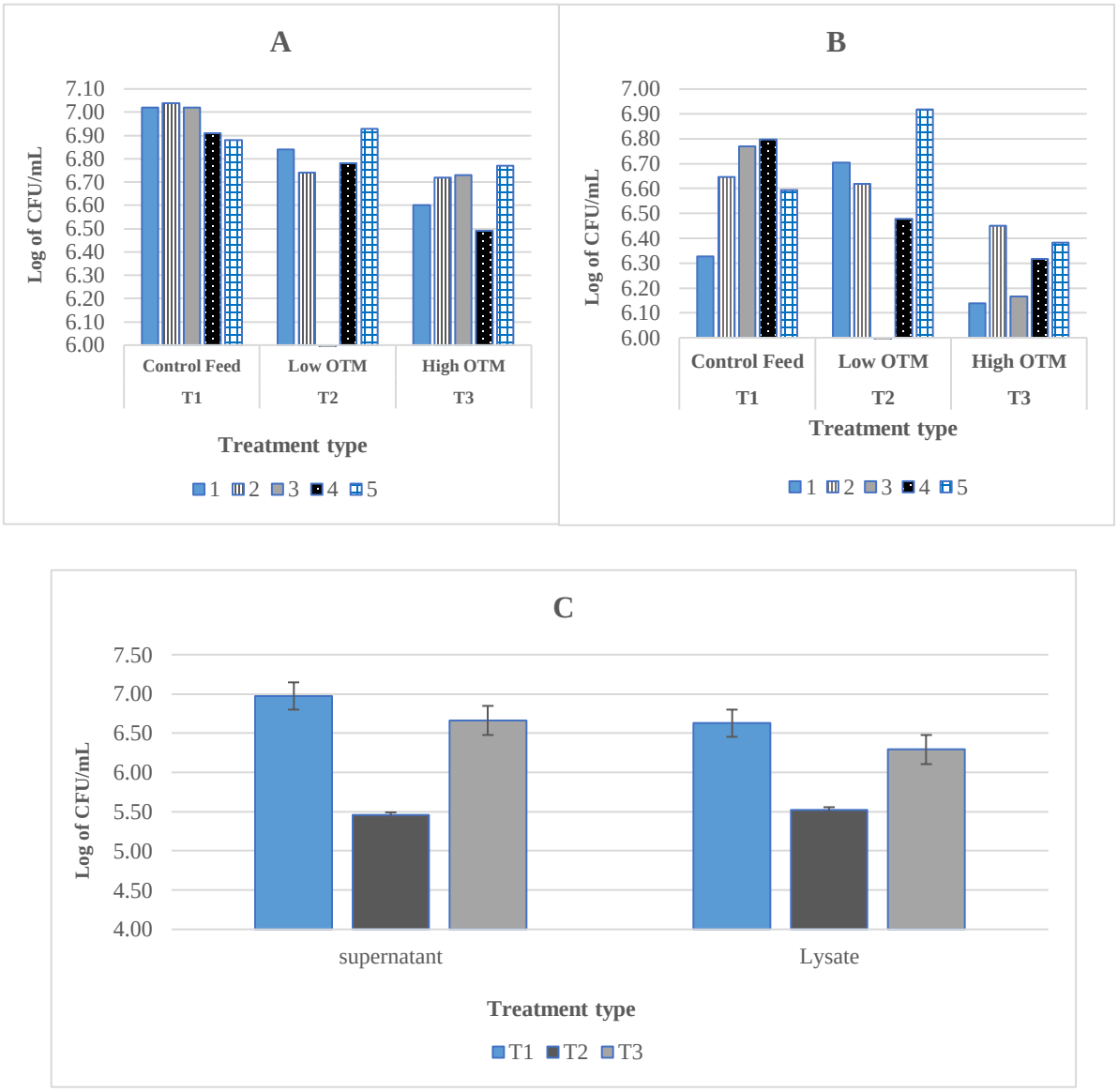

Figure 3.12: The effect of trace elements supplementations on the activity of peripheral blood monocytes (PBMCs). This figure illustrates the efficiency of incorporating OTM into broilers' feeds to evaluate their impact on the performance of the isolated and differentiated monocytes. (T1) represents birds raised on standard feed (control). (T2) and (T3) groups represent birds which were raised on feed supplemented with low (1000 mg/Kg), and high (1500 mg/Kg) levels of commercial OTM, respectively. Panel A is supernatant, Panel B is cell lysate. Panels A and B are for triplicates from individual birds (1-5) while Panel C combines all birds for each treatment.

3. 4 Discussion

This study was designed to investigate the intracellular persistence of S. agnetis, isolate 908, which was identified on our research farm at the University of Arkansas as a particularly virulent isolate. Although this isolate is from our research farm, similar isolates have been reported in chickens in Denmark (Poulsen et al., 2017). Several species of Staphylococci have been associated with bacterial chondronecrosis with osteomyelitis (BCO) leading to lameness, a serious animal welfare issue in the broiler industry. The pathogenic potential of any bacterium to cause a disease is reflected by the presence of virulence-associated factors which are essential for bacterial pathogenicity. Previous observations that many Staphylococcus strains can survive engulfment by macrophages (Baughn and Bonventre, 1975; Jakab and Green, 1976; Kubica et al., 2008), and intracellular survival has also been documented in the development and progression of chronic osteomyelitis in humans (Garzoni and kellery, 2009).

We have demonstrated that chicken macrophage cell line or HTC cells were able to internalize both S. agnetis isolates 908 and 1379 but showed finite capacity for intracellular killing of both bacterial isolates. Sustained phagocytosis for longer time periods and increased bacterial load or MOI overwhelms intracellular killing (Figure 3.2). S. agnetis 908 isolate showed hyper virulence as compared to 1379 because this chicken isolate was able to survive and kill chicken macrophages even when low doses of bacteria were used to infect macrophage. Isolate 908 has killed most of macrophage cells and been released to the extracellular environment within two days postinfection while after two days the cattle isolate showed susceptibility to be killed by macrophages. Persistence of the cattle isolate can be enhanced by increasing bacterial load or MOI (Figure 3.3). These data suggest that pathogenicity depends on the nature of the bacterial species, the number of bacteria used for co-culturing, and time of exposure. The chicken isolate

displayed the same pattern of intracellular survival/ killing activity when co-cultured for six days. Chicken macrophage failed to kill 908 isolate after six days post infection. This suggests that chicken isolate can persist via a cycle including cell lysis and bacterial re-uptake resulting in bacteraemia which play a vital role in bacterial dissemination and infection of the rapidly growing leg bones leading to chronic osteomyelitis. On the other hand, the cattle isolate has shown a similar pattern of susceptibility to be killed by chicken macrophages until day six when a burst of viable bacteria was shown in both intra and extra cellular environment (Figure 3.6). These finding demonstrate that 908 is considered as hyper virulent isolate while 1379 is the hypo virulent one because this bacterium use antiphagocytosis strategies that allow some bacterial cells to survive intracellularly but they are not sufficient to allow complete bacterial escape from infected macrophages into the growth medium until day six. These data may indicate that S. agnetis 1379 isolate persist inside chicken macrophages in continuously constant numbers for several days until the exhausted macrophages allow bacterial cells to escape into the growth media. Therefore, 1379 isolate has delayed phase of killing during which a reduction in the macrophage viability has been observed. A burst of viable bacteria in the culture supernatant may indicate that bacteria either multiplied rapidly after being internalized inside infected macrophages, with large number of the growing bacteria being liberated into the media after macrophage cell lysis, or that small number of the survivors escaped intracellular confinement and multiplied robustly in the media and consequently kill chicken macrophages in the process. In the case of S. agnetis 908, macrophages continued to accumulate further viable intracellular bacteria starting from day 2 until day 6, and the presence of high bacterial count in both culture supernatant and lysate supports the idea of bacterial persistence through extended waves of host cell lysis and bacterial re-uptake by macrophages (Figure 3.6). Comparing the survival/killing

activity of S. agnetis 908 with two other chicken BCO isolates, S. hyicus 1401 and S. agnetis 1416, these other isolates were sensitive to the killing mechanisms employed by chicken macrophages (Figure 3.7). Different rearing systems were used to induce BCO in order to study the etiology and pathology of BCO (Wideman et al., 2012; Wideman and Prisby, 2013). S. agnetis 908 was identified as the main virulent isolate associated with BCO lameness in our research farm (Al-Rubaye et al., 2015). This isolate is likely the result of years of inducing BCO. Based on our established tissue culture methods to assess intracellular survival activity, this hyper virulent strain possesses the genetic determinants or altered gene expression to promote intracellular bacteria survival and macrophage killing. Comparing the genomes of cattle S. agnetis 1379 and our chicken S. agnetis 908 has revealed that three plasmids and 50 genes in 908 that are not in 1379 (unpublished). Therefore, we used S. agnetis 1379 as a control in our studies due to its susceptibility for being killed by macrophages in three days postinfection.

The same pattern of survival/killing activity of S. agnetis 908 was observed when it was used to infect murine macrophages. However, S. agnetis 1379 showed more persistence inside murine macrophages compared to chicken macrophages. This may indicate that there is differential expression of the genes that regulate macrophages response after being exposed to this cattle isolate. Accordingly, activation of a particular signaling pathway(s) occurs and this may lead to inhibition of bactericidal activity inside murine macrophages which makes this isolate more adaptable to survive.

To investigate the impact of presence or absence of serum proteins such as complement proteins on the persistence of both S. agnetis 908 and 1379, both isolates were used to infect chicken macrophages and incubated in normal and heat-inactivated sera. Our observation that S. agnetis 1379 infection of chicken macrophages in the presence of heat-inactivated serum resulted in

significantly higher level of bacterial persistence than its ability to survive the killing exerted by macrophages incubated in normal serum. This suggests that complement proteins play an important role in the killing of 1379 isolate by bactericidal activity of the infected chicken macrophage. Consequently, the opsonin-dependent uptake of 1379 bacterial cells results in macrophage activation and killing of intracellular bacterial cells, while this bacterium escapes intracellular killing in macrophages when opsonin-independent receptors that facilitate in vitro bacterial uptake were utilized in the recognition of the cattle isolate. The presence or absence of the complement proteins has no impact on the survival/ killing activity of S. agnetis 908 which indicates that this isolate is able to modulate an intracellular persistence regardless of presence/absence of opsonins.

Many studies reported that utilization of complement receptors by intracellular pathogens to enter the macrophages, permits these pathogens to evade phagocytic pathways that trigger the respiratory burst (Wright and Silverstein, 1983). Furthermore, another study revealed that involvement of complement receptors in the inhibition of TNF-α synthesis during pathogen-macrophage interactions (Irani and Maslow, 2005). Moreover, an opsonin-independent fashion plays a vital role in the binding and entry of Listeria monocytogenes allowing a successful infection of permissive host macrophages. This fashion of bacterial adherence occurs during the very early stages of infection before mounting a successful antibody or complement-mediated host defense against this pathogen which offers intracellular persistence advantages for Listeria spp. (Pierce et al., 1996). It has been reported that receptor preference is crucial for intracellular pathogens' entry because it defines their intracellular fate by determining subsequent intracellular response of the infected host cells (Small et al., 1994; Irani and Maslow, 2005).

In the present study, the interactions between chicken macrophages and S. agnetis isolates showed that these macrophages possess mechanisms for both the opsonin-dependent and opsonin-independent bacterial uptake, and that receptor preference depends on the particular bacterial isolates.

Evolution of pathogenic variants is frequently driven by their enhanced virulence or fitness, probably associated with the acquisition of novel genes or mutations. Detection of the genes responsible for the hyper-virulent phenotypes is critical for evolving improved techniques for disease prevention and control. Also, development of genomic sequencing, assembly, and annotation technologies has greatly impacted our understanding of pathogens evolution and accordingly genotype-phenotype relationships. Direct genome evolution (DGE) was applied as a powerful and high throughput strategy to identify the genes necessary for survival in macrophages. Comparing the genomes of cattle S. agnetis 1379 with our chicken S. agnetis 908 has revealed the identification of 50 genes and 3 plasmids in 908 that are not in 1379. The list of the identified genes contains numerous potential candidates for virulence determinants including surface proteins and metabolic enzymes (unpublished). Transferring the genetic materials from hypervirulent strain to the hypo virulent one by applying DGE approach was successful. This success was indicated by the presence of hybrid/ transformed population after three rounds of selection through chicken macrophages. This produced a culture that kills chicken macrophages. Also, using less total DNA or an alkaline-SDS plasmid purification from S. agnetis 908 to transform the 1379 strain further refined which genetic determinants confer macrophage killing. We observed that the transformants with the plasmid preparation can transfer macrophage killing. Further work from our group has potentially identified two copies of the gene for

DeoxyRibose-Phosphate Aldolase as the actual determinants. These genes are on the main 2.5 Mbp chromosome and are likely being mobilized by prophage (Chen et al., 2018).

Understanding the role of macrophage killing in the virulence of this emerging chicken pathogen will impact not only BCO in poultry but also other bacterial infections as bacterial persistence inside macrophage may be a critical component for systemic dissemination of many bacterial pathogens in domestic animals. Moreover, identifying the candidate genes that are associated with enhanced virulence can also translate to human medicine regarding S. aureus infections. In human infections with S. aureus, circulating bacteria use receptors to home to sites where there is tissue damage where they adhere and produce biofilm leading to necrosis (Smeltzer and Gillaspy, 2000). Therefore, these results will be directly relevant to this particular human pathogenesis model as we believe that the model for BCO in poultry may be analogous to what is observed in humans, but the causative species of Staphylococcus may be different.

Efficient lameness management will improve animal welfare and augment overall animal performance. In the poultry industry, there are constant attempts to investigate innovative alternatives to improve flock health and productivity. Looking at broilers diet, and how this impacts their immune response is one strategy to be used to combat health issues in broilers. There is a growing demand for antibiotic-free poultry products (Centner, 2016). This will require production of broilers with robust immune response to a variety of pathogens. Therefore, poultry producers are developing techniques to improve flock health and performance by means other than antibiotics or drugs to sustain an optimal immune response. Overuse of antibiotic in animals' diet contributes to the emergence of antibiotic-resistant bacteria as a serious health threat in the poultry industry.

Virtually, all animals' diets are supplemented with trace minerals such as manganese (Mn), zinc (Zn) and copper (Cu) (Richards et al., 2010; Perez et al., 2017). These minerals are essential for a wide variety of physiological processes to maintain animal development and health. The biological importance of these minerals is represented by their involvement in DNA repair, skeletal and tissue development, antioxidant defense, feed efficiency, well feathering, enzyme structure and function, and appropriate immune response and function. Moreover, there is a growing interest in more bioavailable mineral sources such as organic trace minerals (OTM) which show more stability to pH variations along the gastro-intestinal tract, providing more digestibility/absorption, leading to better animal performance when compared to inorganic trace minerals (IMT) (Araújo et al., 2019).

Previous studies have shown that the bacteria that cause BCO lameness in broilers may translocate into the blood via the gastrointestinal tract or the pulmonary system (Al-Rubaye et al., 2017). Therefore, our pathogenic S. agnetis translocate across epithelial barriers, gain access to the blood, colonize weak points in the growth plate, and stimulate necrosis leading to lameness (Wideman and Prisby, 2013; Al-Rubaye et al., 2017). Accordingly, an inflammatory response is developed when a microbial pathogen makes it past the epithelial barriers and enters the tissue (Abbas et al., 2014). Tissue-resident macrophages sense the infection and start proper leukocyte recruitment from blood to the site of infection and tissue injury. Accumulated leukocytes include heterophils and monocytes/macrophages are activated at the site of infection to destroy pathogens by the process of phagocytosis.

A study conducted by Sirri et al., (2016), has revealed that adding OTM supplements can reduce BCO lameness lesion scores in low levels of BCO lameness. Since our chicken S. agnetis isolate has shown its ability to kill chicken macrophages, we designed this experiment to assess the role

of adding two different levels of OTM supplements to the pelleted feed on the performance of isolated and differentiated monocytes collected from lame birds. As compared to standard feed (ITM), OTM supplementation enhances chicken macrophage performance represented by increased bacterial killing/ containment inside infected macrophages. The enhanced performance of the infected macrophages suggests the importance of adding OTM in poultry nutrition due to their impact on the enhanced macrophage bactericidal activity. Therefore, these minerals from organic sources can be used as regulators of the fate and function of macrophages. Several studies have shown that breeder diets supplemented with Zn boost immunity of their progeny (Kidd et al., 2000; Virden et al., 2004). It also plays important role in developing proper disease resistance and its imbalanced amount results in bacteraemia (Kidd et al., 1992), and parasitic infectious diseases (Fraker et al., 1982). Another study has reported that incorporation of zinc-methionine into the diet of turkeys enhances macrophage recruitment and attachment and improves its ability to eliminate systemic E. coli (Kidd et al., 1994). Moreover, dietary Zn-met improves mononuclear-phagocytic performance to clear both Salmonella arizona and S. enteritidis infections in young turkeys. On the other hand, Cu is also an important dietary supplement in poultry (Miles et al., 1998). It has been demonstrated as growth promoter due to its antimicrobial activity. Inadequate dietary Cu supplementation causes reduced ability to generate superoxide anion by infected macrophages to kill the ingested microbes (Percival, 1998). The other essential trace element in supporting proper immune function in broiler chickens is Mn (Virden et al., 2003). This element plays important roles as a cofactor for superoxide dismutase (SOD) activity which is crucial for macrophage and heterophil integrity.

Both high and low levels of OTM are more efficient than ITM due to its higher bioavailability in providing adequate trace minerals (Zn, Mn, Cu) to enhance the immune capacity of the broiler

chickens. This may result in alleviating BCO lameness and those levels of reduction would be of great interest to the broiler commercial sector.

CHAPTER 4 Summary

Lameness is a significant problem resulting in millions of dollars in lost revenue annually. In commercial broilers, the most common cause of lameness is bacterial chondronecrosis with osteomyelitis (BCO). A wide range of opportunistic bacteria were found to be involved in causing BCO in broiler folks. Therefore, we designed a protocol for developing a rapid and reliable molecular diagnostic assay for the detection and identification of the major bacterial species isolated from blood and BCO lesions from the lame birds. The comparative analysis of the of the high resolution melting (HRM) profiles of the different Staphylococcal species have shown that using EvaGreen (EG) in the amplification reaction was the more suitable dye for HRM curve analysis when compared to LCGreen (LCG) and SYBRGreen I because EG gives more distinctive curves. Based on our results, EG is considered as a promising dye for qPCR and other related application. The result of amplifying V2 region using qPCR-HRM analysis has shown that this region was the best diagnostic target for species- specific identification when compared to the other examined regions. This suggests that V2 has a considerable sequence diversity among different Staphylococcal sp. except for distinguishing between the two closely- related isolates S. agnetis and S. hyicus.

The present study also confirms that raising young birds on suspended wire flooring has been proved to successfully induce lameness attributable to BCO. Our results showed that the stressed birds that were raised on wire flooring model are susceptible to bacteraemia, and that birds raised on litter flooring have significantly lower bacterial levels in their blood than those of the birds raised on wire. Moreover, our data revealed that the initial bacterial population composed of primarily Staphylococcus spp. with a few other genera detected on some occasions. The number of viable bacteria was reported to be increased with age of susceptible birds. The

signs of BCO lameness did not develop until after 44 days of age in the wire group when birds gain more body weight with the sustainable footing instability that create insufficiently strong legs to support their heavy bodies. Using qPCR-HRM analysis we found that bacterial species distribution at d 49 begins to shift and by d 56 (last day of the experiment), we primarily identified S. agnetis. This bacterial species was rarely detected in healthy birds until d 49 and was the predominant species in the circulation of the lame birds at d56. This indicates that S. agnetis has a competitive advantage in both colonization and immune evasion. The proposed pathogenesis model resembles the one used by S. aureus associated with osteomyelitis in human. Following bacterial attachment to the exposed, damaged tissues of the affected birds, S. agnetis at later stages of the disease is released in large quantities from the necrotizing tissues into the bloodstream again and this explain why lameness is chronic and persistent disease.

The negative impact on economy, duo to lameness, can be improved by determining specific risk factors and adopting efficient management strategies that influence the overall level of leg problems and consequently can reduce lameness in broilers' industry. The presence of bacteria (Bacteremia) in the blood of the lame and apparently healthy broilers has been reported. Isolation of Staphylococcus agnetis from blood samples suggests that, this bacterial species has already disseminated to the majority of tissues and body organs. This newly described pathogen, S. agnetis, has been reported to be overrepresented in BCO lesions from lame broilers at the University of Arkansas poultry research farm. Chronic and persistent infection caused by microbial pathogens is due to their ability to produce multiple virulence factors which impaired the development of proper immune response. One strategy used by pathogens to survive and persuade infection is using defensive pathways that enable them to evade the killing mechanisms applied by phagocytes. In the present study, we tried to understand the role of macrophage

killing in the virulence of this emerging pathogen as macrophage survival may be a critical component for systemic dissemination of bacterial pathogens. Therefore, we established tissue culture method to assess a virulent determinant. We have found that our chicken isolate S. agnetis 908 is resistant to killing by chicken macrophage cells in tissue culture. In fact, this isolate is internalized (phagocytosed) by the macrophage but within two days the infected macrophages die and release the bacterium. Unlike isolate 908, the cattle S. agnetis 1379 or even chicken BCO isolates of S. agnetis 1416 from food commercial farm and the closely related species S. agnetis 1401 are both killed by the chicken macrophage cells in the same time span. Thus, we have hyper and hypo virulent strains of Staphylococci that showed a great disparity in sensitivity to macrophage killing. Comparing the genomes of cattle S. agnetis 1379 with our chicken S. agnetis 908 has revealed the identification of 50 genes and 3 plasmids in 908 that are not in 1379. The list of the identified genes contains numerous potential candidates for virulence determinants including surface proteins and metabolic enzymes. We developed a system for Direct Genome Evolution (DGE) to identify the genes necessary for bacterial persistence. The results of transferring the total DNA from isolate 908 (resistant) to transform isolate 1379 (susceptible) have shown increase in survival activity of the transformants/ survivors compared to the original 1379. To further refine which genetic determinants confer macrophage killing, we observed that the transformants with purified 908 plasmids but not transformants with genomic 908 DNA can transfer macrophage killing. There is ongoing work to investigate the correlation between two potential genes (Aldolase 1 and 2), which are found to be coded by one of the three 908 plasmids in the transformants/survivors, with the pathogenesis of chicken S. agnetis 908 isolate. This study also focuses on the growing demands for antibiotic- free poultry products as well as production of broilers with robust immune response to a variety of pathogens as poultry

procedures are turning into the use of better techniques to improve flock health and performance by means other than antibiotics or drugs to sustain an optimal immune response. We assess the process of adding two different levels of OTM supplements to the pelleted feed on the performance of isolated and differentiated monocytes collected from lame birds. As compared to standard feed (ITM), OTM supplementation enhances chicken macrophage performance represented by increased bactericidal activity of the infected macrophages against our hyper virulent 908 isolate. Therefore, minerals of the organic sources can be used as regulators of the fate and function of macrophages. Enhancement of the immune capacity of the broiler chickens may result in alleviating BCO lameness and those levels of reduction would be of great interest to the broiler commercial sector.

This study was designed to make fundamental progress in understanding key components to the innate immune response against invading pathogens, relevant not only to poultry but also to humans. In addition to the direct benefit in the poultry industry in understandind a disease constituting a major animal welfare issue, studying the pathogenesis of S. agnetis in chicken BCO is an excellent model for juvenile osteomyelitis in humans since this model will be informative on new approaches to reduce human Staphylococcus infections such as septicemia and osteomyelitis.

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