Research Strategies for Prophylaxis of Enterococcal Spondylitis in Broilers

Thesis

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Abstract

Enterococcal spondylitis (ES) is a clinical syndrome caused by improper colonization of *Enterococcus cecorum* in the free thoracic vertebra (FTV) and adjacent notarium or synsacrum. Enterococcal spondylitis is a devastating disease that results in lameness which can lead to lowered feed efficiency, decreased weight gain, and mortality. Because of the relatively recent discovery and recognition of the pathogenesis for ES, research models are still under development. Induction of ES through *E. cecorum* bacterial translocation, which occurs when the intestinal barriers are damaged or weakened by stressors like pathogens, heat, dietary ingredients, etc. may accurately represent production setting conditions. Some diet additives that have been known to have antinutritional effects and cause intestinal barrier damage include rye high in non-starch polysaccharides (NSPs), animal-based proteins, and poor-quality soybean meals (pqSBM). These ingredients may be able to induce translocation of *E. cecorum* in broilers and lead to cases of ES. *Enterococcus cecorum* is a normal component of gastrointestinal flora of chickens and has been found to make up the majority of enterococcal and streptococcal flora in older birds. Since *E. cecorum* is part of normal gastrointestinal (GI) tract, finding methods to prevent translocation to other areas of the body can be challenging. Thus, promoting and maintaining the intestinal barrier is integral. Probiotics have been documented to influence gut barrier integrity via increased

tight junction gene expression. Since the gut barrier prevents transfer of potential pathogens like *E. cecorum* into the blood stream and probiotics have been shown to increase gut barrier integrity, probiotics may be able to prevent instances of ES. Two separate experiments were conducted, a series of 4 *in vitro* assays were used to determine the effects of two commercial *Bacillus* sp. probiotics, GutCare® and Ecobiol® on growth of known pathogenic strains of *E. cecorum* and an *in vivo* trial to determine if diets containing rye, meat and bone meal, and poor-quality soybean meal could result in *E. cecorum* translocation to the FTV and induce ES. The *in vitro* assays included agar overlays, agar diffusion, cell-free supernatant inhibition, and *in vitro* digestion. The agar overlays showed GutCare® produced significantly larger zones of inhibition (ZOI; $p<0.05$) than Ecobiol®, but Ecobiol® still had significantly larger ZOIs than *E. cecorum* only controls. The cell-free supernatant inhibition assay found no differences between growth of *E. cecorum* and probiotic groups. Agar diffusion assays found GutCare® to again have significantly larger ZOI ($p<0.05$) when compared to Ecobiol® and controls and Ecobiol® was only found to have significantly higher ZOIs than the control in one of two trials. Finally, in the *in vitro* digestion assay, there were no differences found between treatments in the first trial but the second and third trial found significantly increased levels ($p<0.05$) of *E. cecorum* recovery in the GutCare® challenged group when compared to other treatments. The *in vivo* experiment consisted of 5 dietary treatment groups, inoculated control (IC), 10% rye (rye), 10% rye + meat and bone meal (R-MBM), 10% rye + meat and bone meal + poor-quality SBM (R-MBM-S), and meat and bone meal + poor-quality SBM (MBM-S). Body weight and body weight gain data

found that the treatments containing pqSBM (R-MBM-S and MBM-S) had the lowest body weights ($p<0.05$) at both d15 and d35 compared to other treatment and on d35 R-MBM was also significantly smaller than the IC and Rye treatment groups. Percent body weight gain showed that growth in the R-MBM-S and MBM-S groups was heavily suppressed from d0-15 but from d15-35 maintained similar % growth as the other treatments. At d15, FTV bacterial recovery found the presence of *Enterococcus* sp. in only R-MBM-S and MBM-S groups ($p<0.05$), but no differences were found in the total bacteria recovered and on d35, there were no differences in levels of *Enterococcus* sp. and total bacteria in the FTV. Fluorescein isothiocyanate dextran (FITC-d) blood serum concentrations were elevated ($p<0.05$) in the R-MBM-S and MBM-S as compared to the R-MBM group, but no treatments showed differences when compared to the controls. On d35, no differences in serum FITC-d levels were observed. By the end of the experiment ES was determined to not have been successfully induced due to only 2 abnormal FTV's found. However, there were many cases of bacterial chondronecrosis with osteomyelitis (BCO) on the femoral head observed in the rye and R-MBM groups. The results of these experiments found that GutCare® outperformed Ecobiol® in both the agar overlay and agar diffusion tests which suggests that GutCare® could be a good candidate for future *in vivo* testing of *E. cecorum* inhibition and ES prevention. The *in vivo* experiment suggested that pqSBM was able to induce early leaky gut, but it also suppressed bird growth to a level that may not be conducive to the induction of ES, which typically affects heavy birds. The rye group maintained a similar BW as the IC group and had many cases of BCO on the femoral head which, like ES, is caused by bacterial

translocation indicating the possibility of rye to induce ES though a future experiment may need to be extended to d49 or d56 and possibly a second dose of *E. cecorum* given at d14. Overall, these studies demonstrate a potential new research model for studying ES and suggest that probiotics may serve as effective prophylactic agents to prevent bacterial lameness diseases of broilers.

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Over the last 2 years as a Master's student and 4 years working in Dr. Lisa Bielke's lab, I have learned countless lessons, developed lifelong friendships, and grown academically, professionally, and personally more than I ever expected.

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I got to start this crazy journey at the same time as Michael Trombetta. Michael and I were more like work brother and sister than anything else, clueless 19-year-olds being guided through the lab by our pseudo-father and mother, Audrey and Kaylin. Michael, Audrey, Kaylin and I could always find a way to make even the most tedious work go quickly, whether it was playing and singing to Disney princess radio on sample days, watching Hercules for the 100th time while counting oocysts, or making up endless jokes. I will look fondly back on those days for the rest of my life.

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Chapter 1. Introduction

Enterococcal spondylitis and several other lameness disorders of poultry are caused by bacteria that colonize the gut. One of the major commensal bacteria of concern is *Enterococcus cecorum* (*E. cecorum*). Within recent years, *E. cecorum* has be found as the cause for a variety of bacterial infections in broiler chickens ranging from pericarditis and splenomegaly to osteomyelitis and spondylitis (Kense and Landman, 2011; Jung and Rautenschlein, 2014). There are theories that *E. cecorum* strains are rapidly evolving while becoming more pathogenic and antimicrobial resistant (Borst et al., 2012). These bacteria make their way into the circulatory system when leaky gut exists, eventually find a place to colonize such as the free thoracic vertebra (FTV) or epiphysis of weight-bearing long bones and establish abscesses. By the time lameness is observed in birds, the disease-causing events have already passed and treatment becomes difficult. Thus, prevention by controlling gut inflammation and decreasing levels of bacteria known to colonize bone structures are key to controlling these diseases. In order for advancements towards treatments and preventatives for these diseases, a reliable model needs to be produced. Since *E. cecorum* relies on bacterial translocation to induce disease, known insults to gastrointestinal immune system may best serve as an *E. cecorum* infection induction method. One prominent insult is diets that include antinutritional additives including diets with high non-starch polysaccharide levels, animal-based protein sources,

and poor-quality soybean meal. After a model is established, methods of prevention can be established.

With the banned use of antibiotics, probiotics have become a front runner as possible treatments and prevention for a plethora of diseases. Probiotics selected for activity against species like *E. cecorum*, that can be included in feed as direct fed microbials (DFM), historically have good ability to manage a variety of bacteriological diseases, especially *Bacillus* species due to their notorious enzyme production capabilities. This thesis describes the role of enteric inflammation, leaky gut, and pathogens in development of lameness diseases in broilers, as well as the potential for probiotics to prevent enterococcal spondylitis, describes *in vitro* assays conducted to determine the ability of two *Bacillus* sp. probiotics to inhibit growth of *E. cecorum*, and to determine if the inclusion of feed additives that have known negative effects on gastrointestinal health and immunity can result in increased levels of *E. cecorum* translocation and enterococcal spondylitis.

Chapter 2. Review of Literature

Inflammation and Immune System

The gastrointestinal (GI) tract serves as one of the most important immune organs in the body by working to maintain homeostasis and reacting to threats with potential to disrupt this state. The intestinal immune system is presented with more threats than the systemic immune response as it has to defend maintain mucosal integrity in the face of a diverse array of microflora, dietary components, and is affected by external stressors such as environmental temperatures or air quality. The gastrointestinal immune system is made up of several barriers, most of which are part of the innate immune system including macrophages, granulocytes, dendritic cells, and epithelial cells (Cardoso Dal Pont et al., 2020; Kogut et al., 2018). Cells of the innate immune system express pattern recognition receptors (PRRs) that can respond to microbial threats, specifically pathogen-associated molecular patterns and damage-associated molecular patterns (Cardoso Dal Pont et al., 2020). Once activated, PRRs signal innate immune responses such as secretion of pro-inflammatory cytokines, complement fixation, and macrophage activation. The intestinal barrier is made up components comprising of mucus, phospholipids, tight junctions, lymphocytes, and gut microbiota (Stewart et al., 2017). When functioning properly, the intestinal barrier prevents luminal contents, including bacteria, from entering the blood stream in an uncontrolled manner, which can result in inflammation and potentially

disease (Stewart et al., 2017). The GI barriers contain the microbiota and potential pathogens within the luminal side of intestinal epithelium and try to eliminate any microbes that make it past the barrier (Kogut et al., 2018).

It is important to maintain the intestinal barriers as poor integrity has been found to have an impact on bone health, as measured through *E. cecorum* in the FTV and tibia strength (Bielke et al., 2017). Barriers of the GI tract start with the outer mucus layer that covers the entire epithelium and contains microbiota. Next is the inner mucus layer which, in a healthy GI tract, does not contain bacteria, but has antimicrobial proteins and IgA produced by B-cells that can kill penetrating bacteria (Hooper, 2009). The mucus layers, made of mucins, are secreted by specialized cells along the epithelium called goblet cells (Schroeder, 2019). Beyond mucin production, goblet cells are also thought to aid in immune regulation and they act as protectors of the luminal epithelial cells (Dao and Le, 2022). The IgA reduces epithelia-adherent bacteria in the inner mucus layer by initiating immune system activation and blocking toxin and virus activity. IgA can also travel to the outer mucus layer and shape the microbial composition (Hooper, 2009). Another major role of the immune system is to maintain the diverse microbial community and tolerate commensal bacteria (Kogut et al., 2018).

Constant exposure to pathogenic and non-pathogenic insults results in activation of the GI innate immune system in an attempt to maintain homeostasis. When there is a deviation from homeostasis, inflammation occurs (Chovatiya and Medzhitov, 2014). The purpose of inflammation is to signal need for repair of damaged tissue after injury or insult. Inflammation can be a result of both a stress and a defensive response brought on

by changes in regulating variables such as oxygen and protein folding, detected by sensors whereas defense response is initiated by insults that can disrupt homeostasis (Chovatiya and Medzhitov, 2014). Inflammatory reactions can vary greatly and are adaptable depending on the condition they are responding to.

The types of inflammation can be broken down into four categories defined by Kogut et al., 2018 as physiological, pathological, sterile, and metabolic. Physiological inflammation occurs when the immune system is fending off pathogens and preventing them from reaching the microflora while also maintaining tolerance for commensal microbes. During physical inflammation there is a symbiotic relationship between the GI immune system and the microbiome, each influences the other's function, composition, and development. When a microbial infection or injury to tissue occurs, physiological inflammation transforms into pathological type, typically considered a "true inflammation" brought on by several triggers, two of which are the presence of microbe-specific molecules (MAMPs) from microbial pathogens and the release of nucleic acids into the extracellular space after cell death and tissue injury. Sterile inflammation occurs in situations where there are non-microbial environmental stressors. Oxidative stress, microbiota-derived components, and metabolic stimuli such as non-starch polysaccharides (NSPs) in the diet can all lead to sterile inflammation, which can be chronic due to the extended nature of exposure to this class of irritants. A major dietary stressor for poultry is rye grain, which contains large amounts of insoluble NSPs not readily digested by domestic poultry. While sterile inflammation is typically low-grade and long-term, it can result in an increased susceptibility to infections, lowered feed

intake, and muscle catabolism. The final type is metabolic inflammation which occurs when an excessive amount of nutrients are consumed, resulting in a metabolic surplus, leading to metabolic disfunction. As a byproduct of the poultry industry's drive towards larger, faster growing broilers, over consumption of nutrients has resulted in negative impacts on the gut immune regulation, dysbiosis of the gut microbiota, and excess fatty acids and carbohydrates leading to systemic inflammation and metabolic disease that can serve as precursors to infectious diseases, especially in cases of increased GI permeability that allows bacteria to transfer from the mucosal surface to blood circulation.

Nutrition

The poultry industry strives to produce fast-growing, efficient, large birds and one of the most important factors needed to achieve that is proper nutrition. Cardoso Dal Pont et al. in 2020 outlined several potentially damaging feed components outlined below.

Non-starch polysaccharides (NSPs) are carbohydrates found in plants and cannot be digested by monogastric animals, though some can be broken down by typical gut microbiota, those of poultry typically do not efficiently break down fiber to digestible forms (Cardoso Dal Pont et al., 2020). NSP's are split into several categories including chemical groups like cellulose, non-cellulosic polysaccharides, and pectin polymers and types of solubility. Some common soluble NSPs used in feed are rye, wheat, barley, and oats. When soluble NSPs come in contact with the mucus lining of intestines they form a thick slime called hydrocolloids that increase viscosity and decrease nutrient absorption by lowering contact of feed with enzymes, enterocytes, and digestive secretions. The lowered nutrient absorption then affects both feed conversion ratio (FCR) and body

weight gain (BWG). This increased viscosity slows feed passage which can lead to an increased growth of bacteria, including pathogens (Slominski, 2011). Some of these effects can be mitigated by the use of probiotics that produce enzymes capable of breaking down NSPs.

Another feed component that can result in GI problems when improperly fed is protein. Protein is a necessary nutrient and serves many vital functions but when overfed or comes from low quality sources, intestinal disease and inflammation can occur. One function of protein is to provide nitrogen to colonic microorganisms but when too much protein is available there can be an increase in putrefactive fermentation products including ammonia and phenols. These products have carcinogenic, genotoxic, and cytotoxic effects and an increase within the ceca has been shown to cause shorter villus heights and larger crypt depths, which can lower nutrient absorption (Qaisrani et al., 2014). The source of protein also can play an important factor in GI health of birds. Protein that is from animal sources, such as meat and bone meal, can predispose birds to necrotic enteritis due to increased growth of *Clostridium perfringens* (Dahiya et al., 2006).

Soybean meal (SBM) is a widely used protein source for broiler feeds but without careful monitoring of production, SBM could result in dietary problems. Undercooked SBM can contain several anti-nutritional factors including trypsin inhibitors and urease. Trypsin inhibitors can cause many harmful effects in broilers as they lower proteolytic activity, which decreases free amino acid levels (Han et al., 1991). They can also result in pancreatic hypertrophy in young birds due to increased pancreatic secretions (Han et al.,

1991). Soybean meal also varies greatly between locations grown, variety, and methods of storage and, one concern from this variability is lectin levels. Lectins are a glycoprotein that, when ingested, bind to enterocytes and disrupt the brush border membrane and enzymes which results in lowered nutrient digestibility (Fasina et al., 2003). Just like raw SBM, overcooked SBM can have detrimental effects in birds. Some of the major concerns with overcooking include, lowered amino acid availability, lowered protein nutritional value, lysine derivatization, and sulfur and methionine oxidation (Lee and Garlich, 1992). There is also a potential link to lowered chick growth when fed overcooked soybean meal (Lee and Garlich, 1992). Taken together, all of these factors related to regionality, strain, and cooking quality stress the importance of high quality SBM in promoting bird health by affecting inflammatory factors.

Enterococcal Spondylitis

Enterococcal spondylitis (ES) is a clinical syndrome caused by improper colonization of *Enterococcus cecorum* within free thoracic vertebra (FTV) and adjacent notarium or synsacrum. This disease manifests as paraparesis or paralysis of hind limbs due to an inflammatory mass of *E. cecorum* infection on the FTV that compresses the thoracolumbar spinal cord, it can also infect and deteriorate the femoral head (Borst et al., 2017b). Clinical signs of ES include hock-sitting or dog-sitting where the bird sits far back on its tail with outstretched legs, being alert but hunchbacked, bilateral lameness, and wing-walking (Robbins et al., 2012). Due to this manifestation, ES is a devastating

disease that results in lameness which can lead to lowered feed efficiency, decreased weight gain, and mortality.

Enterococcal spondylitis is a rather new disease with the first appearances occuring in the 2000's. There is speculation that changes in management practices have made broilers more likely to be exposed to pathogenic *E. cecorum* and that they are more susceptible to infection due to increasingly larger and faster growing birds. The FTV functions as the only vertebra with weight-bearing articulations and, therefore, is under constant stress (Borst et al., 2017a). Birds with ES typically begin to show clinical signs between 6 and 10 weeks and can result in up to 15% mortality (Martin et al., 2011). These are typically a result of dehydration or starvation from lack of mobility. Additionally, processing plant condemnation rates increase due to dehydration and scratching that results from lowered mobility in birds with ES (Jung et al., 2018).

Probiotics

In 2014 the International Scientific Association for Probiotics and Prebiotics published a consensus statement defining probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill et al., 2014). With a reduction in antibiotics usage in poultry production, especially subtherapeutic levels used for growth promotion and disease prophylaxis, probiotics have become a popular means to improve health and reduce enteric inflammation. Probiotics have been found to reduce mortality, improve body weight gain and decrease feed conversion ratios (Jin et al., 1997). Introduction of probiotics to the GI tract have also been reported to

reduce intestinal permeability (Bajagai et al., 2016). Those used in poultry diets typically include one or more of the following genera of *Bifidobacterium, Lactococcus, Lactobacillus, Bacillus, Streptococcus,* and the yeast *Candida* (Jha et al., 2020). When choosing a probiotic, some factors to consider include adherence to GI mucosa, ability to survive GI conditions, and competitive exclusion of pathogens. *Bacillus subtilis* has been found to help strengthen the immune system and improve gut health and *Bacillus amyloliquefaciens* also improves gut health as well as growth performance (Jha et al., 2020). In a study by Jha et al. in 2020, probiotic fed broilers showed improved intestinal barrier function and larger crypt to depth ratios than their non-probiotic fed counterparts. The study also observed that *Bacillus subtilis* fed broilers inoculated with *Escherichia coli* maintained higher digestibility of fiber, crude protein, and gross energy.

Bacillus subtilis secretes several enzymes, including lipase, amylase, and protease which aid in digestion and nutrient utilization (Chen et al., 2009). Additionally, *B. subtilitis* for production of antimicrobial compounds, notably bioactive metabolites with direct activity against other bacteria called bacteriocins (Caulier et al., 2019). A previous study found that, in an *in vitro* setting, *Bacillus sp.* cell-free supernatants (CFS) were able to inhibit growth in some strains of *E. cecorum* (Medina Fernández et al., 2019). *Bacillus amyloliquefaciens* produces enzymes that are able to aid in the digestion of antinutritional factors, such as NSPs, found in feed while also being tolerant of bile and acid in the GI tract, potentially reducing NSP-associated enteric inflammation (Farhat-Khemakhem et al., 2018). Some strains of *B. amyloliquefaciens* have been identified as producers of antimicrobial peptides that make them good potential candidates for probiotics against

infectious diseases (Arias et al., 2013). This infers that probiotics may be able to act as a growth inhibitor to *E. cecorum* while also strengthening intestinal barriers.

Enterococcus cecorum

Enterococcus cecorum is a Gram-positive, catalase-negative cocci bacteria that is commensally found in many different species including *Gallus gallus*. It was initially categorized as a *Streptococcus*, but within the last 20 years *E. cecorum* has become an "emerging pathogen" and is causing great concern among the poultry industries (Dolka et al., 2016). Associations between *E. cecorum* and osteomyelitis were first reported in North America (Gingerich, 2009) and Belgium (Herdt et al., 2009) in 2009 and cases have also been noted in Germany, Hungary, Iran, Poland, South Africa, and Switzerland (Jung et al., 2018). More recently, there are signs that *E. cecorum* may be causing disease earlier in life. A study conducted in 2014 found that *E. cecorum* can be isolated from yolk sacs of birds at just 3 days of age (Jung and Rautenschlein, 2014). Flocks with pathogenic strains of *E. cecorum* have also shown increased incidence of sepsis related death at 2-3 weeks of age (Jung et al., 2018).

Enterococcus cecorum infections can be broken down into two phases, septic phase and skeletal infection. The septic phase is often subclinical and can reside in the yolk sac or spleen but have been associated with increases in mortality noted around 2-3 weeks post hatch (Jung et al., 2018). In addition, lesions containing pure cultures of *E. cecorum* can occur on the heart (pericarditis), liver (perihepatitis), and spleen (splenomegaly) (Kense and Landman, 2011; Jung and Rautenschlein, 2014). During the skeletal infection phase, mortality rate is much higher and broilers take on a more clinical

form of the disease. Lameness is most commonly caused by lesions on the FTV and necrosis of the femoral head but can also be present in the stifle, hock, and synovitis (Jung et al., 2018).

Cases of enterococcal spondylitis and osteomyelitis appearing several decades after *E. cecorum* was first characterized suggests that a new *E. cecorum* strain may be the cause of a spike in ES cases (Boerlin et al., 2012). Though the pathogenicity *E. cecorum* it mostly unknown, one of the leading theories is that clones of *E. cecorum* are vertically spread with increased pathogenicity as well as antimicrobial resistance (Borst et al., 2012). Enterococci are known to develop antimicrobial resistance and have been observed in *E. cecorum* strains isolated from chickens (Cauwerts et al., 2007). There are also more similarities between the *E. cecorum* strains found in pathogenic *E. cecorum* infected birds than there are between *E. cecorum* strains present in healthy birds (Boerlin et al., 2012).

Molecular epidemiology studies suggest that pathogenic strains of *E. cecorum* have emerged with higher mannitol metabolism and multi-antibiotic resistance (Borst et al., 2012). Isolate variations in antimicrobial resistance suggest that some were acquired and emphasize the need for non-antibiotic control strategies in poultry flocks. Furthermore, genomic comparison of commensal and pathogenic *E. cecorum* isolates revealed virulence genes typically associated with streptococci and *E. feacalis*, including cell wall associated proteins that allow bacteria to adhere to host extracellular matrices (Borst et al., 2015). This may be an important pathogenicity factor that allows pathogenic strains of *E. cecorum* to bind to cartilage and bone in FTV and femoral head regions.

Thus, control of enteric inflammation resulting in mucosal permeability becomes a critical factor in control of lameness diseases, including ES.

In-vitro Assays

Assays conducted in laboratories can help determine ability of probiotics to perform desired functions before moving them to animal studies. *In-vitro* tests tend to cost less, are quicker to results, and can provide important information regarding characteristics of bacterial probiotics candidates such as levels of bacteriocin production, germination capacity, and activity against pathogens. The assays described below are common techniques used to characterize probiotics to determine their capacity to act against pathogens before transferring to bird studies.

i) Cell-free Supernatant Inhibition Assay

The cell-free supernatant (CFS) inhibition assay can be used to test if extracellular components produced by probiotics can inhibit the growth of pathogens. *Bacillus sp.* produce exogenous products like enzymes and many antimicrobial compounds including bacteriocins, peptide antibiotics, and lipopeptide antibiotics (Abriouel et al., 2011). These antimicrobial compounds can kill or inhibit the growth of pathogens such as *E. cecorum*. By filtering out the bacteria and only leaving the products produced, the effect of the products can be tested against the pathogen without the influence of direct competitive exclusion.

ii) *Agar Overlay*

Agar overlay is an assay technique that can test if a bacterial strain produces compounds that can slow or halt the growth of another bacterial strain (Hockett and Baltrus, 2017). Agar overlays were first characterized by Gratia in 1936. This technique is often used by scientists looking for bacteriophages, bacteriocins, and antimicrobial products. When using this technique, the goal is to identify differences in the growth of the overlaid bacteria, detected by a halo, around the underlaid bacterial colony.

iii) Agar Diffusion Assay

Agar diffusion assays have a very similar goal to the agar overlay to test if the products of one bacteria will produce changes in the growth of a second bacteria. In this assay, the bacteria that is attempting to be inhibited is spread across an agar plate and the second bacteria is dropped into a hole punched in the agar that allows extracellular components to be diffused into the agar on a filter paper disk (Finn, 1959). This test works as an indicator for low level antimicrobial compounds produced by the diffused bacteria and susceptibility of the bacteria that was spread on the plate.

iv) Gut Digestion Assay

Gut digestion assays allow researchers to partially simulate the effect of the GI tract on various consumables including feed, probiotics, and pathogens. The assay simulates three of the main stages of digestion in poultry, crop, proventriculus, and intestine, and at each stage the assay mimics components the GI tract using several factors including, pH, enzymes, agitation, temperature, time, and oxygen availability (Sharma et al., 2022). With samples taken at the beginning of the assay as well as after each step, researchers can map out the growth or lack of growth for bacteria included

within the test. Gut digestion assays can also be used to test the ability of a bacteria to change the growth of other bacteria either through competitive exclusion or production of bactericidal compounds.

Bacterial Translocation

Bacterial translocation or "leaky gut" is described as the movement of viable indigenous bacteria from the GI tract to elsewhere in the body (Berg, 1995). The route of passage from the GI tract to circulation begins with either intracellular or extracellular passage through the intestinal barriers. From this passage the bacteria travels through hepatic circulation to the liver (Berg, 1995). From the liver the bacteria then can travel to various locations in the body including the FTV and femoral heads. Three major causes of bacterial translocation were outlined by R. D. Berg as intestinal bacterial overgrowth, deficiencies in the host immune defenses, and increases permeability of the intestinal tract from injury to the intestinal mucosa.

There are many immune system components within the GI tract, and failure of any of these components can result in bacterial translocation. Secretory immunoglobulin A prevents bacteria from physically contacting the mucosal epithelium to prevent bacterial penetration (Berg, 1995). Another layer of defense includes macrophages and T cells in the lamina propria and lymphoid organs. Peyer's patches are also able to sample antigens and activate B- and T-cells.

Mucus produced by goblet cells, acid and enzymes, the epithelial cell barrier, which includes the tight junctions, enterocytes, goblet cells, enteroendocrine cells, transit-amplifying cells, planet cells, and stem cells (Zhang et al., 2019), and bowel motility all

play important roles in the GI tract defense against bacterial translocation (Wiest and Rath, 2003). Any physical disruptions or injury to the mechanics listed above can result in translocation. Tight junctions bind intestinal epithelial cells together with occludin and tricellulin, claudins, junction adhesion molecules, and coxsackie virus and adenovirus receptors (Awad et al., 2017). Tight junctions are responsible for regulating paracellular passage of nutrients from the luminal to the basolateral space (Awad et al., 2017).

Physical damage to the GI tract immune barriers can result from many events. Oxidative stress often results from a combination of stressors that commercial broilers are subjected to including, heat stress and feed toxins. Products of oxidative stress can damage the mucous membrane and cause peroxidation of the cell membrane lipids and lipoproteins (Mishra and Jha, 2019). Parasites such as *Eimeria* that reproduce in the epithelial layer of the GI tract also cause serious physical harm and cell death that adds to incidence of bacterial translocation. Certain feeds such as those containing NSPs cause changes in digesta viscosity which has been found to increase bacterial translocation (Tellez et al., 2015).

Fluorescein isothiocyanate-dextran (FITC-d) can be used as an indicator of intestinal permeability. The FITC-d commonly used with broiler intestinal permeability detection has a molecular size of about 4-kDa is large enough to only pass through a compromised intestinal barrier (Liu et al., 2021). The fluorescent molecule is administered as an oral gavage dose of 4.16 mg/kg and 2 hours post-gavage blood samples are collected (Vicuña et al., 2015). Fluorescence can then be compared using a

standard curve and measurements taken with a microplate reader. Higher levels of fluorescence can be equated to increased paracellular permeability (Liu et al., 2021).

Conclusion

In recent years, ES, as well as other *E. cecorum* based diseases, have become a key threat to bird health, growth efficiency, and profits in the broiler industry. With consumer pressure and new legislation requiring poultry to be raised without the use of antibiotics, researchers need to find ways to treat diseases in new ways. *Enterococcus cecorum* is naturally found in the GI tract but has become increasingly more pathogenic and is not showing any signs of slowing down. As an opportunistic pathogen, *E. cecorum* needs to be able to translocate from the GI tract to elsewhere in the body, making gut barrier integrity extremely important for preventing disease. Broilers encounter many predisposing factors that allow bacterial translocation such as heat stress, dietary stressors, and unrelated disease/infection.

There is not currently a good model for the induction of clinical ES, especially in birds under 5 weeks, which can make research on potential prevention methods very difficult. There are several potential feed inclusions that could induce leaky gut in broilers. Rye has high concentrations of NSPs that create highly viscus digesta which lowers nutrient absorption and increases pathogenic bacteria growth. High protein levels as well as animal derived proteins such as meat and bone meal can produce excess putrefactive fermentation products that result in decreased villi height to crypt depth ratios and may predispose birds to another opportunistic disease, necrotic enteritis. Also, low quality SBM can contain trypsin inhibitors, urease, lectins, low amino acid

availability, and low value protein. The addition of these feedstuffs many be able to induce ES in broilers and act as a model for further research involving potential preventatives and treatments for ES.

Probiotics have been found to improve the integrity of the intestinal barrier and competitively exclude pathogens such as *E. cecorum*. Two of the probiotics that may act to inhibit *E. cecorum* growth are *Bacillus subtilis* and *Bacillus amyloliquefaciens*. It is logical to think that the introduction of probiotics may be able to limit the growth of *E. cecorum* as well as prevent its ability to translocate to the FTV and other areas of the body, thus decreasing lameness diseases such as ES.

Chapter 3. *In vitro* evaluation of *Bacillus* direct fed microbials for activity against *Enterococcus cecorum*

Abstract

Enterococcus cecorum is a commensal bacteria found in the gastrointestinal tract of most poultry species. In recent years, the pathogenicity of *E. cecorum* has increased and has been found to cause various diseases including enterococcal spondylitis. Pathogenesis of the diseases, with infection occurring long before clinical signs, indicates that prophylaxis, instead of therapeutic antibiotics, is the best method of control. Probiotics have shown many beneficial effects in aiding poultry growth, digestion, and immune health and may be able to limit or inhibit the growth of pathogens such as *E. cecorum* as a method to prevent development of disease. This series of experiments sought to test the ability of two commercially available probiotics, GutCare® and Ecobiol®, to inhibit or slow the growth of known pathogenic strains of *E. cecorum*. Four *in vitro* assays were performed: agar overlays, agar diffusion, cell-free supernatant inhibition, and *in vitro* digestion. The agar overlay assay found that *E. cecorum* strains challenged with GutCare® produced the largest zones of inhibition (ZOI; $p<0.05$) and the *E. cecorum* strains tested against Ecobiol® also had significantly larger ZOI than control plates, but a lower ZOI than GutCare®. In the cell-free supernatant (CFS) assay no differences were found between the *E. cecorum* control and probiotic groups. Agar diffusion assays also found significantly larger ZOIs in the *E. cecorum* strains challenged with GutCare® than the other two treatments and the first trial found the ZOI of *E. cecorum* strains challenged with Ecobiol® to be significantly higher than the *E. cecorum*

only control, but no differences were discovered between them in the second trial. In the *in vitro* digestion assay no differences between treatments were found in the first trial but in the second and third trials the *E. cecorum* strains challenged with GutCare® was found to have significantly higher levels of *E. cecorum* recovery than the other treatments. While there was variability between the assays, it appears that GutCare® was able to inhibit the growth of the *E. cecorum* in both the agar overlay and agar diffusion assays better than Ecobiol® which may make GutCare® a suitable probiotic to inhibit or lower *E. cecorum* levels in the GI tract of broilers and may also prevent enterococcal spondylitis from developing.

Introduction

Enterococcus cecorum is a species of bacteria historically identified as harmless within the gastrointestinal tract (GIT) of chickens. However, the number of Enterococcal spondylitis (ES) incidents has been on the rise in broilers after six weeks of age, with more recent reports of birds as young as two weeks of age (Jung et al., 2018). The bacteria find their way to the free thoracic vertebra causing an abscess which forms between cartilage and bone push on the spinal cord, causing lameness and arching of affected bird's back with legs splayed, referred to as dog sitting. As an opportunistic pathogen, *E. cecorum* must translocate from the gastrointestinal (GI) tract to blood circulation as part of the pathogenicity cycle. Some reports of flock history and research models describe previous incidents of enteric disease or inflammation that may serve as predisposing factors to ES (Wideman and Prisby, 2013; Borst et al., 2017a).

Enteric inflammation models have been established and utilized to produce bacterial translocation (BT), measured as increased aerobic bacterial recovery in the liver (Kuttappan et al., 2015; Latorre et al., 2018). This can lead to a bacteremia phase of infection with *E. cecorum* that leads to a skeletal phase in which bacteria colonize within free thoracic vertebrae (FTV). Notably, a predisposing osteochondrotic condition has been linked to increased rates of ES in broilers (Chen et al., 2018). However, osteochondrosis alone does not necessarily lead to ES, and presumably, if bacterial translocation of *E. cecorum* can be prevented, cases of ES will subsequently remain low.

As such, probiotics with the ability to reduce enteric inflammation and/or decrease incidence of *E. cecorum* with the GI tract, may prevent ES. *Bacillus* species, historically noted for enzyme production that include bacteriocins, may serve as candidate probiotics to decrease levels of *E. cecorum*. *In vitro* tests characterizing potential functional probiotics, especially strains known to produce bacteriocins, can provide valuable information regarding spectrum of activity, germination cycles, and effective dosing ranges before *in vivo* testing begins. The following studies sought to characterize two probiotic strains, Ecobiol® and GutCare® (Evonik Industries, Essen, Germany) for their anti-*Enterococcus* activity as potential functional probiotics for the prevention of ES in broiler flocks.

Materials and Methods

Bacterial Isolates and Preparation

Three *E. cecorum* isolates from field cases of ES were provided by the Texas A&M Veterinary Medical Diagnostic Laboratory including, *E. cecorum* 11 TXs, *E.*

cecorum 11 TXb, and *E. cecorum* 09 TXs. In the cell-free supernatant inhibition assay and agar overlays *E. cecorum* 11 TXs and *E. cecorum* 11 TXb were used and tested independently. In the agar diffusion test and *in vitro* digestion assays all three strains were tested as a combined *E. cecorum* using equal inclusions from each strain. Prior to each experiment, isolates individually stored at -80 °C were thawed and grown independently in tryptic soy broth (TSB) at 37 °C overnight in anaerobic conditions. Cultures were spectrophotometrically measured to an absorbance estimated to equal approximately 1 x 10^8 CFU/ml, then diluted based on the specifications of the assay, and actual CFU/g retrospectively determined by serial dilution plating, reported in Tables 1, 2, 3, and 4. In the agar diffusion and digestion assays all three strains of *E. cecorum* were combined equally after being measured at ~1 x 10^8 CFU/ml before further dilution. *Bacillus subtilitis* (GutCare®, Evonik Industries, Essen, Germany) and *Bacillus amyloliquifaceans* (Ecobiol®, Evonik Industries, Essen, Germany) were provided in commercial spore form at a concentration of 2 x 10^9 CFU/g and 1 x 10^9 CFU/g, respectively. Spores were directly used in the *in vitro* digestion assay to simulate direct fed microbials. In all other assays, aliquots stored in a -80 °C freezer were thawed, added to culture medium specified by the assay, and grown overnight in a 37 °C aerobic forced air incubator. For the agar diffusion assay, concentrations of the probiotic were serial dilution plated on tryptic soy agar (TSA) to determine inoculation concentrations and are reported in Table 3. In the digestion simulation assay, probiotic strains were weighed to provide the needed concentration of bacteria and retrospectively plated on TSA to confirm inclusion rate, reported in Table 4.

Agar Overlay Tests

Ability of each probiotic strain to independently inhibit growth of *E. cecorum* was measured with agar overlay tests in which *Bacillus* was pre-grown on TSA and followed by an overlay of TSA containing *E. cecorum*. Probiotics were tested independently with a 10 µL drop of aliquoted probiotic in the center of TSA plates and *Bacillus* were incubated overnight at 37 °C in an aerobic incubator to produce a colony. Overlay agar containing 1 mL of overnight *E. cecorum* culture and 10 mL warm liquid TSA were poured over the *Bacillus*-containing agar plate to create second layer of agar and plates were incubated at 37 °C in anaerobic conditions. Zones of inhibition were measured after 24h of incubation and pathogen inhibition scores (PIS) calculated as $\frac{diameter\ of\ inhibition\ zone}{diameter\ of\ bacterial\ colony} = PIS.$

Enterococcus cecorum 11 TXb and *Bacillus* combinations were tested four times against *E. cecorum* 11 TXb and three times against *E. cecorum* 11 TXs with 15 agar overlay plates per treatment. Zones were measured in three different places on each plate, averaged to calculate a mean zone per plate, with data reported as mean of zones per treatment, per experiment.

Cell-free Supernatant Inhibition Assay

To test the effect of probiotic secreted proteins and metabolites against *E. cecorum*, a cell free supernatant assay was conducted against the wild-type isolates. *Bacillus* probiotics were tested individually against each strain of *E. cecorum*. To begin, each probiotic was prepared in TSA, as described above, then centrifuged for 15 min at 5,000 x *g* to remove large components, followed by filtration at 0.2 µm to create a cell free supernatant (CFS), which was diluted in sterile saline. Cell-free supernatants were plated

on TSA and incubated overnight at 37 °C aerobically to confirm that no viable cells remained. *E. cecorum* was grown as described above and diluted in sterile saline to ~1x10^4 CFU/mL. Four groups were established: i. medium only; ii. medium + 1% v/v *E. cecorum*; iii. medium + 10% v/v CFS; and iv. medium + 1% v/v *E. cecorum* + 10% v/v CFS. All combinations were placed into a 96-well plate with 16 replicates of each treatment at a volume of 300 µL per well and incubated anaerobically at 37 °C while OD at 625nm was measured with a BioTek plate reader every 30 min until treatment ii *E. cecorum* control reached an optical density (OD) of 0.4. Percent inhibition was calculated as the percent decrease in OD of each treatment compared to ii *E. cecorum* control.

Agar Diffusion Test

Similar to CFS, agar diffusion test measures the ability of non-membrane bound components of a probiotic culture to inhibit growth of *E. cecorum*, but via zones of inhibition instead of OD. Instead of TSB and TSA, LB-Kelly broth and Caso-yeast agar were used for growth of *Bacillus* probiotics and the diffusion test. Each treatment consisted of four replicate holes per plate and 10 replicated plates per treatment. 100 µL of overnight cultures of *E. cecorum* measured at an OD600 value of 1.0, were evenly spread across 4mm thick agar plates of Caso-yeast agar, then four holes were punched into each agar plate using the back of a sterile 1000 µL pipet tip. In the control group, 100 µL of sterile 0.9% saline was added to each hole and in the probiotic treatments 100 µL of overnight probiotic cultures were added to each hole. The plates were then anaerobically incubated overnight at 37 °C and zones were measured in three different

places on each hole (12 measurements/plate), averaged to calculate a mean zone per plate, with data reported as mean of zones per treatment, per experiment.

In Vitro Digestion Assay

The *in vitro* digestion model used, with some modifications, was based on previous publications and was completed in quintuplicates (Zyla et al., 1995; Annett et al., 2002; Latorre et al., 2015). For all the gastrointestinal compartments simulated during the *in vitro* digestion model, samples were placed on a standard orbital shaker (19 rpm; VWR, Houston, TX, USA) to simulate GI movement and mixing of feed content with tubes held at an angle of 30° inclination to facilitate proper blending of feed particles and enzyme solutions. The first GI compartment simulated was the crop, in which 5 g of feed and 10 ml of 0.03M hydrochloric acid (HCL, EMD Millipore Corporation, Billerica, MA, USA) were placed in 50 mL sterile polypropylene centrifuge tubes and mixed vigorously, then adjusted to reach a pH value around 5.2, followed by addition of 0.1 g probiotic spores to probiotic treatments. Tubes were then incubated for 30 min. Following, all tubes were removed from the incubator and advance to the proventricular stage with addition of 3000 U of pepsin per g of feed (Sigma-Aldrich, St Louis, MO, USA), and 1.5M HCl added to each tube to reach a pH of 1.4–2.0. All tubes were incubated for an additional 45 min. The third and the final step intended to simulate the intestinal section of the GI tract via inclusion of 6.84 mg of 8× pancreatin (Sigma-Aldrich, St Louis, MO, USA) in 6 mL of 1.0M sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA), and the pH was adjusted to range between 6.4 and 6.8 with 1.0M sodium bicarbonate. Then, $1x10^7$ CFU of the *E. cecorum* strain combination was added to each tube. All tube

samples were further incubated for 2 h. After the final incubation, samples from each tube were serially diluted, plated on TSA and incubated anaerobically overnight to quantify total *E. cecorum*.

Statistical Analysis

All statistics were analysed using one-way ANOVA in JMP Pro 16.0.0 using Tukey-Kramer for comparing the means with a significance of $p<0.05$.

Results

Agar Overlay Tests

The results of the agar overlays were extremely consistent throughout each replicate and when comparing both strains of *E. cecorum*. In each test *E. cecorum* challenged with GutCare® had the largest ($p<0.0001$) ZOI and, *E. cecorum* challenged with Ecobiol® had a smaller ZOI ($p<0.0001$) than GutCare®, as shown in Table 1.

Cell-free Supernatant Inhibition Assay

Percent inhibition, measured as changes in OD compared to the control wells, was not significantly different for any completed tests (Table 2). Each assay started with approximately 10^3 CFU/well and continued to an OD of 0.4 for control wells, at which time Ecobiol® produced a non-significant ($p>0.05$) increase growth of *E. cecorum* TXs by 8.38±4.44% and 2.85±2.84% and TXb by 6.52±2.88% and 3.25±2.79% in trials one and two, respectively. Similar results were noted for GutCare® with a lack of significant changes in growth of both *E. cecorum* strains.

Agar Diffusion Test

Much like the agar overlay, in the agar diffusion test the *E. cecorum* combined challenged with GutCare® had the statistically largest ($p \leq 0.0001$) ZOI at 4.86±0.11mm and 5.08±0.07mm in both experiments, shown in Table 3. However, in trial 1 the *E. cecorum* combined challenged with Ecobiol® had a statistically larger ZOI than the *E. cecorum* combined only control ($p=0.0326$) but in trial 2 no differences were observed.

In Vitro Digestion Assay

In two of the three digestion assays, Ecobiol® inclusion resulted in a lower ($p<0.05$) CFU of *E. cecorum* than GutCare®, but this was not different from non-probiotic control samples (Table 4).

Discussion

The two assays that measured ZOI, agar overlay and diffusion, showed promising results, especially for the effectiveness of GutCare® in the prevention of *E. cecorum* growth. However, these results did not carry through to CFS (Table 2) and digestion assays (Table 4), suggesting that the mechanism of action may be related to membrane bound components that require significant growth of probiotic. Notably, the digestion assay does not continue to a cecal stage because of the complexity associated with mimicking microbial activity in this region, and this may be a primary site of action for probiotics.

Agar overlay assays have become a common method for screening of probiotics against pathogens, at least as an initial step for selection (Vicente et al., 2007; Galarza-Seeber et al., 2015; Mandal et al., 2021). Here, overlays of Ecobiol® or GutCare®

showed activity against both wild-type strains of *E. cecorum*, suggesting at least a competition for nutrients and possibly the production of bacteriocins or other components that inhibit growth of the pathogen. Though this assay works well for screening a large number of probiotic candidates, the GI tract is a complicated ecosystem and one *in vitro* mechanism of action may not be a good indicator of *in vivo* activity, especially since agar overlays provide a much longer period of growth than would be available to non-colonizing bacteria within intestines. The final stage of the digestion assay, at which point *E. cecorum* is added to tubes, involves anaerobic incubation, a condition under which both probiotics are known to have limited growth. When combined with the results of other experiments presented here, active metabolism appears to be a critical component of competitive activity for these probiotics, and an inability to mimic pockets of aerobic conditions within the gut may have contributed to the results presented here, thus, should not exclude these probiotics from advancing to *in vivo* testing stages, especially given the successful ZOI associated with agar overlay and diffusion experiments.

The CFS and agar diffusion assays tested activity of secreted, or non-membrane bound, components of GutCare® and Ecobiol® against *E. cecorum*. These both depend on pre-growth of the probiotic for a relatively long period of time compared to digestion but contribute to knowledge regarding mechanism of action and contribute to feeding strategies. Agar diffusion inhibition of *E. cecorum* without the presence of actively growing *Bacillus* resulted in zones of inhibition that imply the mechanism of action is not related to nutrient competition and may occur through bacteriocins (Table 3). However, a

lack of decreased growth of *E. cecorum* in the CFS tests is not consistent with agar diffusion results. Perhaps phenotype changes in *E. cecorum* from agar to broth conditions affected the ability of *Bacillus* probiotic by-products to act against the pathogens.

A lack of reduction of *E. cecorum* in the CFS assays may be attributed to the use of a different growth medium, which could induce different phenotypes for probiotics and pathogens alike. Additionally, these were conducted in broth instead of agar, which may also induce a different phenotype. This stresses the importance of bacterial phenotype for efficacy assays, and given the inhibition results of other assays, indicates that Ecobiol® and GutCare® do, in fact, have anti-Enterococcal activity.

In conclusion, Ecobiol® and GutCare® exhibited anti-*E. cecorum* activity in agar overlay and agar diffusion assays, which each test different mechanisms of action. When probiotics were directly plated with pathogens in five agar overlay tests, both probiotics created ZOI. Similarly, agar diffusion tests in which CFS was allowed to diffuse into agar before incubation with *E. cecorum*, also created ZOI around the inoculation point. This anti-Enterococcal activity justifies further investigation for ability of these probiotics, provided as direct fed microbials, for prevention of lameness diseases associated with *E. cecorum* infection.

Table 1. Zones of Inhibition (mm) created by Ecobiol® and GutCare® against wild-type *E. cecorum*. A 10µL drop of probiotic was grown overnight on a TSA followed by overlaying additional TSA containing *E. cecorum*, followed by additional overnight incubation and measuring the ZOI in three locations per plate from the edge of the probiotic colony to the edge of the zone. Mean values represent mean of 10 ZOI (mm) ± standard error. ^{a,b,c} values with different superscripts are significantly different ($p<0.05$).

	ZOI (mm)				
Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
E. cecorum 11 TXb only	0.00 ± 0.00^{c}	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
E. cecorum 11 TXb vs Ecobiol®	6.27 ± 0.22^{b}	-	4.84 ± 0.06^{b}	6.133 ± 0.08^{b}	6.21 ± 0.06^{b}
E. cecorum 11 TXb vs GutCare®	7.13 ± 0.21^{a}	-	5.57 ± 0.08^{a}	7.21 ± 0.08^{a}	6.86 ± 0.07^{a}
E. cecorum 11 TXb CFU/plate	3×10^{8}	-	1×10^{8}	9×10^{7}	2×10^{8}
E. cecorum 11 TXs only	-	0.00 ± 0.00^{c}	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
E. cecorum 11 TXs vs Ecobiol®	-	3.49 ± 0.19^{b}	-	3.98 ± 0.05^{b}	4.42 ± 0.14^{b}
E. cecorum 11 TXs vs GutCare®	-	6.24 ± 0.30^{a}	-	6.94 ± 0.11^{a}	7.24 ± 0.11^{a}
E. cecorum 11 TXs CFU/plate	-	1.5×10^{8}	-	2.3×10^{8}	8×10^{8}

Table 2. Percent inhibition of *E. cecorum* in cell-free supernatant inhibition assay with Ecobiol® and GutCare®: 16 replicate wells per treatment were prepared and optical density (OD) measurements were measured until *E. cecorum* only control wells reached an OD of 0.4. Percent inhibition was calculated as percent decrease in OD compared to the *E. cecorum* control. OD values were converted and presented as % inhibition ± standard error.

	% Inhibition	
Treatment	Trial 1	Trial 2
E. cecorum 11 TXs Control	0	0
E. cecorum 11 TXs vs Ecobiol®	-8.38±4.44	-2.85±2.84
E. cecorum 11 TXs vs GutCare®	3.21±6.05	-2.57±2.32
E. cecorum 11 TXs at start per well	3×10^3	4×10^3
E. cecorum 11 TXb Control	0	0
E. cecorum 11 TXb vs Ecobiol®	-6.52±2.88	-3.25±2.79
E. cecorum 11 TXb vs GutCare®	-1.92±2.88	5.87±3.16
E. cecorum 11 TXb at start per well	2.5×10^3	3×10^3

Table 3. Zone of Inhibition formed by GutCare® and Ecobiol® against wild-type *E. cecorum* strains in an agar diffusion assay: 100µL of *E. cecorum* was spread on a TSA plate, then 4 holes were punched in the agar of each plate, and 100µL of probiotic cultures were placed in each well prior to overnight incubation. Zones of inhibition were then measured from the edge of the well to the edge of the zone in 3 locations for each hole (12 per plate). Data is presented as zone of inhibition (mm) ± standard error. [a,b,c] values with different superscripts are significantly different ($p<0.05$).

	Zone of Inhibition (mm)	
Treatment	Trial 1	Trial 2
E. cecorum only	0^{c}	0^{b}
E. cecorum vs GutCare®	4.86 ± 0.11^{a}	5.08 ± 0.07^{a}
E. cecorum vs Ecobiol®	2.52 ± 0.37^{b}	0.19 ± 0.11^{b}
E. cecorum CFU/plate	2.4×10^{6}	2.1×10^{6}
GutCare® CFU/well	5.6×10^{6}	3.6×10^{7}
Ecobiol® CFU/well	3.8×10^{6}	1.9×10^{7}

Table 4. Effect of GutCare® and Ecobiol® probiotics on levels of *Enterococcus cecorum* after *in vitro* digestion assay completed: Total *E. cecorum* present in tube after the completion of the *in vitro* digestion assay in Log_{10} and the initial amount of *E. cecorum* added to each tube. Data presented as Log_{10} of *E. cecorum*/tube ± standard error. [a,b] values with different superscripts are significantly different ($p<0.05$).

	Final *E. cecorum* (Log_{10})		
Treatment	Trial 1	Trial 2	Trial 3
E. cecorum only	6.99±0.09	6.33±0.12[b]	6.48±0.03[b]
E. cecorum vs GutCare®	6.82±0.17	8.03±0.15[a]	7.66±0.32[a]
E. cecorum vs Ecobiol®	7.16±0.08	6.56±0.04[b]	6.42±0.04[b]
E. cecorum added	6.99	6.61	7.20
Initial GutCare® per tube	-	8.63	8.69
Initial Ecobiol® per tube	-	8.30	8.39

Chapter 4. Evaluation of poor-quality diets on the induction of lameness diseases and bacterial translocation in broilers

Abstract

Enterococcal spondylitis (ES) is an emerging disease in broilers that is caused by an infection of *Enterococcus cecorum* (*E. cecorum*) in the free thoracic vertebrae (FTV). The *E. cecorum* must permeate the intestinal barriers via leaky gut to translocate to the FTV. In order for researchers to find adequate preventative measures and treatments for ES, a reliable model must be developed. This experiment examined combinations of three feed additives that are known to disrupt the gastrointestinal mucosal integrity and may cause leaky gut. In this experiment five treatments including inoculated control (IC), 10% rye (Rye), 10% rye + meat and bone meal (MBM) (R-MBM), 10% rye + MBM + poor quality soybean meal (pqSBM) (R-MBM-S), and MBM + pqSBM (MBM-S). On day of hatch (DOH), all birds received an oral gavage dose of *E. cecorum*. A predetermined subsample of 15 birds/pen were weighed on DOH, d15, and d35 for BW, BWG, and %BWG data. On d15 and d35, 3 birds/pen were sampled for FITC-d blood serum concentration, total FTV bacterial count, and *Enterococcus* sp. FTV count. From d21 to d42 of the experiment, mortalities and euthanized lame birds were necropsied to determine if macroscopic FTV abnormalities or bacterial chondronecrosis with osteomyelitis (BCO) on the femoral head were present. At d15, significant differences ($p<0.05$) were found between the two treatments containing pqSBM (R-MBM-S and

MBM-S) and the other three treatments (IC, Rye, R-MBM) where the pqSBM treatments had lowered BW, BWG, %BWG, and presence of *Enterococcus* sp. in the FTV whereas other treatments had none present. However, there were no differences in total bacterial recovery from the FTV. At d35, the R-MBM-S and MBM-S treatments continued to have the statistically lowest BW and BWG, but the R-MBM treatment was in the middle and statistically lower than CC and Rye treatments but higher than R-MBM-S and MBM-S. From d15 to d35 there were no differences in %BWG, FTV *Enterococcus* sp. recovery, and total FTV bacterial recovery. Also, at d15 there were no differences in FITC-d levels when compared to the IC group, but R-MBM-S was statistically higher than the Rye and R-MBM treatment groups. By d35 there were no differences in the FITC-d levels between treatments. Very few birds were found to be lame by d42 in all treatments and only one bird from R-MBM and one bird from Rye were found to have FTV abnormalities, though all but one mortality were found to have BCO on the femoral head. These results suggested that the diets containing the pqSBM may have an early effect on increasing bacterial translocation, but it may be too detrimental to growth performance to induce ES due to a possible need for birds to get heavy. Another concern is that the project may have been too short to have larger incidences of ES and possibly should have been extended to 49 or 56 days to produce heavier broilers.

Introduction

Enterococcal spondylitis (ES) is an increasingly prevalent disease that is caused by an infection of *Enterococcus cecorum* (*E. cecorum*) in the free thoracic vertebrae (FTV) of broiler chickens. The inflammatory mass on the FTV compresses the

thoracolumbar spinal cord and also can infect and cause necrosis of the femoral head, resulting in bacterial chondronecrosis with osteomyelitis. Enterococcal spondylitis presents clinical symptoms such as hock-/dog-sitting, bilateral lameness, wing-walking, and hunchbacked posture while remaining alert. *Enterococcus cecorum* is a Gram-positive cocci bacterium that is commensally found in most poultry species, though some may contain pathogenicity factors that contribute to development of disease (Borst et al., 2015).

In order to prevent *E. cecorum* from reaching the FTV, broiler GI tracts must maintain a strong intestinal immune system and intact barriers. These barriers can be affected by many different insults to including heat stress, disease and diets (add reference). Some dietary ingredients that can cause degradation of intestinal barriers are rye, animal derived proteins, and poor-quality soybean meal (pqSBM) (Han et al., 1991; Dahiya et al., 2006; Slominski, 2011; Qaisrani et al., 2014; Cardoso Dal Pont et al., 2020). Rye contains high volumes of non-starch polysaccharides (NSPs) which are not well-digested by chickens (Latorre et al., 2014). These NSPs create a highly viscus slime-like mucus that can harbor pathogenic bacteria, increase bacterial translocation, and have negative effects on both feed conversion ratio (FCR), and BWG (Cardoso Dal Pont et al., 2020). Animal derived proteins such as meat and bone meal (MBM) have been found to cause enhanced growth of pathogenic bacteria (Dahiya et al., 2006). Finally, pqSBM can include several antinutritional factors and effects such as, trypsin inhibitors, lowered amino acid availability, lowered protein nutritional value, and lowered growth performance (Han et al., 1991; Lee and Garlich, 1992). These ingredients, while

generally detrimental to growth performance, may allow for the development of a model for inducing ES by creating GI conditions favoring pathogens and promoting leaky gut. The following study investigated multiple diet formulations for development of leaky gut, bacterial translocation to FTV, lameness, and growth performance.

Materials and Methods

Animal Handling and Housing

Day of hatch male Ross 708 broilers were acquired and the experiment conducted under approved animal care protocols from the Ohio State University Institutional Animal Care and Use Committee. Birds were housed in floor cages on clean pine shavings while feed and water were provided *ad libitum*. Room temperature was kept in an age-appropriate range throughout the experiment, while the first week of lighting was continuous with an additional hour of darkness added each week until a 20:4 light:dark period was achieved.

***E. cecorum* Preparation**

Enterococcus cecorum 11 TXb and *E. cecorum* 11 TXs were prepared in tryptic soy broth (TSB) and incubated anaerobically overnight at 37 °C. The approximate bacterial concentration was measured using spectrophotometry (Spectronic 200E, Thermo Scientific) and then diluted 10,000-fold. The actual concentration was retrospectively confirmed by spread plating serial dilutions on TSA and confirmed as $1x10^4$ CFU/mL.

Poor-quality Soybean Meal Preparation and Diets

Poor-quality SBM was produced by combining uncooked SBM, normal SBM, and overcooked SBM in a 1:1:1 ratio. The overcooked SBM was produced by cooking normal SBM in an autoclave for 120 mins based on conditions set by McNaughton and Reece (1980). Rye was included at 10% and formulations are included in Table 5.

In vivo Testing of Poor-Quality Diets on Enterococcal Spondylitis Frequency in Broiler Chickens

This study consisted of 1000 Ross 708 broiler chickens that were randomly assigned to one of 5 treatment groups. Each treatment group consisted of 4 pens with 50 birds/pen totaling 200 birds per treatment. Treatments (Table 5) included were Inoculated Control (IC), 10 % Rye (Rye), 10% Rye + R-MBM, 10% Rye + MBM + pqSBM (R-MBM-S), and MBM + pqSBM (MBM-S). Birds were fed a starter (d0-14), grower (d14-35), and finisher (d35-42) of their respective treatment diet. On DOH, 15 birds per pen were selected at random for body weight measurements throughout the study and were tagged with numerical tags. An additional 6 randomly selected birds were tagged for sampling at either d15 or d35. All birds received a 0.25 mL oral gavage dose of ~$1x10^4$ CFU *E. cecorum* and placed in their designated pen. On d15 and d35, all birds with numerical tags were weighed, and 3 birds per pen dosed with fluorescein isothiocyanate dextran (FITC-d) at 4.17 mg/kg of bird weight two hours prior to sampling. Blood was collected for serum FITC-d analysis (Vicuña et al., 2015) and FTVs were aseptically collected into 0.9% saline and weighed to calculate dilution. Samples were then serial diluted on TSA and CHROMagar™ and incubated aerobically and anaerobically, respectively, at 40 °C to determine total bacterial count and presence of *Enterococcus* sp. At the first sign of lameness, defined as chickens that do not voluntarily walk or show

abnormal posture, birds were marked for observation, and marked birds that were still lame after 24 hours were euthanized and necropsied to determine cause of lameness. Additionally, mortalities were necropsied for observation of abnormal femoral heads or FTV abscess. On d42, the project ended.

Fluorescein Isothiocyanate Dextran Assay

Fluorescein isothiocyanate dextran was administered via oral gavage at 4.17 mg/kg of bird two hours prior to euthanasia and blood collection (Vicuña et al., 2015). After CO2 asphyxiation, blood was collected by severing the femoral artery and allowed to clot at room temperature in a dark container to limit exposure to light. Serum was separated from the blood by centrifugation at 1200 x g. Levels of FITC-d were measured on a black 96-well plate using a 485nm excitation wavelength and 528 nm emission wavelength (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT). Measurements were then compared back to negative serum collected from each treatment group (1 bird/pen) using procedure outlined by (Vuong et al., 2021).

Statistical Analysis

All statistics were analysed by one-way ANOVA in JMP Pro 16.0.0 using Tukey-Kramer for comparing the means with a significance of $p<0.05$.

Results and Discussion

Fifteen birds per pen were weighed to determine the effect of the feed treatments on BW (Table 6), BWG (Table 7), and %BWG (Table 8). For BW and BWG, by d15 the treatments containing the pqSBM (R-MBM-S and MBM-S) were significantly smaller than the other treatments. Body weight by d35, BWG from d15-d35 and d0-35 all had

similar results with IC and Rye were the heaviest, followed by R-MBM ($p \leq 0.001$) and then R-MBM-S and MBM-S having the lowest BW ($p < 0.0001$). Percent body weight gain provided a different insight into the growth of the birds between timepoints, the d0-d15 results showed IC was significantly higher than the three groups whose diets contained MBM ($p < 0.0001$) but similar to the rye group ($p = 0.9977$). The rye group was similar to the R-MBM group ($p = 0.1028$) and the two groups containing low quality soy (R-MBM-S and MBM-S) were the smallest ($p < 0.0001$). An interesting change happened between d15 and d35 as there were no differences in %BWG between any groups. The overall differences (d0-d35) showed the IC and rye having the largest ($p < 0.002$) %BWG, R-MBM being in the middle, and MBM-S and R-MBM-S having the lowest ($p < 0.0001$) %BWG. The lack of differences in d15-d35 were not enough to make up for the large differences from d0-d15 when looking at the overall %BWG.

Based on the data for BW, BWG, and %BWG the two diets containing pqSBM led to lower body weight parameters. Similarly, birds that consumed the R-MBM treatment were smaller than the IC and rye groups at d35, which confirms similar reports in which rye decreased growth performance of chickens (Fernandez et al., 1973; Lázaro et al., 2004). Since historically ES affects larger birds, the significant decrease in body weight of some groups may have resulted in lowered incidence of clinical ES specifically in the R-MBM-S and MBM-S groups.

On d15 and 35, FTV were collected to enumerate bacterial recovery in the FTV, as this is a potential indicator of enteric permeability and the status of *Enterococcus* colonization. On d15 there were no differences in total bacterial recovery on the TSA

plates (Table 10) but on the CHROMagar™ plates the IC, Rye, and R-MBM did not have any *Enterococcus*, while the R-MBM-S and MBM-S had a Log_{10} of 4.7 CFU/g and 4.3 CFU/g, respectfully (Table 9), suggesting that MBM and pqSBM may contribute to the pathogenesis of infectious lameness diseases. However, by d35 there were not any differences in the total and *Enterococcus sp.* found in the FTV, which may be a result of natural leakiness of the GI mucosal surface as animals age and the number of potential insults increases. While not statistically different, the MBM-S was numerically lower at a p-value of <0.87 with Log_{10} 2.7 than the other treatments, Log_{10} 3.4-3.8. It is important to note that the two treatments that had *Enterococcus sp.* present at d15 had numerically lower concentrations of *Enterococcus sp.* at d35 and that the other treatments that had no *Enterococcus sp.* at d15 had similar concentrations to the other treatments by d35.

Similar to the *Enterococcus sp.* recovery, FITC-d analysis (Table 11) found differences between some treatments at d15 but not d35. At d15 the IC group was not significantly different from any other group. Of the remaining groups, R-MBM-S was significantly higher than Rye and R-MBM and MBM-S was significantly higher than R-MBM. No differences were found at d35 between groups.

There were very few incidences of lameness observed in the birds and only 28 necropsies were performed with 16/28 euthanized for decreased mobility, and included 14% IC birds, 36% rye, 43% R-MBM, 4% R-MBM-S, and 4% MBM-S. All but one bird (R-MBM ascites) necropsied bird had BCO on the femoral head. However, only two birds had FTV abnormalities, one from R-MBM had a small abscess and one from the rye group had a friable FTV that easily separated during observation.

While there was only two abnormal FTVs in the project, there was a notable amount of BCO on the femoral head, or bacterial chondronecrosis with osteomyelitis, which may indicate *E. cecorum* infection in the proximal head, or at least a dietary effect that promoted bacterial infection, and culturing of abnormal femoral heads for bacteria should be considered in future experiments. Outside of ES, this project was able to show that pqSBM can have very detrimental effects on growth rate and enteric permeability during the first 2 weeks of life and it has lasting effects on BW and BWG. It is also interesting to note that while %BWG was much lower in birds given pqSBM from d0-d15, there were no differences in %BWG from d15-d35. While there were several indicators that the feed treatments were able to produce leaky gut and conditions suitable for the induction of ES, this experiment failed to produced ES birds within the 42 days of this study. It is possible that the treatments R-MBM-S and MBM-S, which showed promising results for both the presence of *Enterococcus sp.*in the FTV and higher FITC-d levels at d15, may not have been able to reach a high enough body weight by 42 days of age to cause the FTV to develop ES. Extending the project to 49 or 56 days in length may also provide more chance for ES cases to be seen as birds historically have not started to develop ES until they are between 35 to 42 days old and this project ended at d42. The rye treatment group was able to maintain a similar BW to the IC group, also every necropsy performed on the group found BCO on the femoral head and one of the two abnormal FTVs from the project came from the rye group. Based on these results the rye treatment may be the best to move on to further studies on the effectiveness of a probiotic to prevent ES in broilers.

Table 5. In-vivo experiment diets: Treatment diets were made using none, one or more of the following additives, 10% rye, MBM, and pqSBM. This chart provides a visual of the diets used and their inclusions in each treatment.

	Inoculated Control			10 % Rye			10 % Rye + MBM			10 % Rye + MBM + pqSBM			MBM + pqSBM		
Ingredient	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
Ground Corn	53.97%	56.73%	60.72%	43.40%	46.29%	50.28%	49.21%	52.14%	56.15%	49.21%	52.14%	56.15%	59.69%	62.59%	66.58%
Soybean Meal 47%	38.99%	35.27%	30.75%	38.64%	34.76%	30.24%	31.36%	27.46%	22.93%	10.45%	9.15%	7.64%	10.62%	9.33%	7.82%
Raw Soybean Meal	-	-	-	-	-	-	-	-	-	10.45%	9.15%	7.64%	10.62%	9.33%	7.82%
Overcooked Soybean Meal	-	-	-	-	-	-	-	-	-	10.45%	9.15%	7.64%	10.62%	9.33%	7.82%
Rye	-	-	-	10.00%	10.00%	10.00%	10.00%	10.00%	10.00%	10.00%	10.00%	10.00%	-	-	-
Pork MBM	-	-	-	-	-	-	5.00%	5.00%	5.00%	5.00%	5.00%	5.00%	5.00%	5.00%	5.00%
Blended Fat	3.37%	4.59%	5.45%	4.35%	5.52%	6.38%	2.48%	3.63%	4.49%	2.48%	3.63%	4.49%	1.53%	2.70%	3.56%
Dicalcium Phosphate 18.5%	1.64%	1.58%	1.39%	1.63%	1.57%	1.38%	0.37%	0.32%	0.13%	0.37%	0.32%	0.13%	0.39%	0.33%	0.14%
Ground Limestone	0.93%	0.92%	0.87%	0.93%	0.92%	0.87%	0.36%	0.35%	0.30%	0.36%	0.35%	0.30%	0.36%	0.35%	0.30%
Salt	0.42%	0.43%	0.36%	0.42%	0.42%	0.35%	0.20%	0.20%	0.13%	0.20%	0.20%	0.13%	0.20%	0.20%	0.13%
DL-methionine	0.30%	0.25%	0.23%	0.31%	0.26%	0.24%	0.32%	0.27%	0.25%	0.32%	0.27%	0.25%	0.31%	0.26%	0.24%
L-threonine	0.13%	0.02%	0.01%	0.05%	0.03%	0.02%	0.08%	0.06%	0.05%	0.08%	0.06%	0.05%	0.07%	0.05%	0.04%
L-lysine HCL	0.06%	0.02%	0.02%	0.06%	0.03%	0.04%	0.13%	0.10%	0.10%	0.13%	0.10%	0.10%	0.12%	0.08%	0.09%
Choline chloride (60%)	0.05%	0.05%	0.05%	0.06%	0.05%	0.05%	0.09%	0.05%	0.05%	0.09%	0.05%	0.05%	0.07%	0.05%	0.05%
Sodium bicarbonate	-	-	-	-	0.01%	0.01%	0.26%	0.27%	0.27%	0.26%	0.27%	0.27%	0.25%	0.26%	0.26%
Provimi - Turkey Starter Premix	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%	0.15%
Total	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Table 6. Body weight of broilers at 0, 15, and 35 days of age: Body weights were collected from 60 pre-selected birds per treatment at different timepoints. Data is displayed as BW (g) ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	BW (g)		
	d0	d15	d35
IC	37.84±0.37	450.64±10.53[a]	2014.74±33.53[a]
Rye	38.34±0.39	454.37±10.72[a]	2031.45±35.00[a]
R-MBM	38.16±0.35	419.89±8.49[a]	1826.69±32.86[b]
R-MBM-S	37.71±0.41	314.48±8.94[b]	1446.42±38.82[c]
MBM-S	37.68±0.45	333.21±6.25[b]	1498.18±26.26[c]

Table 7. Body weight gain of broilers at d0-15, d15-35, and d0-35: Body weights were collected from 60 pre-selected birds per treatment at different timepoints and BWG was calculated between time points and overall, for the experiment. Data is displayed as BWG (g) ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	BWG (g)		
	d0-d15	d15-d35	d0-d35
IC	412.92±10.38[a]	1561.25±27.32[a]	1977.02±33.44[a]
Rye	415.94±10.59[a]	1576.51±27.60[a]	1992.45±34.85[a]
R-MBM	381.67±8.43[a]	1410.68±26.34[b]	1792.35±32.82[b]
R-MBM-S	276.73±8.88[b]	1130.37±31.50[c]	1408.62±38.75[c]
MBM-S	295.51±6.12[b]	1164.28±21.70[c]	1460.45±26.08[c]

Table 8. Percent body weight gain of broilers at d0-15, d15-35, and d0-35: Body weights were collected from 60 pre-selected birds per treatment at different timepoints and %BWG was calculated between time points and overall, for the experiment. Data is displayed as %BWG ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	%BWG		
	d0-d15	d15-d35	d0-d35
IC	1094.32±25.50[a]	355.35±7.36	5221.45±95.00[a]
Rye	1083.73±26.42[ab]	354.30±7.60	5196.44±88.45[a]
R-MBM	1002.16±22.96[b]	338.78±5.10	4708.14±91.65[b]
R-MBM-S	736.04±23.92[c]	363.25±8.83	3742.14±104.44[c]
MBM-S	787.65±16.66[c]	351.41±5.58	3886.75±68.99[c]

Table 9: *Enterococcus* sp. bacterial count (LOG^{10} CFU/g) found in the free thoracic vertebrae at d15 and d35 on CHROMagar™: The FTV was aseptically collected on d15 and d35 and added to 0.9% sterile saline then serial plated on CHROMagar™ and calculated as LOG_{10} CFU/g. Data is displayed as *Enterococcus* sp. LOG_{10} CFU/g ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	CHROMagar™ (LOG_{10} CFU/g)	
	d15	d35
IC	0 ± 0^{b}	3.81±0.12
Rye	0 ± 0^{b}	3.84±0.11
R-MBM	0 ± 0^{b}	3.41±0.34
R-MBM-S	4.85 ± 0.34^{a}	3.74±0.35
MBM-S	4.3 ± 0.44^{a}	2.68±0.58

Table 10: Total bacterial count (LOG_{10} CFU/g) found in the free thoracic vertebrae at d15 and d35 on Tryptic Soy Agar: The FTV was aseptically collected on d15 and d35 and added to 0.9% sterile saline then serial plated on TSA and calculated as LOG_{10} CFU/g. Data is displayed as total bacteria LOG_{10} CFU/g ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	TSA (LOG_{10} CFU/g)	
	d15	d35
IC	3.22±0.45	3.12±0.48
Rye	3.37±0.35	3.66±0.38
R-MBM	3.46±0.34	3.51±0.33
R-MBM-S	2.77±0.63	3.92±0.14
MBM-S	3.04±0.46	3.95±0.14

Table 11: Concentration in ng/mL of fluorescein isothiocyanate dextran in blood serum samples at d15 and d35: FITC-d was administered at 4.17 mg/kg of bird weight two hours prior to serum being collected. Serum was then analyzed using a Synergy HT and then compared back to negative serum from the treatment group the sample came from. Data is displayed as FITC-d concentration (ng/mL) ± standard error. Superscripts denote statistical differences ($p<0.05$) between treatments.

Treatment	FITC-d (ng/mL)	
	d15	d35
IC	264.31±41.98^{abc}	184.37±25.12
Rye	176.16±38.75^{bc}	306.69±62.82
R-MBM	177.18±34.03^{c}	210.51±23.79
R-MBM-S	381.15±47.87^{a}	232.95±15.87
MBM-S	355.45±45.04^{ab}	228.4±19.28

Chapter 4. Thesis Conclusions

Enterococcus cecorum has become increasingly pathogenic in recent years, causing the emergence of enterococcal spondylitis in broilers. These *E. cecorum* caused diseases are likely to continue to progress in coming years and finding ways to treat and prevent *E. cecorum* infections is very important. Some methods that may aid in the prevention of *E. cecorum* based infections may be strengthening of the gastrointestinal immune system and the inclusion of probiotics that may be able to inhibit *E. cecorum* growth.

The *in vitro* assays tested the ability of two commercial probiotics, GutCare® and Ecobiol®, to inhibit or limit the growth of known pathogen *E. cecorum* strains. The assays showed varying results but, in the agar overlay assays, both probiotics inhibited the growth of the *E. cecorum* strains and the GutCare® outperformed Ecobiol®. In the agar diffusion assays, GutCare® again was the most effective at inhibiting the growth of the *E. cecorum,* but Ecobiol® only showed inhibition in one of the two replicates. However, no growth inhibition was found in the cell-free supernatant inhibition assay and digestion assay. Based on these results, I think that GutCare® showed more promise as a possible prevention for *E. cecorum* based disease and over Ecobiol®.

The *in vivo* assay aimed to establish a potential model for the induction of enterococcal spondylitis that can be used to test potential treatments and preventatives for

ES. Rye, poor-quality soybean meal, and meat and bone meal were used as diet additives based on known antinutritional effects that then have in broilers that could result in increased incidence of bacterial translocation. The two treatments that contained poor-quality SBM showed increased bacterial translocation of *Enterococcus* sp. on d14, but those effects were lost by d35. The birds in the pqSBM treatments also grew significantly less than birds in the other treatments and based on prior articles (Martin et al., 2011), ES primarily occurs in heavy birds. While the 10% rye group did not show elevated levels of bacterial translocation, there were several incidences of BCO on the femoral head, and they were the only group to maintain a similar BW as the control group. Since very few FTV abnormalities were observed, there may be cause to look into studies the go beyond d42 and allow birds to grow larger to possible increase ES observations. Also, since the FTV *E. cecorum* levels lowered in the two pqSBM treatments between d14 and d35 there may be a benefit to providing an additional inoculation of *E. cecorum* at d14.

Based on these observations, the probiotic GutCare® may be a good candidate for further research to see if GutCare® can lower *E. cecorum* growth in an *in vivo* setting possibly with birds that are further challenged with rye or a different ratio make up of pqSBM.

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